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DISSERTATION

Titel der Dissertation

Development of vaccines and antibody-based therapies against *Streptococcus pneumoniae* using conserved antigens

Verfasserin

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Abstract

Despite the availability of antibiotics and polysaccharide-based vaccines *S. pneumoniae* remains the main cause of community-acquired pneumonia worldwide. Although the currently available conjugated vaccines have proven efficacy against vaccine serotypes, there are disadvantages of polysaccharide-based vaccines, and there is an urgent need for alternatives.

Two promising candidates for a protein-based vaccine against pneumococcus, StkP and PcsB, were identified by the ANTIGENome technology.

StkP, a eukaryotic type serine/threonine protein kinase, seems to play a regulatory role in gene transcription. To learn more about the interaction partners of StkP, we performed microarray analysis with two strains, TIGR4 and 6B, comparing WT and Δ*stkp* strains. We could confirm that StkP is a key player in the pneumococcal cell cycle by regulating the expression of several important gene products, although effects of this regulatory function seems to be dependent on the genetic background to a great extent.

PcsB is a predicted murein hydrolase and is an immunodominant antigen during human infection. In a microarray comparison between WT and Δ*pcsB* strains, with two different genetical backgrounds (TIGR4 and PJ1324) the main difference we found was the greatly increased transcript levels for two LysM domain proteins, which play a role in the cell wall metabolism. This strong upregulation in the expression might represent a compensatory mechanism for the bacterium lacking the PcsB protein. Except for the two LysM domain proteins, only a few genes were affected and then to a lower extent (2-10 folds). These results suggest that there is a very selective change in global transcription as a consequence of the lack of PcsB.

Three additional vaccine candidates indentified by the ANTIGENome: SP2027, SP0609 and SP2194 were also analysed. Immunization with these proteins protects mice in a sepsis model and two pneumonia models against different *S. pneumoniae* serotypes. The only phenotypes observed with deletion mutants were a reduced virulence for ΔSP2027 and a lower affinity to eukaryotic cells with ΔSP0609. Due to its role in adhesion, SP0609 could be considered as a vaccine candidate while anti-SP0609 antibodies may reduce intranasal colonization by *S. pneumoniae* and thus reduce invasion into the host.

Since vaccine induced immune protection against specific microbes takes weeks to develop, passive immunization is indispensable when acute protection or treatment is required. In order to develop an antibody-based therapy, we generated mouse mAbs against the five antigens and two additional (PspA and SP1650) ones, and tested them for their potential as anti-infective agents in various *in vitro* and *in vivo* assays. Out of the tested antigens just one, the anti-PspA elicited cross-protection using different strains. Anti-PspA mAb showed strong surface staining and opsonophagocytic killing activity as well.

With a powerful protein-based vaccine which is intended to prevent infections caused by all serotypes in elderly and children and with effective monoclonal antibody targets for passive immunization in case of emergency we hope to reduce in the future the 1.5 million deaths annually worldwide caused by pneumococcus.

Kurzfassung

Trotz der Verfügbarkeit von Antibiotika und Impfstoffen, die auf Polysacchariden basieren, bleibt *S. pneumoniae* weltweit die Hauptursache für Pneumonie.

Obwohl die momentan verfügbaren konjugierten Impfstoffe Wirksamkeit gegen die vom Impfstoff gedeckten Serotypen gezeigt haben, gibt es Nachteile, die solche auf Polysacchariden basierende Impfstoffe mit sich bringen und daher gibt es einen dringenden Bedarf an Alternativen.

Zwei vielversprechende Kandidaten, StkP und PcsB, wurden mittels ANTIGENome Technologie für einen Protein-basierten Impfstoff gegen Pneumococcus identifiziert.
StkP, eine eukaryotische Typ Serin/Threonin Proteinkinase, scheint eine regulatorische Rolle in der Gentranskription zu spielen. Um mehr über die Interaktionspartner von StkP zu erfahren, führten wir Microarray Analysen mit 2 Stämmen durch, TIGR4 (Serotype T4) und PJ1324 (Serotype 6B) um den Wildtyp-Stamm und den Δ*stkp*-Stamm zu vergleichen. Wir konnten bestätigen, dass StkP eine Schlüsselfunktion im Zellzyklus der Pneumococcen spielt, indem die Expression einiger wichtiger Genprodukte reguliert wird, obwohl die Effekte der regulatorischen Funktion zu einem großen Ausmaß vom genetischen Hintergrund abhängig zu sein scheint.

PcsB ist eine vorausgesagte Murein Hydrolase und ist während der Infektion im Menschen ein immundominantes Antigen. Die Microarray Analysen bei Vergleich von Wildtyp und Δ*pcsB* Stämmen in 2 verschiedenen genetischen Hintergründen (TIGR4 und 6B) ergaben, dass der Unterschied größtenteils ein erhöhtes Transkriptionslevel zweier LysM Domänenproteine ist, welche eine Rolle im Zellwandmetabolismus spielen. Die starke Hochregulation in deren Expression repräsentiert möglicherweise einen Kompensationsmechanismus für Bakterien, denen das PcsB Protein fehlt. Es gab nur wenige Gene die zu einem geringeren Ausmaß (2-10 fach) betroffen waren. Diese Resultate deuten darauf hin, dass das Fehlen der PcsB Expression, eine sehr selektive Veränderung in der globalen Transkription zur Folge hat.

Drei zusätzliche Impfstoffkandidaten, identifziert mittels ANTIGENome Technologie, wurden analysiert: SP2027, SP0609 und SP2194. Immunisierung mit diesen

Proteinen schützt Mäuse im Sepsis-Modell und zwei Pneumonia-Modellen gegen verschiedene *S. pneumoniae* Serotypen.
Weiters zeigte die Mutante ΔSP2027 eine verringerte Virulenz und die Mutante ΔSP0609 hatte eine reduzierte Affinität zu eukaryotischen Zellen.

Aufgrund seiner Rolle in der Adhäsion, könnte SP0609 als Impfstoffkandidat berücksichtigt werden. Antikörper spezifisch für SP0609 könnten die intranasale Kolonisierung durch *S. pneumoniae* und weiters die Invasion des Wirtes verhindern.
Da Impfstoff induzierte Protektion gegen spezifische Mikroben Wochen braucht, um sich zu entwickeln, ist passive Immunisierung unverzichtbar wenn akute Protektion und Behandlung erforderlich ist. Um eine auf Antikörper basierende Therapie zu entwickeln, haben wir neben PspA und SP1650 monoklonale Maus Antikörper gegen fünf Antigene generiert, und diese auf ihre anti-infektiöse Wirkung in in vitro und in vivo Experimenten getestet. Von den getesteten Antigen-spezifischen Antikörpern, zeigte nur einer, nämlich anti-PspA, Kreuzprotektion bei der Verwendung unterschiedlicher Stämme. Die anti-PspA monoklonalen Antikörper zeigten ein starkes positives Signal in Experimenten zur Oberflächenfärbung und in Opsonophagocytose-Killing Assays.

Mit einem auf Protein basierenden Impfstoff, welcher vor Infektionen, hervorgerufen durch alle Serotypen bei sowohl Älteren als auch Kindern schützen soll, und mit effektiven monoklonalen Antikörpern zur passiven Immunisierung im Falle eines akuten Notfalles, hoffen wir in der Zukunft die rund 1.5 Millionen Todesfälle weltweit verursacht durch Pneumococcen, zu verringern.

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Introduction

Streptococcus pneumoniae

Bacteriology

Streptococcus pneumoniae are Gram-positive, lancet-shaped cocci. They are non-motile, form pairs (diplococci), but may also occur as single or in short chains. Pneumococcus is alpha hemolytic when cultured on blood-agar, bile-soluble and aerotolerant and mesophil, living optimally at temperatures between 30 and 35 degrees Celsius. Individual cells are between 0.5 and 1.25 micrometers in diameter.

Like other streptococci, they lack catalase and ferment glucose to lactic acid. Pneumococci hydrolyze inulin, and their cell wall composition is characteristic both in terms of their peptidoglycan and their teichoic acid.

The capsule. Since Pasteur noted the capsule in the first published description of pneumococcus, it played a central role in many historic observations regarding genetics, immunology and pathogenesis. A capsule composed of polysaccharide forms the outermost layer of the pneumococcal cells. It represents a diverse group; the simplest types are linear polymers with repeat units comprising two or more monosaccharides, the more complex structures contain up to six monosaccharides, linkages, and side chains (Fischetti 2006). The capsular polysaccharide represents one of the most important pneumococcal virulence factors, there are just a few exceptions for virulent unencapsulated strains (Crum, Barrozo et al. 2004).

Neufeld and Händel described the pneumococcal capsular types 1 and 2, allowing the introduction of rational serotherapy of pneumococcal infection (Neufeld 1910). Today there are more than 90 immunologically distinct serotypes known, of which some possess distinct epidemiological properties. Certain serotypes are more likely to be associated with nasopharyngeal colonization than to cause invasive disease. Compared with transient or infrequent colonizers, serotypes carried at high rates by young children may rapidly elicit age-associated natural immunity to invasive disease. Other serotypes seem to be of disproportionate importance as causes of disease in very young infants, in older children, in immunocompromised individuals, or in elderly people. Some serotypes seem to be associated with particular disease syndromes, such as complicated pneumonias in children,

or with higher rates of hospitalization in children or mortality in adults, or some are consistently responsible for outbreaks in certain populations (Hausdorff, Feikin et al. 2005).

The expression of capsule reduces entrapment in the luminal mucus, allowing the pneumococci access to epithelial surfaces (Nelson, Roche et al. 2007). Inside the host, this polysaccharide capsule serves as a biochemical barrier, protecting the pneumococcus from phagocytosis and killing by inhibiting complement deposition and consequently complement-activation (Hammerschmidt, Wolff et al. 2005).

There is a correlation between increased amounts of capsular polysaccharide and greater virulence in mice (Mac and Kraus 1950). The transparent phenotype, which produces lower amounts of capsular polysaccharide, was shown to be more efficient in colonizing mucosal surfaces of the nasopharynx and in residing on surfaces, whereas the opaque phenotype with larger amount of polysaccharides is more virulent in systemic infections (Hammerschmidt, Wolff et al. 2005).

The cell wall. The cell wall of *S. pneumoniae* is composed of peptidoglycan covalently linked to chains of an unusually complex teichoic acid, which contain as structural components phosphoryl choline residues, and the wall is about 6 layers thick. This is an essential element in the biology of *S. pneumoniae* since the choline specifically adheres to choline-binding receptors that are located on human cells. In most case chains of the capsular polysaccharide are attached by covalent bonds to the underlying peptidoglycan (Fischetti 2006). The cell wall is the cause of the intense inflammatory reaction during pneumococcal infection, since it stimulates the influx of inflammatory cells, activates the complement cascade and the cytokine production (Bruyn and van Furth 1991).

The pili. The Gram-positive pili, are hair-like structures that extend from the surface, have recently been described in many strains of *S. pneumoniae*. They are made of extended polymers formed by a transpeptidase reaction involving covalent cross-linking of LPxTG motifs containing subunit proteins assembled by specific sortases (Barocchi, Ries et al. 2006). Pili are present in some but not all clinical isolates, are encoded by the rlrA islet, which includes the genes for the three pilus subunits (RrgA, RrgB, and RrgC) (Hava and Camilli 2002; Barocchi, Ries et al. 2006). The recent finding that pneumococcal pili contribute to adherence to the upper respiratory tract, to virulence and to elicit host inflammatory responses (Barocchi, Ries et al. 2006) by increasing the formation of large

amounts of TNF (tumor necrosis factor) by the immune system during invasive infection. The pilus antigens are protective in mice (Gianfaldoni, Censini et al. 2007).

Surface Proteins. Surface-exposed proteins are key players during the infectious process of pathogenic bacteria, mediating the pneumococcal attachment to eukaryotic cells. On the basis of functional genomic analysis, it is estimated that the pneumococcus contains more than 500 surface proteins. Three clusters of surface proteins can be distinguished by genome analysis: the membrane–associated lipoproteins (42 in R6 and 47 in TIGR4), the CBP family (10 in R6 and 15 in TIGR4), and proteins with an LPxTG motif (13 in R6 and 19 in TIGR4). Bioinformatic analysis of the pneumococcal genomes also indicates the presence of incomplete biosynthetic pathways, which is consistent with the inability of this pathogen to carry out respiratory metabolism, and also explains the high number of ATP binding cassette (ABC) transporters (Bergmann and Hammerschmidt 2006) (Figure 1).

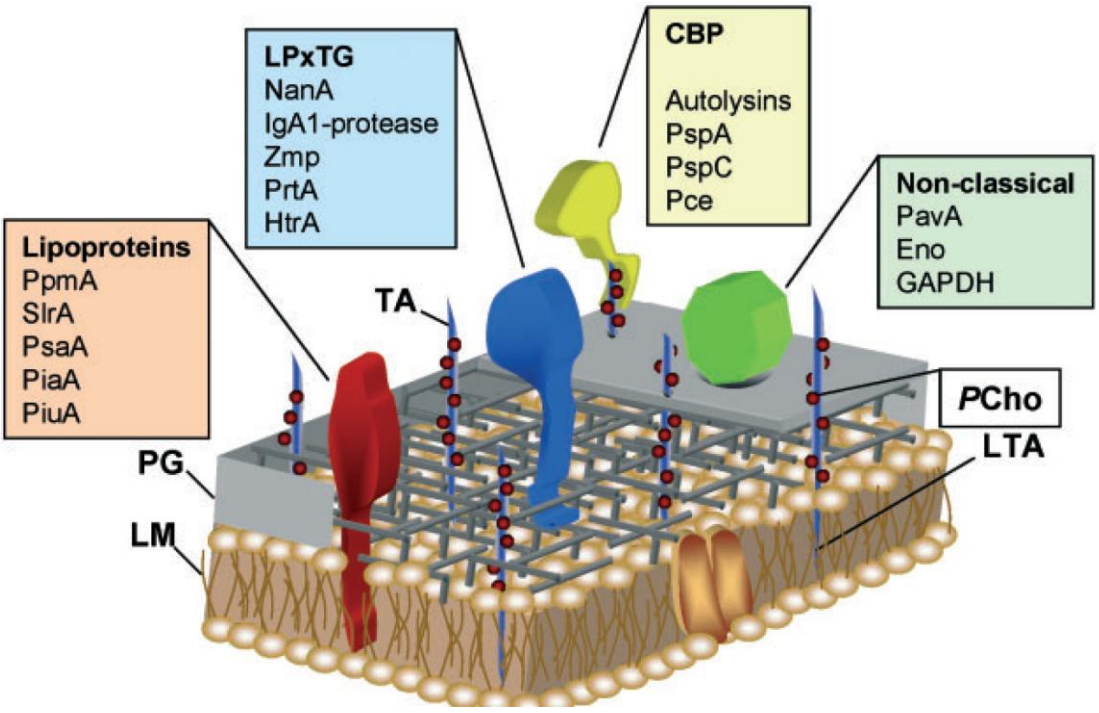

Figure 1: Schematic model of the pneumococcal outer cell wall and surface-exposed proteins. The pneumococcal cell wall consists of a phospholipid membrane (LM), peptidoglycan (PG), and teichoic and lipoteichoic acids. Phophorylcholine (PCho) anchors the choline-binding proteins (CBPs) non-covalently on the cell wall. (Bergmann and Hammerschmidt 2006)

The choline-binding proteins (CBP) share a common C-terminal choline-binding domain while the N-termini are distinct, indicating that their functions are different. The

CBP family includes such important determinants of virulence such as PspA (pneumococcal surface protein A), LytA, B, and C (three autolysins), and CbpA (choline binding protein A, also referred to as PspC), which mediates adherence to lung epithelia.

The lipoproteins are essential for substrate transport and bacterial fitness. A lipoprotein is the pneumococcal surface adhesin A (PsaA) which is part of an ABC manganese transporter and plays a significant role in pneumococcal adhesion and colonization (Anderton, Rajam et al. 2007). PiuA, PiaA, which are components of iron-uptake ABC transporters, have been shown to be required for full virulence (Brown, Gilliland et al. 2001).

LPxTG proteins are covalently anchored to the peptydoglycan backbone of the cell wall after cleavage of the LPxTG sequence by a transpeptidase, designated a sortase (Nobbs, Vajna et al. 2007). Different sortases process functionally different surface-exposed proteins and have, therefore, different impacts on pneumococcal adherence and virulence (Bergmann and Hammerschmidt 2006). Among the LPxTG proteins are the neuroaminidase, the hyalonurate lyase, the IgA protease, and ZmpC.

Pneumolysin. The pore-forming toxin pneumolysin is a member of a large family of highly conserved, cholesterol binding toxins. These toxins are released from the bacteria as soluble monomeric proteins, which then assemble into prepore oligomers on the surface of cholesterol-containing cell membranes. The prepores then puncture the membrane to form very large pores containing 30-50 subunits. A second biological activity contributing to the virulence of pneumolysin is the activation of the complement pathway (Tilley, Orlova et al. 2005)

History

S. pneumoniae has been profoundly important in our understanding of the human response to infectious diseases. The pneumococcus has pushed biological science to the identification of DNA as the genetic material (Griffith 1928), of polysaccharide-based vaccines, quorum sensing, peptide-based bacterial communication, and many other tenets.

S. pneumoniae was first isolated simultaneously and independently by Louis Pasteur in January 1881 and by George Miller Sternberg. Sternberg sampled his own saliva and successfully grew the bacteria as a culture in September 1880, but did not publish his report until April 1881. With the help of the Gram-stain technique pneumococcal pneumonia could be distinguished from other causes of pneumonia, *S. pneumoniae* was demonstrated to be a major cause of pneumonia. In the early 1900s Neufeld described capsular swelling (quelling) with type-specific antiserum, a discovery that allowed serotyping of the isolates (Neufeld 1910). In 1913 Lister published the development of type-specific antibody (Lister 1913) and then in 1930 Francis shows capsular polysaccharide to be immunogenic in humans (Francis and Tillett 1930). In 1928, Frederick Griffith demonstrated that genetic information could be transferred, turning harmless pneumococcus into a lethal form by co-inoculating the live pneumococci into a mouse along with heat-killed, virulent pneumococci. Following this, in 1944 Oswald Avery and his colleagues demonstrated the transforming factor in Griffith's experiment was DNA, not protein, as was widely believed at the time (Avery, Macleod et al. 1944).

In the past decades the research output was increased as the pneumococcus acquired multiple antibiotic resistance and the scientific and medical communities have been gearing up to develop, introduce and validate novel vaccines to counter this problem.

Host-microbe interactions

S. pneumoniae infection and epidemiology

S. pneumoniae is a normal constituent of the microflora of the human upper respiratory tract and an opportunist pathogen (Tuomanen 2004). Nasopharyngeal colonization occurs in approximately 40% of the healthy children and 5-10% of healthy adults, without causing symptoms (carrier state) or generates milder, self-limited mucosal infections. Children are colonized in the nasopharynx asymptotically for about 4-6 weeks, often several serotypes at a time (Fischetti 2006).

Pneumococcus attaches to nasopharyngeal cells through interaction of bacterial surface adhesins. This colonization becomes infectious if the organisms are carried into areas such as the Eustachian tube or nasal sinuses where it can cause otitis media and sinusitis, respectively. Invasive disease usually arises during the first few days of colonization by a new serotype (Austrian 1986). The question of why bugs so innocent to most, can produce life-threatening invasive infections in a few remains something of guiding paradox in the Gram-positive field. The bacterium can cause pneumonia, usually of the lobar type, paranasal sinusitis and otitis media, or meningitis, which is usually secondary to one of the former infections. Rare manifestations of the pneumococcal disease are osteomyelitis, septic arthritis, endocarditis, and peritonitis (Butler, McIntyre et al. 2004).

Adhesion. Pneumococci adhere tightly to the nasopharyngeal epithelium by multiple mechanisms that for most individuals, appears to result in an immune response that generates type-specific immunity. Upon reaching the lower respiratory tract by aerosol, pneumococci bypass the ciliated upper respiratory epithelial cells unless there is damage to the epithelium (Tuomanen 1986). They progress to the alveolus and associate with specific alveolar cells which produce a choline-containing surfactant. The presence of pneumococci in the alveolus need not lead to inflammation unless bacteria maintain localization to this site and multiply (Talbot, Paton et al. 1996). Adherence to the epithelium occurs though pneumococcal binding to cell-surface carbohydrates (N-acetyl-glycoseamine) and it is mediated by cell-wall associated proteins, like the PsaA. The pneumococcus adheres to

syliated cell surface glycoconjugates (Barthelson, Mobasseri et al. 1998). Then neuroaminidases cleaves the terminal sialic acid, exposing cryptic receptors and enhancing adherence (Paton, Berry et al. 1997). Viruses with neuroaminidase activity, such as influenza, may act synergistically with pneumococcus, priming the respiratory epithelium for adherence. Removing sialic acids from the host targets of pneumococcus might promote bacterial persistence in the respiratory tract and facilitate subsequent internalization (McCullers and Rehg 2002).

CbpA acts as an adhesin in the upper and lower respiratory track (Rosenow, Ryan et al. 1997). It shows increased affinity for immobilized sialic acid on the cytokine-activated human cells (Rosenow, Ryan et al. 1997). CbpA interacts with polymeric Ig receptors on eukaryotic cells, which results in increased migration through the mucosal barrier (Balachandran, Brooks-Walter et al. 2002). The lack of CbpA inhibits the bacteria to multiply in the lungs, although after intravenous administration they show no loss of virulence (Balachandran, Brooks-Walter et al. 2002). CbpA has been shown to interact with the platelet-activating factor receptor, which plays essential role in pneumococcal interactions with cells in the lung that lead to progression from pneumonia to bacteraemia and meningitis (Balachandran, Brooks-Walter et al. 2002). Surface proteins also contribute to adherence to host cells through conferring to the non-specific physiochemical properties of the bacterial surface (e.g. net surface charge, hydrophobicity (Swiatlo, Champlin et al. 2002) and reviewed in (Bogaert, De Groot et al. 2004).

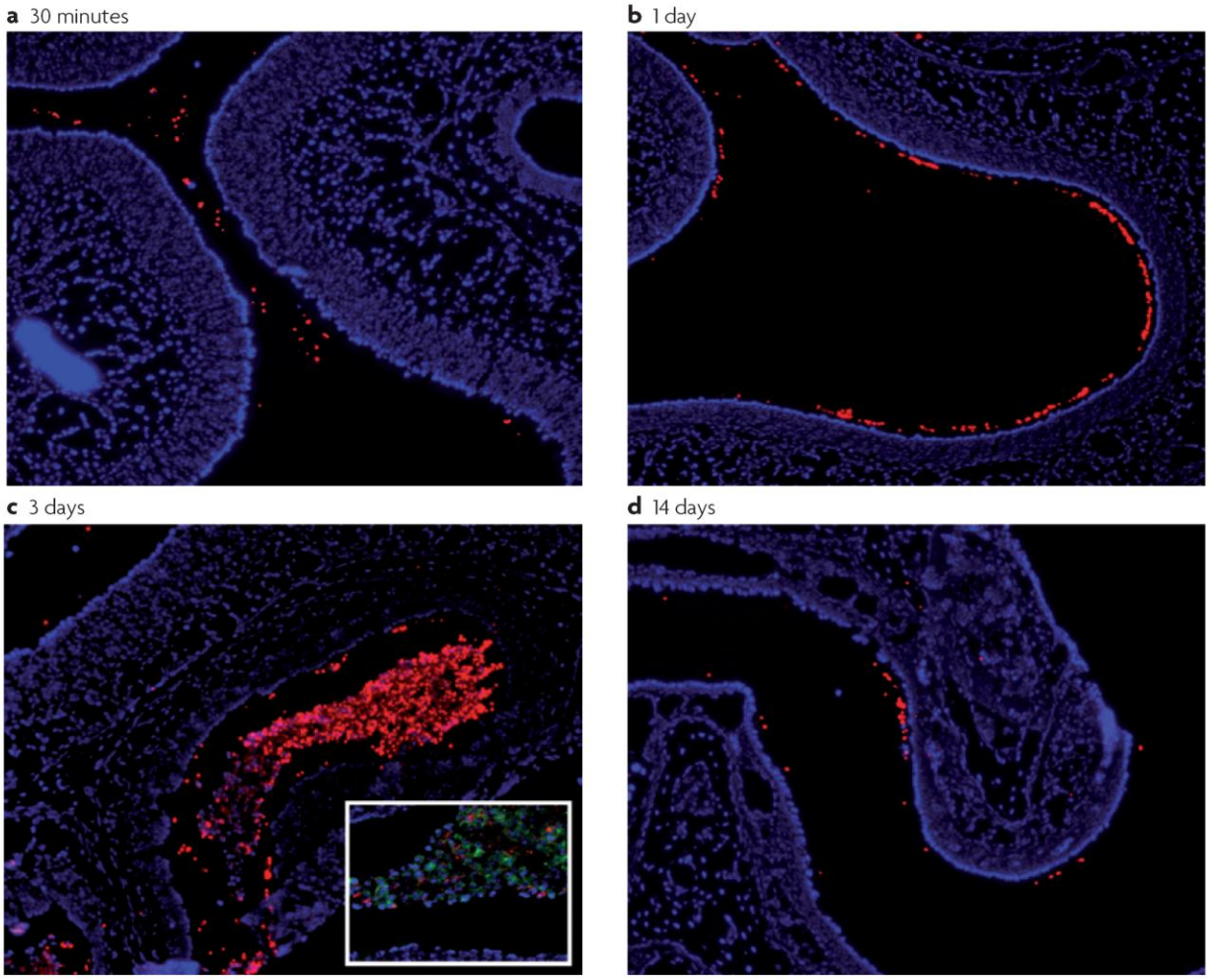

Figure 2: Progression of nasal colonization of mice with a pneumococcal isolate (23F). Bacteria (red) were detected using serotype-specific antisera, mouse tissue (blue) was stained using DAPI, and neutrophils (green) in the section C were stained with an antibody to murine ly6-G. (Kadioglu, Weiser et al. 2008)

Invasion. To generate invasive disease from asymptomatic colonization inflammation is needed. Experimentally, in healthy tissues, it requires approximately 100,000 bacteria/ml to trigger an inflammatory response, which can cause considerable tissue damage (Fischetti 2006). The appearance of inflammatory factors (Il-1, TNF) changes the type and the number of receptors on the epithelial and endothelial cells.

Colonization of, and translocation across the mucosal barrier eventually leads to subcellular dissemination within the host. Once disseminated, the pathogen then requires strategies to circumvent host immunity. The pneumococcus is considered to be an extracellular pathogen; however, the dynamic process of adherence to mammalian cells is

accompanied by cell-specific internalization mechanisms (Hammerschmidt 2006). Phosphorylcholine (ChoP) is a component of teichoic and lipotechoic acids that extends outward from the bacterial cell wall and cell membrane, respectively. Upon exposure to pro-inflammatory stimuli that activates NFκB, platelet activating factor receptor (PAFr) is expressed by mammalian cells (Mutoh, Ishii et al. 1994) and ChoP binds to PAFr (Cundell, Gerard et al. 1995). Pneumococcus binding to the PAFr does not result in the activation of a G-protein-mediated signal transduction pathway as does PAF (Radin, Orihuela et al. 2004). Rather, pneumococcal binding results in activation of ERK kinases consistent with activation by b-arrestin. This results in uptake of the pneumococcus into a vacuole and its transport through the cell. (Radin, Orihuela et al. 2004). PAFr mediated invasion of endothelial cells requires the bacterial protein Choline binding protein A (CbpA) (Ring and Tuomanen 2000) but CbpA does not binds PAFr. The bacteria subvert the endocytosis/recycling pathway of the PAF receptor for cellular transmigration.

In addition, as pneumococci begin to lyse due to autolysis or in response to host defences and antimicrobial agents, they release cell wall components, pneumolysin and other substances that lead to stronger inflammation and cytotoxic effects. Pneumolysin and hydrogen peroxide produced by the bacteria kill cells and induce production of nitric oxide which may play a key role in septic shock. If bacteraemia occurs, the risk of meningitis increases. Pneumococci can adhere specifically to cerebral capillaries as well (through the above mentioned pairings of choline to PAF receptor and CbpA to carbohydrate receptor). Once in the cerebrospinal fluid, a variety of pneumococcal components, particularly cell wall components, initiate the inflammatory response in the brain. Inflammatory and cytotoxic components released by the pneumococcus trigger multiple inflammatory cascades. Pneumolysin and hydrogen peroxide can directly damage eukaryotic cell (Feldman, Anderson et al. 2002). Autolysis is responsible for the spike in inflammation observed immaditely following antibiotic treatment of meningitis (Fischetti 2006).

S. pneumoniae is a leading agent of pneumonia, meningitis and sepsis throughout the world, affecting primarily those individuals at either extreme of the age spectrum. As of July 2009 the World Health Organization (WHO) estimates that globally 735,000 (519,000 – 825,000) HIV negativ child deaths under five years of age occurred during 2000 due to pneumococcal infections. 7% of all cause-child mortality under five was due to pneumococcal infections (World Health Organization 2009). Community-acquired

pneumonia can have mortality rates of more than 20% in patients with concurrent pneumococcal septicaemia, despite the appropriate antibiotic therapy (Balakrishnan, Crook et al. 2000). In countries that have a high prevalence of HIV-1 infection, there has been a significant increase in the rate of pneumococcal pneumonia and associated bacteraemia, and this increase has been most marked in young adults (Redd, Rutherford et al. 1990).

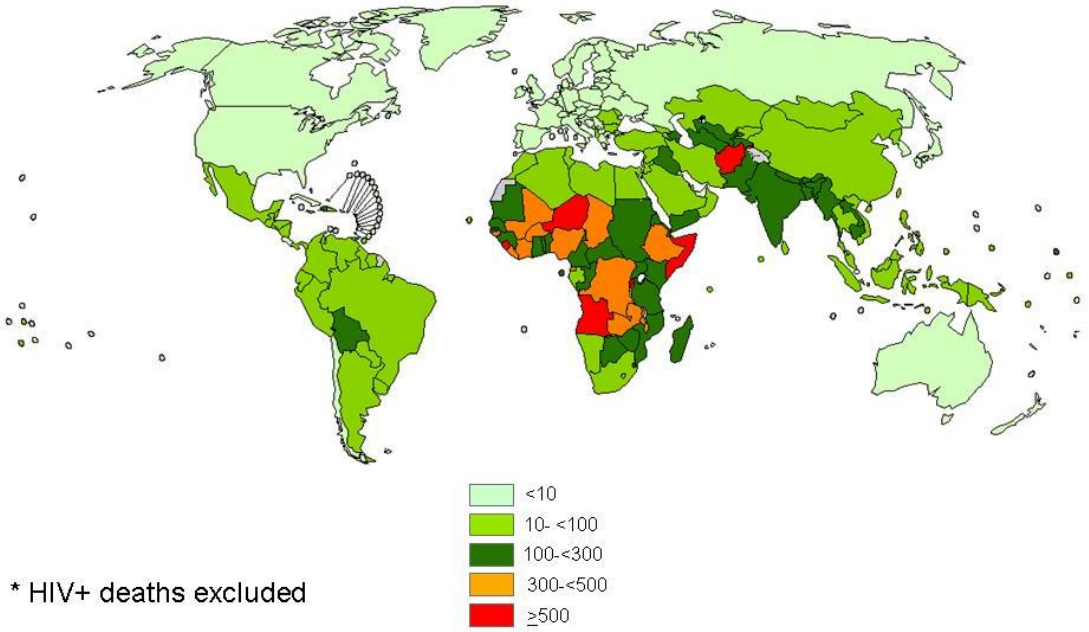

Figure 3: Rate of deaths* caused by *S. pneumonae* in the year 2000 (per 100,000 children under age 5). (World Health Organization 2009)

Host defence

The first line of lung defence is the mucocilary clearance along the upper respiratory tract. Once *S. pneumoniae* breached the physical barriers, activation of epithelial and endothelial cells occurs and results in altered vascular permeability. The innate defence mechanisms get activated, in the first line the alveolar macrophages, and proinflammatory response begins. The inflammatory cells are recruited to the lung, and this infiltration is the hallmark of clinical pneumonia (Figure 4).

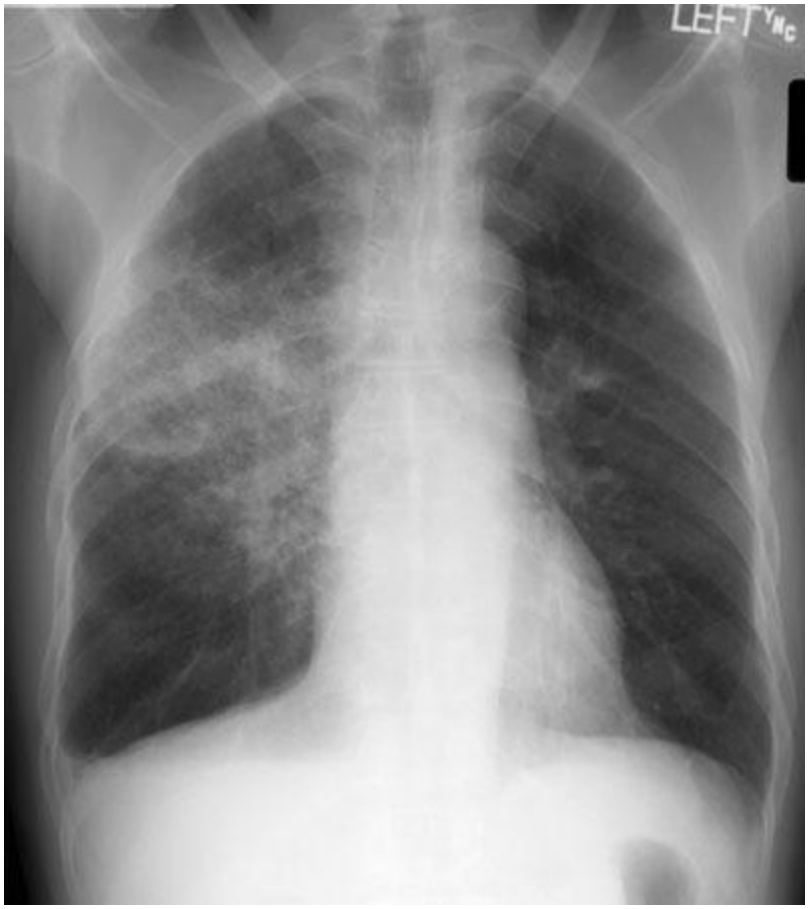

Figure 4: Typical radiographic finding in pneumococcal pneumonia.
X-ray picture of clear right upper lobe infiltrate.
(Source: http://www.meded.virginia.edu/courses/rad/cxr/pathology3chest.html; 23.03.11)

Inflammation is helpful for the host, because it can control the infection, but at the same time it is capable to disrupt alveolar epithelium integrity and thus the gas exchange is impaired. In case of the disequilibrium of the pro-inflammatory and anti-inflammatory mechanisms sepsis syndrome can occur, and generalised tissue injury, vascular permeability means better nutrition for the bacteria hence its greater access to sugars, proteins, lipids and fatty acids. It is important to distinguish the role of inflammatory responses in eliminating bacteria from inflammatory processes that may lead to secondary harmful effects during septic shock

The mechanism of clearance is determined by the interaction of type-specific antibodies (IgA, IgM, IgG), complement, and neutrophils or phagocytic cells from lung, liver and spleen. The rates of bacterial acquisition and carriage depend on age, geographical area, genetic background and socioeconomic conditions. A poor mucosal immune response

might lead to persistent and recurrent colonisation and consequently infection, whereas a brisk local immune response involving phagocytes (neutrophils and macrophages), B cells (antibodies against pneumococcal polysaccharides and proteins) and T cells, to the pathogen will eliminate colonization and prevent re-colonization (Bogaert, De Groot et al. 2004).

For the clearance of bacteria the host needs to improve the AM function, excess of the extracellular fluid, and recruitment of neutrophils and monocytes to increase phagocytosis. The innate immune system recognizes pathogens by sensing so-called pathogen-associated molecular patterns via its pattern recognition receptors (PRRs) on phagocytic cells, mainly macrophages and neurtrophils. Binding of microbial ligands triggers signalling cascades in the host cell that activate phagocytes to internalize and destroy the invaders and to express pro-inflammatory mediators such as cytokines that activate inflammatory partner cells, and chemokines that attract them to the site of infection.

The best studied innate immune receptors are the Toll-like receptors (TLRs). Cell-wall components, lipotheichoic acid and peptidoglycan of pneumococci are recognized by Toll-like receptor 2 (TLR2, acting together with the pattern recognition receptor membrane CD14 (mCD14)), pneumolysin via TLR4 and unmethylated CpG in the endosome via TLR9. Cross-linking of TLR2 triggers intracellular signalling that activates transcriptional regulators such as NFκB. NFκB expression results in production of proinflammatory cytokines (Koedel, Angele et al. 2003; Ku, von Bernuth et al. 2007). Nucleotide-binding oligomerization domain (Nod) receptors in the cytoplasma also bind to intracellular peptidoglycan and may modulate inflammation. (Opitz, Puschel et al. 2004).

Pneumococci that are not ingested and killed by neutrophils and macrophages during the inflammatory reaction in the alveoli may escape into the systemic circulation, causing bacteremia (Austrian 1981). The major immune mechanism of pneumococcal clearance from the blood protecting the host against infection is the opsonization of pneumococci by type-specific antibody (either IgG or natural or aquired IgM) (Guckian, Christensen et al. 1980). Any deficiences in either part of this system result in a predisposition to pneumococcal infection (Guckian, Christensen et al. 1980; Johnston 1981; Ram, Lewis et al. 2010; Shriner, Liu et al. 2010). In adults, antibodies to pneumococcal polysaccharides are predominantly of the IgG2 subclass. In children, however, antibodies

to the pneumococcal polysaccharide are predominantly of the IgG1 subclass (Freijd, Hammarstrom et al. 1984). The antibodies are induced by infection, by colonization, and by vaccination with polyvalent pneumococcal vaccine. Antibodies have a variety of mechanisms of action: blocking the function of the target antigen by e.g. inhibiting an enzymatic activity or adhesion of a pathogen to host cells, neutralization viruses and secreted factors such as toxins, binding to surface located antigens leading to opsonisation and Fc-receptor mediated phagocytosis via professional phagocytes, activation of the complement cascade and cell mediated cytotoxicity.

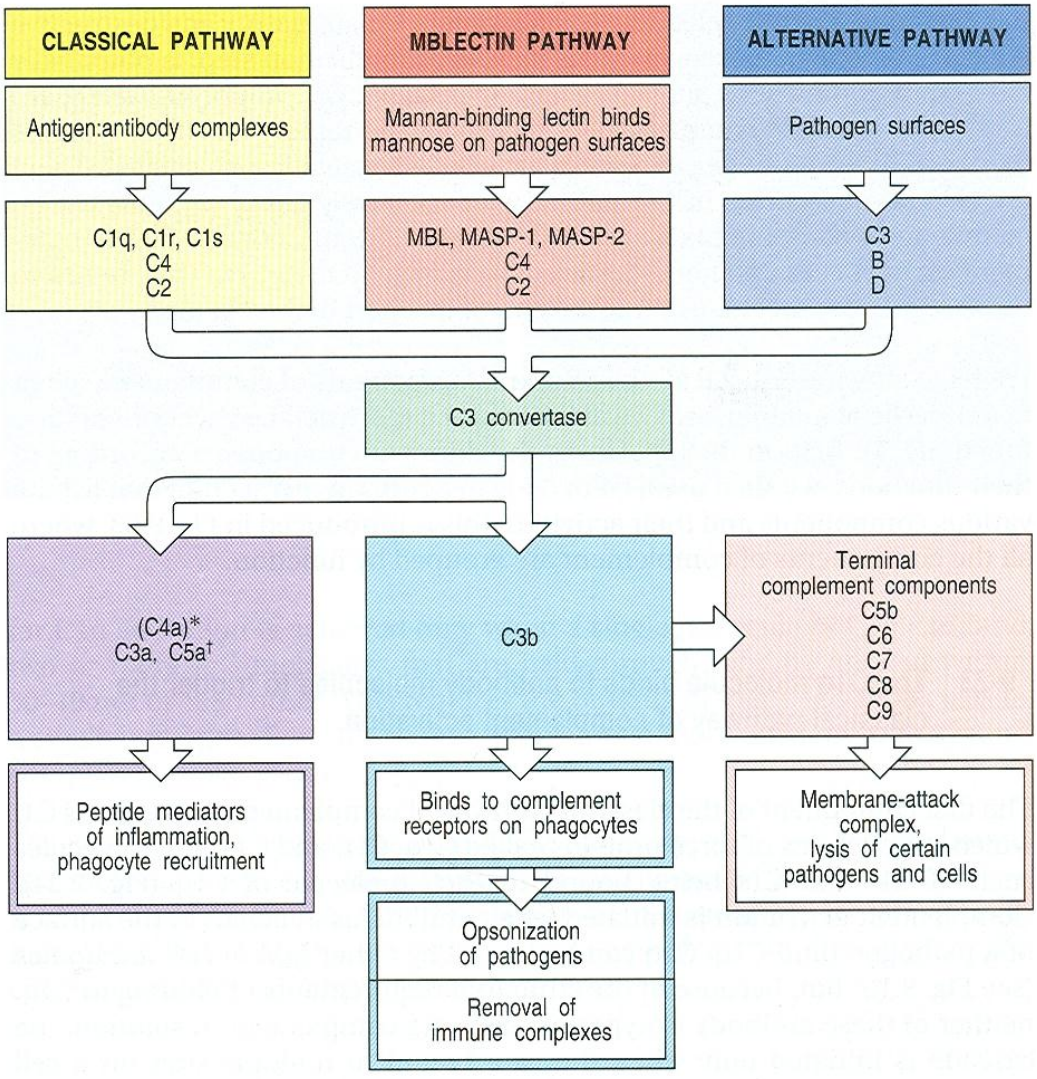

Figure 5: Shematic overview of the complement system (Murphy, Travers et al. 2008)

Pneumococci are *in vitro* able to activate both the classical pathway and the alternative pathway (Figure 5) of the complement system; this results in both cases is the

binding of C3b fragments to the bacteria (Winkelstein 1981). The classical pathway is activated by pneumococci covered with antibodies specific to bacterial proteins on the cell surface, of either the IgM or the IgG class but not in the absence of such antibodies (Mitchell, Andrew et al. 1991; Ren, Szalai et al. 2003). Activation of the alternative pathway of the complement system by pneumococci takes place *in vitro* in the absence of type-specific antibody (Winkelstein, Shin et al. 1972). Cell wall also activates complement via the lectin-binding pathway.

Role of T-cells on pneumococcal infection is clarified as well. T-cells are recruited to the lungs of mice that are infected intranasal with *S. pneumoniae*. Th1 cells improve the inflammatory response and the macrophage activation. Th2 plays role in antibody response and Th17 facilitates recruitment and activates mucosal antimicrobial peptides. $CD4^+$ T cells contribute to early host resistance to infection, as shown by an early rapid increase in T-cell infiltration to areas that are subject to increased pneumococcal invasion. In this case pneumolysin is responsible for the pattern of T-cell infiltration (Kadioglu, Gingles et al. 2000; Kadioglu, Coward et al. 2004). In another example, human T-cell responses are described to PspA following natural exposure (Baril, Dietemann et al. 2006). Mucosal immunization with whole cell vaccine, cell wall polysaccharide or pneumococcal proteins induces an antibody and serotype independent protection against colonization that is dependent on IL-17A producing CD4+ T-cells in mice (Malley, Trzcinski et al. 2005; Basset, Thompson et al. 2007). $CD4^+$ T cells are required for efficient clearance of nasopharyngeal pneumococcal colonization in naive mice, as $CD4^+$ T-cell-deficient mice failed to clear pathogen colonization (van Rossum, Lysenko et al. 2005) and mucosal antibody production against pneumococcal proteins, such as pneumolysin and CbpA, was T-cell dependent (Zhang, Bernatoniene et al. 2006). It has to be considered that future vaccines should include conserved pneumococcal protein antigens that are capable of inducing $CD4^+$ T-cell immunity.

The spleen and liver are the major organs involved in the clearance of intravascular microorganisms (Rogers 1960). Complement opsonization is required for effective hepatic clearance (Friedman and Moon 1980), splenic clearance of pneumococci continues in the absence of complement (Brown, Hosea et al. 1981).

Prevention and vaccine

Current pneumococcal vaccines are exclusively targeted on the capsular polysaccharide (CPS) of *Streptococcus pneumonia*, and these vaccines provide strictly serotype-specific protection. Given the more than 90 different capsular types of pneumococci, a comprehensive vaccine based on polysaccharide alone is not yet feasible. Thus, vaccines based on a subgroup of highly prevalent types have been formulated. The adult vaccine formulation is comprised of polysaccharides purified from the 23 most prevalent serotypes, and these stimulate B cells to produce protective antibodies. The number of serotypes in the vaccine has increased from four in 1945, to 14 in the 1970s, and finally to the current 23-valent formulation (25 mg of each of serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F). These serotypes represent 85-90% of those that cause invasive disease, and the vaccine efficacy is estimated at 60% (Hausdorff, Feikin et al. 2005).

In vaccine formulations designed for infants and young children with relatively immature B cells, polysaccharides and oligosaccharides from seven to 13 of the most prevalent types are chemically linked to carrier proteins. These conjugates activate T cells to provide sufficient immunological help to elicit antibody production, and to stimulate immunological memory. In the United States, a heptavalent pneumococcal conjugate vaccine (PCV7 representing serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F with diphtheria CMR_{197} protein, from Pfizer Inc.) has been recommended since 2000 for all children aged 2-23 months and for at-risk children aged 24-59 months. Since 2009 6 more serotypes were added (Prevnar 13) to create a broader protection. The four-dose series is given at 2, 4, 6 and 12-14 months of age. Protection is seen against invasive pneumococcal infections, especially septicemia and meningitis. In 2009 another pneumo-vaccine, Synflorix (GlaxoSmithkline) appeared on the European market. It is a conjugate vaccine as well, against 10 serotypes with non-typeable Haemophilus influenza protein D conjugate, with three doses added at 2-4-6 months of age with a booster dose at 11-18 (Wysocki, Tejedor et al. 2009). However, children exposed to a serotype not contained in the vaccine are not afforded any protection. This limitation and the ability of capsular-polysaccharide conjugate vaccines to promote the spread of non-covered serotypes, led to research into vaccines that would provide species-wide protection (Whitney, Farley et al. 2003).

Antimicrobial treatment that inhibits susceptible strains provides a selective advantage to resistant strains or species. Likewise, vaccine-induced immune responses that reduce carriage of certain pneumococci will provide an advantage to their competitors, whether these are pneumococcal strains that do not carry epitopes against which the vaccine is directed or other species.

In the US the PCV7 vaccine had a profound effect on the proportion of vaccine and nonvaccine serotypes recovered from patients, but did not change the percentage of vaccinated or nonvaccinated children who carry any serotype of *S. pneumoniae* in the nasopharynx (Frazao, Brito-Avo et al. 2005; Garbutt, Rosenbloom et al. 2006). Since the carriage rate has remained stable among vaccinated children, and there is a competition between different pneumococcal serotypes to colonize hosts, the proportion of nonvaccine serotypes that are potentially capable of causing disease has increased (Mbelle, Huebner et al. 1999).

Another problem is the potential of pneumococci to change their capsular locus, and hence their serotype, through recombination. This may favour the emergence of successful strains, originally of vaccine serotype, that have acquired a nonvaccine capsule through serotype switching (Coffey, Enright et al. 1998). The impact of these processes on disease will depend on the ability of the recombined strains to cause disease, which is largely unknown (Hanage, Kaijalainen et al. 2005).

Protein based vaccines

Due to the limitations of current polysaccharide-based pneumococcal vaccines (see above) there is an urgent need to develop alternative pneumococcal vaccines that do not suffer from the shortcomings seen with polysaccharide-based pneumococcal vaccines. The most promising approach to date is to develop protein based vaccines with antigens that contribute to virulence and are common to all serotypes. The T-cell dependent protein candidates should have no tendency to spontaneous mutation, as antigens they should be highly immunogenic and elicit immunological memory in all age groups including children below two years and elderly, should be cheap to produce, should provide a serotype independent protection and no replacement phenomenon should occur.

To design the best possible combination of protein antigens for a subunit vaccine a systematic search for protective pathogen-specific antigens is inevitable. Traditionally vaccine antigens were chosen based on their role in virulence and pathogenesis. Several candidates have been identified, including non-toxic derivatives of pneumolysin (Paton, Lock et al. 1983) , choline-binding proteins, such as PspA (Wu, Nahm et al. 1997), and CbpA (Balachandran, Brooks-Walter et al. 2002), metal-binding lipoproteins, such as PsaA (Talkington, Brown et al. 1996; Seo, Seong et al. 2002), PiaA and PiuA (Jomaa, Terry et al. 2006), the poly-histidine triad proteins PhtB and PhtE (Adamou, Heinrichs et al. 2001) and the neuraminidase NanA (Simell, Jaakkola et al. 2006).

As genomic extension of this strategy, *in silico* predicted, surface located proteins from the available genome were subjected to validation as vaccine candidates (reverse vaccinology). Several hundred of recombinant proteins were tested in animal models of protection to identify vaccine antigens first from *Neisseria meningitides*, and later on from other important pathogens (Montigiani, Falugi et al. 2002; Adu-Bobie, Capecchi et al. 2003). Mouse immune sera against the recombinant proteins are used in Western blot analysis of total bacterial lysates by two-dimensional electrophoresis and sera derived from exposed individuals identifies immunogenic proteins that are applied directly to peptide sequencing and bioinformatics for gene identification (Covert, Spencer et al. 2001; Montigiani, Falugi et al. 2002). Phage display technologies are widely used in combination with other selection methods (e.g. human serology, binding to host molecules) to combine them in an expression library of polypeptides of different size (Santamaria, Manoutcharian et al. 2001; Mintz, Kim et al. 2003).

Intercell has developed a novel method, named ANTIGENome technology that combines the advantage of full genome coverage and serological antigen identification using human immunogenicity as primary screening and selecting parameter. Briefly, the complete genome of the pathogen is expressed in the context of *E. coli* outer membrane proteins LamB and FhuA as peptides on the bacterial surface. The libraries are exposed to selection with pathogen-specific human antibodies derived from well characterized donor sera (Etz, Minh et al. 2001; Etz, Minh et al. 2002; Henics, Winkler et al. 2003). Antibodies in serum and other body fluids represent the immunological fingerprint the pathogen induces *in vivo.* The identified antigens are further validated using a comprehensive strategy to retrieve the most promising candidates for vaccine development from the

'ANTIGENome' (Meinke, Henics et al. 2005). Selected candidate proteins are expressed in recombinant form and analyzed for protection in animal models.

Two of the three lead candidates for Intercell´s pneumococcal vaccine are StkP and PcsB. StkP (annotated as SP1732 in the TIGR4 pneumococcal genome), a eukaryotic type serine/threonine protein kinase was identified as one of the most promising vaccine candidates. StkP was found to be exceptionally conserved among clinical isolates (>99.5% identity) and cross-protective against different pneumococcal serotypes in murine lethal sepsis and pneumonia models. It is transcribed from the same operon as its corresponding phosphatase PhpP (Novakova, Saskova et al. 2005). The presence of specific antibodies in human sera suggested that StkP was expressed during pneumococcal disease and colonization. Based on our previous studies and also others' with gene deletion mutant strains, StkP seems to have important non-redundant functions in bacterial multiplication (Echenique, Kadioglu et al. 2004; Giefing, Meinke et al. 2008). In addition to the dramatic decrease in *in vitro* growth and virulence we observed, electron microscope analysis revealed a rather elongated cell shape instead of cocci in the *ΔstkP* mutant strain (Giefing, Meinke et al. 2008) Deletion of *stkP* results in a phenotype more sensitive to various stress conditions (including elevated temperature, oxidative stress, osmotic pressure and acidic conditions) and was also reported to play a regulatory role in gene transcription (Saskova, Novakova et al. 2007). Although serine/threonine kinases have been identified in a wide range of bacterial species, so far no common regulatory function in a specific biosynthetic pathway has been described for the homologous proteins.

PcsB (annotated as SP2216 in the TIGR4 genome) plays a major role in the bacterial life cycle, is essential for pathogenicity of *S. pneumoniae* and for proper cell division. Lacking PcsB causes antibiotic sensitivity, growth defects, enhanced protein secretion and altered morphology, affecting the cell wall composition (Ng, Kazmierczak et al. 2004). Deletion mutant cells displayed cluster formation due to aberrant cell division, reduced growth and antibiotic sensitivity that were fully reverted by transformation with a plasmid carrying *pcsB* (Giefing-Kroll, Jelencsics et al. 2011). PcsB is localized at the cellular poles in the early logarithmic growth phase, excluded from the septum in a similar manner as PBP3 and LytB, which are reported to be required for daughter cell separation. Its potential role in organizing cell division is hydrolyzing substrates of PBPs, interacting with SP0437, FtsZ and SP1772. In addition to defects in septum placement and separation,

absence of PcsB induced increased release of several proteins, such as enolase, MalX and SP0107 LysM domain protein (Giefing-Kroll, Jelencsics et al. 2011).

Therapy

The morbidity and mortality of infections caused by *S. pneumoniae* remain high despite appropriate antibiotic therapy. Since 1940, penicillin has been the drug of choice for the treatment of pneumococcal infections. Efforts aimed at identifying new antibiotics were once top research and had priority among pharmaceutical companies. The potent broad spectrum drugs provided extraordinary clinical efficacy, but the success, however, has been compromised.

The first clinical isolate resistant to penicillin was described in 1967, it was recovered from a patient in Papua New Guinea (Hansman, Glasgow et al. 1971). The tremendous increase in antibiotic usage worldwide has strongly contributed to the emergence of multidrug resistant pneumococci. The alarming event in the epidemiology of antibiotic-resistant pneumococci was the outbreak in 1977 in South Africa of pneumococcal diseases caused by multidrug-resistant strains (Klugman and Koornhof 1988). These strains were found to be tolerant to primarily against β-lactams (penicillin - with a 1,000-fold increase of the minimal inhibitory concentration) and macrolides (erythromycin) and to clindamycin, tetracycline and chloramphenicol as well. The incidence of resistance to penicillin increased from <0.02 in 1987 to 3% in 1994 to 30% in some communities in the United States and 80% in regions of some other countries in 1998 (Breiman, Butler et al. 1994). Adjunct or new ways of therapies are urgently needed.

The antibody-based therapy

The reintroduction of antibody-based therapy is an option that should give serious consideration. The first serum therapy was introduced in the 1890s for the treatment of diphtheria, and it was in 1891, when Klemperer´s protected rabbits with immune serum against pneumococcal infection (Klemperer and Klemperer 1891). At the beginning of the next century (usually animal) serum was widely used against bacterial (as pneumonia, meningitis, scarlet fever) and viral infections (as measles, mumps, and poliomyelitis). The efficacy of serum therapy varied with the type and severity of infection. In some cases (diphtheria) the antibody therapy continues to be used today as well. When sulphonamides were introduced in 1935, antimicrobial therapy became the standard therapy for many infections. Serum was expensive, difficult to use, caused "serum-sickness" (a syndrome characterized by rash, proteinuria and arthralgias in significant percentage of the patients) and was less efficient than sulphonamides. But for certain diseases (e.g. scarlet fever and pneumococcal pneumonia) combination of serum and chemotherapy gave good results: sulphonamides made pneumococci more susceptible to antibody-mediated phagocytosis (reviewed in (Casadevall and Scharff 1995). However the side effects of serum made the potential benefits of combination therapy marginal. The use of antibody therapy is now limited to viral and toxin neutralization and replacement therapy in patients with immunoglobulin deficiencies.

By elucidating Gram-positive virulence factors and the host defenses they exploit, novel therapeutic targets for treatment or prevention of invasive human are revealed. These novel treatments envision alternatives to direct microbial killing, such as blocking disease progression by neutralizing specific virulence factors, boosting key innate immune defenses or pharmacologic augmentation of host innate immune defenses, and could be used in conjunction with classical antibiotic or vaccine strategies (Nizet 2007).

Adaptive immunity to extracellular bacteria, such as the *S. pneumoniae*, is largely conferred by antibody. Antibodies specific for both bacterial polysaccharide and protein antigens was shown to protect the host from infections with otherwise lethal pneumococcal strains (AlonsoDeVelasco, Verheul et al. 1995). Antibody binding to the bacterial surface can activate complement either through the classical (IgM and IgG) or alternative (IgA) pathways. Subsequent binding of antibody and C3b to Fc and C3b receptors expressed by

phagocytic cells results in opsonophagocytosis and rapid killing of ingested bacteria. The associated generation of other complement fragments can indirectly mediate protection by enhancing other aspects of the innate response, as well as adaptive immunity (Pepys 1974; Winkelstein 1981).

Monoclonal antibodies

In 1975 Kohler and Milstein (Kohler and Milstein 1975) succeed in generating mouse monoclonal antibodies, the "magic bullets", first envisaged by Paul Ehrlich 80 years earlier establishing the hybridoma technology. Monoclonal antibodies, derived from the progeny of a single immune cell, are pure and available in potentially unlimited quantities. Since then a prodigious number of different monoclonal antibodies have been generated, they have been exploited in almost every branch of biomedical research. Novel techniques of genetic engineering and expression are now available to reduce the immunogenicity of rodent mAbs in humans, minimizing the side effect of serum therapy. The classical hybridoma technology first was difficult to apply on human cells because human hybridomas or immortalized cell lines do not produce high levels of antibody and *in vivo* immunization of humans is not feasible for many antigens. Mouse-human chimerics started to be expressed with murine variable regions joined to constant regions from human of humanized mAbs. A few attempts were tested, however human anti-chimeric antibody responses were observed (Bell and Kamm 2000). By protein engineering humanized antibodies can be generated, where only the complementarity-determining regions (antigen binding loops) are integrated into a human variable framework, together with murine key framework residues that support the conformations of the loop (Jones, Dear et al. 1986). Fully human antibodies can be produced by phage display or in genetically engineered mice, in which the native immunoglobulin repertoire is replaced with human genes. Immunization of such transgenic mice results in a human antibody response, from which hybridomas that produce human antibodies can be generated. (McCafferty, Griffiths et al. 1990; Morrison 1992; Lonberg 2008; Altshuler, Serebryanaya et al. 2010). Phage antibody technology can successfully mimic the immune system by cloning large libraries of antibody genes and selecting for binding to a desired target. Once a library is made, it can

be propagated and used repeatedly to isolate antibodies against numerous antigens. The library can be generated from immunized donors (immune library), from non-immunized donors (naïve library) or it can be created of fully synthetic repertoires, which has the potential of encoding antibodies to self-antigens. The screening of antibody libraries enables the isolation of specific antibody clones and engineering of the antibodies with high affinity. The most widely used technique for library screening is based on the display of antibodies on the surface of filamentous bacteriophages (Hoogenboom, de Bruine et al. 1998), on the surface of microbial cells or on a ribosome display (reviewed in (Kim, Park et al. 2005).

There are five classes of immunoglobulins: IgM, IgG, IgE, IgA and IgD. From a biotechnology perspective IgG is the most important class of antibodies. The human IgG1 is the most efficient in complement-dependent cellular cytotoxicity and in antibody-dependent cellular cytotoxicity, and therefore the most suitable for therapeutic use against pathogens or tumor cells. Functionally, an antibody comprises of into two antigen-binding fragments (Fabs) and a constant (Fc) region, which are linked via a flexible hinge region. The variable regions (Fabs) are loosely tethered to the mobile Fc region, the elbow angle can be adjusted as the function requires. To allow the Fab to drift from the Fc the hinge polypeptides are just tethered instead of attached. Fab and Fc needs this freedom of flexibility, since monoclonal antibodies often achieve their therapeutic benefit through two binding events: the variable domain of the antibody binds a specific protein on a target cell, and this is followed by recruitment of effector cells that bind to the Fc (Harris, Larson et al. 1992).

Better penetration characteristic and better clearance from the whole body can be observed with antibody fragments such as Fab, scFv, diabodies and minibodies. These are easier to produce in bacteria in large amounts and are therefore considered to bypass the hurdles associated with mammalian cell based production of whole antibodies (Kim, Park et al. 2005).

Bacterial components that are targeted by anti-infective mAbs should be expressed during human disease, accessible (surface bound or secreted) for functional antibodies and/or effector immune cells, conserved among strains and clinical isolates and protective in animal models mimicking human disease (Nagy, Giefing et al. 2008).

Monoclonal antibody therapy against the S. pneumonia

After analyzing Intercell's *in vitro* validation data and testing the proteins for protection in both sepsis and pneumonia models, seven highly conserved vaccine antigens were identified (StkP, PcsB, PspA, SP0609, SP2027 and SP2194), that could also serve as targets for monoclonal antibodies.

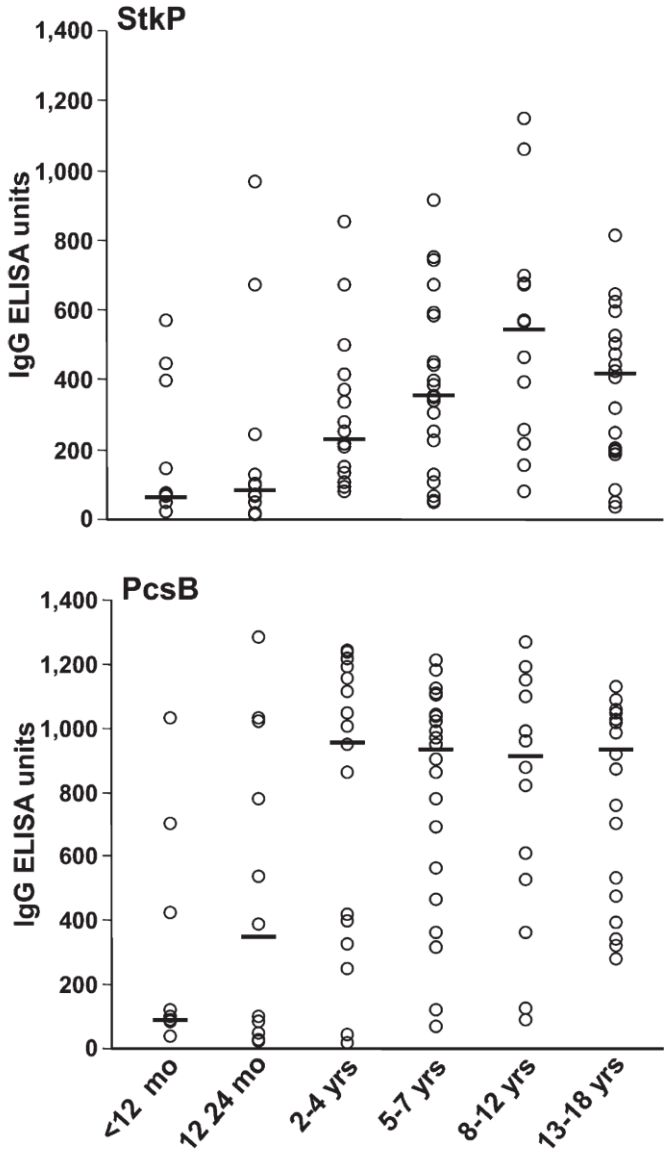

Figure 6: StkP and PcsB antigens are immunogenic in young children. Sera obtained from healthy children were analyzed for anti-pneumococcal IgG levels. Data are expressed as ELISA units (absorbance at 405 nm) at 1:1,000 serum dilutions; horizontal lines represent medians (Giefing, Meinke et al. 2008)

Evidence for that was obtained by testing rabbit and mouse sera in protection against pneumococcal infection. The total specific IgG levels detected in sera from healthy children against PcsB and StkP are relatively high above 12 months and increases with age (Giefing, Meinke et al. 2008) (Figure 6).

We found that transfer of rabbit immune sera specific for StkP-PASTA1–4 protected most of the animals from septic death (Fig. 7).

Specificity was confirmed by the loss of protection upon addition of recombinant StkP-PASTA1–4 to the serum before passive immunization (Giefing, Jelencsics et al. 2010).

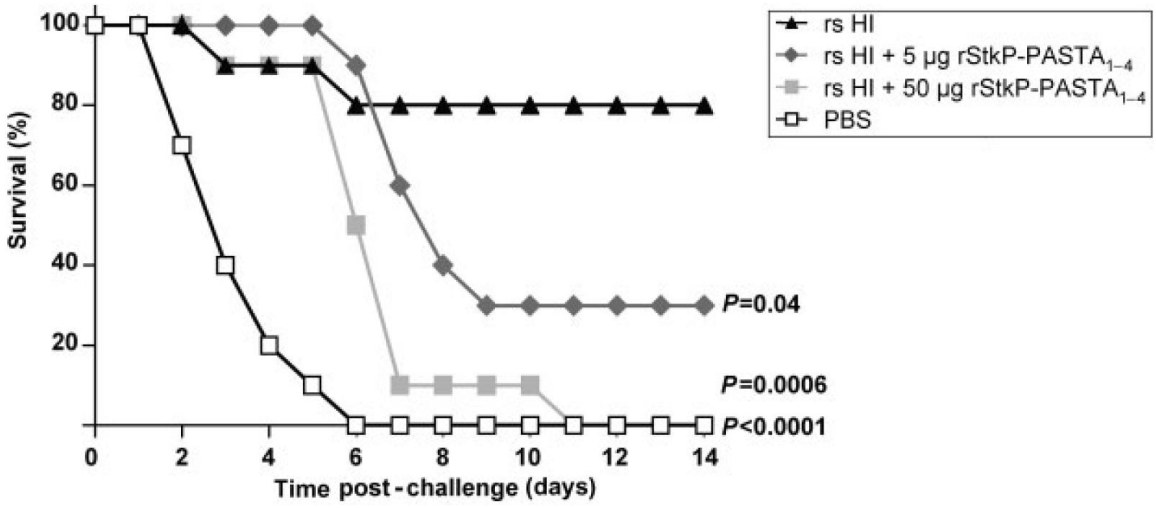

Figure 7: Recombinant StkP blocks protection of anti-StkP hyperimmune serum. Mice received rabbit hyperimmune sera (rs HI) generated with StkP PASTA1– 4 or PBS (negative control) prior to lethal challenge with strain PJ1324 intra-peritoneal and were monitored for 14 days. To neutralize StkP-specific antibodies, sera were pre-incubated with recombinant protein StkP-PASTA1–4 before administration, as indicated (Giefing, Jelencsics et al. 2010).

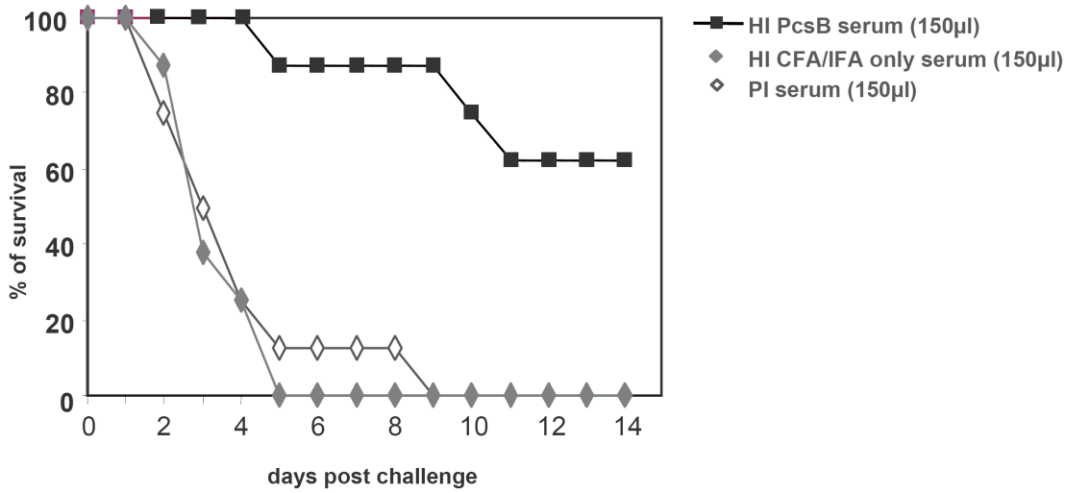

Figure 8: Anti-PcsB serum protects against pneumococcal infection. Mice received rabbit immune sera (HI) generated by immunization with recombinant PcsB with CFA/IFA, or with adjuvant only (negative control), and pre-sera prior to lethal challenge with strain PJ1324 intra-peritoneal and were monitored for 14 days (Giefing, Meinke et al. 2008).

The same pronounced protection was seen when C3H mice were injected with immune sera specific to PcsB against pneumococcal infection (Figure 8). These facts lead us to the conclusion that antibodies against these vaccine antigens might protect against *S. pneumoniae* and thus be a candidate for prevention or therapy based on monoclonal antibodies.

Aims

During my PhD I focused on the characterization of conserved pneumococcal surface proteins with the aim to develop a protein-based vaccine and an antibody-based therapy.

The specific aims are the followings:

- To analyse the gene expression pattern in Δ*stkP* (SP1732) and Δ*pcsB* (SP2216) pneumococcal strains in order to understand their role in the bacterial cell cycle. These two antigens are the main candidates of a protein-based vaccine against *S. pneumoniae.*

- To characterize the SP0609, SP2027 and SP2194 antigens that were selected as potential vaccine candidates, with the main focus on SP0609. Generation of gene deletion mutants lacking the three proteins (individually) and investigate their phenotype *in vitro* and virulence in mice (pneumonia and sepsis model).

- To study the potential of monoclonal antibodies against these proteins, by setting up animal models (pneumonia and sepsis) to assess the protection of murine monoclonal antibodies targeted the above mentioned pneumococcal antigens. Mapping the epitopes of the protective antibodies and elucidating the mode of action (neutralization and/or opsonisation).

Materials and methods

Bacterial strains and culture conditions

TIGR4 (serotype 4) and PJ1324 (serotype 6B) strains were provided by Birgitta Henriques-Normark (Swedish Institute for Infectious Disease control), EF3030 (serotype 19F) and WU-2 (serotype 3) by David Briles (University of Alabama, USA) and 4DS2341-94 (serotype 4) strain by Eddie Ades (CDC, USA). All strains were cultured in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) at 37°C in an atmosphere of 5% CO_2.

Generation of pneumococcal gene deletion strains. Gene deletion strains were generated by competence stimulatory peptide – induced transformation of *S. pneumoniae* strains TIGR4 and PJ1324. The gene-replacement cassettes — the kanamycin gene surrounded by a 1-kb flanking regions of the target genes — were generated by ligation-mediated PCR. For transformation, 2×10^6 pneumococci from the logarithmic phase of growth were incubated in competence medium with 0.5 μg of PCR-generated linear DNA for 3h at 37°C. Transformants were selected on blood agar containing 250 μg/ml kanamycin (Kan). Mutant strains were verified first by PCR amplification of the altered genomic region using primer combinations specific for flanking and integrated fragments. Southern blot analysis confirmed integration into the expected chromosomal region, and Western blot proved the absence of protein expression. Bacteria were cultured in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) including the appropriate antibiotic for mutant strains (Kan).

Complementation of the Δ*pcsB* strain. For complementation extra-chromosomal expression of SP0609 from a plasmid was chosen. Briefly the complete SP0609 gene was cloned into the multiple cloning site of pMU1328 with a fucose-inducible promoter (Chan, O'Dwyer et al. 2003) via EcoRI and XbaI. The plasmid was transformed as described above and erythromycin and kanamycin resistant clones were selected for further analysis.

In vitro assays

RNA isolation. Bacteria grown in THY medium were harvested in the exponential phase (OD 620 nm of ~ 0.4) and total RNA was isolated using the RNeasy Midi Kit (QIAGEN) according to the manufacturer's instructions, performing on-column DNase digestion using the RNase-free DNase set (QIAGEN). The integrity and purity of the RNA was verified by agarose gel electrophoresis, determining 260/280 optical density ratios and performing RT-PCR using the Superscript III First-Strand Synthesis system (Invitrogen) and random hexamers in the standard protocol.

Analysis of changes in mRNA levels. Microarray analysis was performed at Eurogentec (Seraing, Belgium) using Cy3 and Cy5 labelled samples on a *S. pneumoniae* MicroArray (Eurogentec, 2085 ORFs of S. pneumoniae (TIGR4) including data processing and normalization (*S. pneumoniae* Hybridization Service package). Based on these data genes that showed changes of at least 2 fold in mRNA expression were selected for RT-qPCR. qPCR analyses were performed using the LightCycler 480 SYBR Green I Master (Roche). mRNA expression of the selected genes was analyzed in three independent RNA isolations from TIGR4 and PJ1324 wild-type, isogenic *ΔpcsB* and *ΔstkP* strains using gene-specific internal primers. Quantitative differences for each sample were determined using the $2(-\Delta\Delta C_T)$ method (Livak and Schmittgen 2001).

Western blot. Proteins were separated on 4-20% gradient SDS-PAG under reducing conditions and blotted to nitrocellulose membranes using a dry-blotting system (Invitrogen). Blocking and incubation with primary (1:1,000 dilution) and HRP-conjugated secondary antibodies (1:5,000 dilution) was done in 5% milk in PBS containing 0.1% Tween20 for 1 hour at room temperature. Membranes were imaged with chemiluminescent substrate (Chemiglow, Alpha Innotech) and a CCD camera (FluorChem SP, Alpha Innotech).

ELISA. ELISA was performed according to standard protocols (Dryla et al., 2007). Recombinant proteins (1 mg/ml in carbonate buffer) were coated on Maxisorb plates (Nunc) and human sera were used in a 1,000–5,000-fold dilution range. For peptide ELISA biotin-tagged 20 aa long peptides (overlapping by 5 aa) were coated on streptavidin pre-coated ELISA plates (Nunc) at a concentration of 1 mg per ml in PBS and tested with human sera diluted 1:200–1:1,000.

Surface staining of bacteria. Bacteria (4DS 2341-94 and capsule-negative T4) from the early logarithmic growth phase were collected and washed twice in HBSS. Approximately $5x10^5$ CFU in 100 µl HBSS, 2% BSA were incubated with mouse or rabbit sera at 0.5 and 2% final concentrations or with monoclonal antibodies (5µg-1.25µg) for 60 min at 4°C before detection with PE-conjugated goat anti–rabbit IgG (Beckman Coulter) antibodies. Cells were stained with SYTO® 60 (0.05 µl/100 µl HBSS) for 10 min at 4°C. After fixation with 2% paraformaldehyde overnight, surface staining was detected by a flow cytometer (Cytomics FC500; Beckman Coulter), and data were analyzed using analysis software (FCS Express).

OPK assays. *S. pneumoniae* strain PJ1324 was grown until an OD_{620nm} of 0.3 was reached. Bacteria were washed twice with HBSS containing 0.125% BSA and diluted to $3x10^3$ cfu/40 µl. For the opsonization of the bacteria, polyclonal rabbit anti-PspA serum was used in dilutions 1:300, 1:600 and 1:900 and monoclonal anti-PspA antibodies clone 7E6D9F4 and clone 3G3E8E3 were used at an amount of 9 µg and further tested in 3 fold serial dilutions down to 0.012 µg. As complement source 4% baby rabbit complement was used. Opsonization was carried out in a total reaction volume of 100 µl for 30 minutes at room temperature. Differentiated HL60 cells were washed twice with HBSS supplemented with 2 mM glucose, stained with trypanblue to evaluate the percentage of viable cells. Next, $1.2x10^6$ cells per 50 µl were added to the OPK reaction to a final volume of 150 µl. $3x10^3$ bacteria and $1.2x10^6$ differentiated HL60 cells were used to obtain a ratio of bacteria to HL60 of 1:400. For phagocytosis HL60 cells and opsonized bacteria with complement were incubated for 60 minutes at 37°C and shaking at 750 rpm. Controls such as the effect of active versus inactive complement were included. After phagocytosis the reaction was diluted 1:4 and plated on blood agar plates so that ~17% of the initial reaction was plated.

Additionally, to elucidate the contribution of bacterial growth during the experiment, serial dilutions of bacteria after 30 minutes (after opsonization) and 1.5 hours were also plated.

Adhesion assay. Monolayer of nasopharyngeal cells (Detroit 562, approx. 10^8) were incubated in NucleonΔSurface plates (24 wells) with *S. pneumoniae* strains TIGR4 or PJ1324 in Eagle´s Minimal Medium (supplemented with 10% FCS, 1% MEM non-essential aminoacids, 1% sodium pyruvate, 50 µM β-mercaptoethanol) at 37°C in an atmosphere of 5% CO_2 for 2 hours. The wells were washed two times with HBSS and incubated with 100 µl, 1% saponin at room temperature for 5 minutes. The cells were suspended in 900 µl HBSS and serial dilutions were plated onto blood agar plates (BioMerieux). After overnight incubation at 37°C (5% CO_2) colonies were counted and data were expressed as the percentage of colony forming units of the deletion mutant or the pre-incubated strains compared to the wild type (set to 100%). When indicated, before the incubation with the Detroit cells bacteria were preincubated with monoclonal antibodies at RT for 30 minutes.

In vivo assays

Bacterial challenge experiments in mice. All animal experiments were carried out according to Austrian Law (BGB1 Nr. 501/1989).

In pneumonia experiments (5 mice per group, RjOrl:SWISS), mice received 40 µl *S. pneumoniae* WU-2 strain intranasally under anaesthetising by Xylazin (2%, 4 mg/kg bodyweight) and Ketamin (10%, 100 mg/kg bodyweight). On day 3 mice were sacrificed, and lungs were removed. The homogenized lungs were diluted and plated on blood-agar plates. After overnight incubation at 37°C (5% CO_2) colony forming units were counted.

In sepsis experiment C3H/HeNRj mice were injected intraperitoneal with $1x10^3$ *S. pneumoniae* PJ1324 strain in a total volume of 100 µl. Depending of the experiment, mice were pre-immunized with monoclonal antibodies (in a total volume of 100 µl), 3 or 24 hours before the challenge. The animals were then monitored for 14 days, and data was expressed as survival curves. P values were calculated using the Mantel–Cox test.

Monitoring bacterial challenge with the IVIS Imaging System (Caliper LifeSciences). Immunization and challenge of the animals are made with the above mentioned setting of the pneumonia model, using *S. pneumoniae* EF3030 and XEN11 strain (the latter one is a product of the Caliper LS). The monoclonal antibodies used in these experiments were labelled with the XenoLight CF680 fluorescent dye, according to the standard protocol of XenoLight. For the detection of the luminescence or fluorescence of the animals, a CCD camera was used (Spectral Instruments, 850 Series CCD camera), in the Imaging Chamber where the mice inhaled through a nose cone a constant 2.5% of isopropanol, in order to stay asleep. The picture were set and analysed by the Living Image 3.1 program.

Results

Gene expression studies of StkP and PcsB

Differences of the gene expression in the stkP knock-out strain compared to the corresponding wild type. StkP was previously described to play an important role in virulence and competence of pneumococcus (Giefing, Meinke et al. 2008), being a global regulator of gene expression in *S. pneumoniae* affecting approximately 4% of the pneumococcal transcriptome when comparing gene deletion mutants and wild type (WT) strain *in vitro* (Saskova, Novakova et al. 2007). Such effects may depend on the genetic background as we have seen differences in growth rate and virulence.

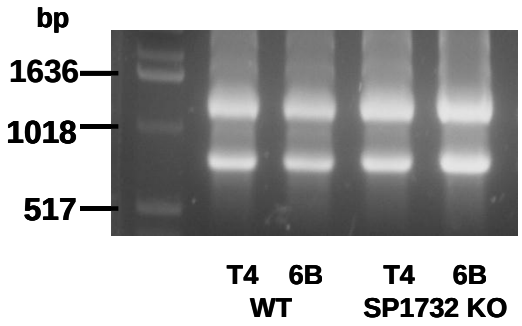

Figure 9: Pneumococcal total RNA samples run on an agarose gel. Quality of the isolated RNA was tested by running the samples on an agarose gel.

To learn more about the interaction partners of StkP we decided to perform microarray analysis. The T4 and 6B WT and StkP deletion mutant strains were cultivated in THY until logarithmic growth phase (OD 620nm ~ 0.4) and total RNA was extracted. The quality of the RNA was tested by electrophoresis (Figure 9) and by reverse transcription for DNA contamination (data not shown). As positive control FtsZ primers were used, both microarray and RT-qPCR analysis showed that the expression level of it is unaltered in the *stkP* mutant strain compared to the wild-type. All primers were designed to have melting temperature of 54°C, and to give a 100 bp product, randomly chosen from the middle of the genes.

In a total of three microarray experiments performed, we found around 500 genes showing altered transcription in the knock-out strains (T4 and 6B). To confirm these results

we selected 80 genes based on significance of the transcription changes (over 2 fold) for further testing with real-time PCR, which was done for each gene two times with both of the genetic backgrounds. Compared to a previous report (Saskova, Novakova et al. 2007;) we found a much smaller set of gene transcripts affected by the deletion of *stkP*. Out of all genes found by microarray and tested by real-time PCR 10 were up-regulated and 20 down-regulated in both the TIGR4 and 6B knock-out strains (

Table **1**). The mRNA of the following proteins were down regulated, LysM domain protein (SP2063), the glucoseamine-fructose-6-phosphate aminotransferase (GlmS, SP0266) and PcsB (SP2216), all three envolved in the cell wall biosynthesis and homeostasis. A beta lactam resistance factor (SP0615), a bacteriocin transport accessory protein (SP1499), competence protein (ComW, SP0018), which is essential for pneumococcal competence, proving the role of Stkp in genetic transformation (Echenique, Kadioglu et al. 2004), magnesium transporter, CorA family, iron-compound ABC transporter proteins (SP1869, SP1870, SP1871-1872) encoding the *piuBCDA* loci, most important iron transporters for iron acquisition during both *in vitro* and *in vivo* growth (Brown, Gilliland et al. 2002).

Upregulated independent from the genetical background were sortases of the pilus assembly (SP0467, SP0468), cell wall surface anchor family protein (SP SP0467), heatshock protein (SP0516) GrpE (SP0516) and DnaK protein (SP0517).

Moreover, in a strain specific manner, 18 genes were up-regulated and 13 down-regulated only in the TIGR4 strain; and 10 up-regulated and 7 down-regulated only in the 6B strain (Table 2), in 13 cases contradicting pattern was observed (which is upregulated in one, is downregulated in the other), confirming the striking differences among pneumococcal serotypes.

In addition, we compared our data set to previously published data by Saskova et al. (Saskova, Novakova et al. 2007), and altogether transcription of only three genes was connected to StkP independently of genetic background and growth conditions: *piuBCDA*, *pcsB* and the gene of the rhodanase-like protein (SP0095).

However, we could confirm that StkP is a key player in the pneumococcal cell cycle by regulating the expression of several important gene products, although effects of this regulatory function seems to be dependent on the genetic background to a great extent.

Table 1: Changes in relative transcript amounts caused by the lack of StkP, gene regulated in the same manner in both strains. Values represent fold changes in the Δ*stkP* strains relative to the wild-type strains. Genes down-regulated at least 2 fold in the Δ*stkP* strain are shown in green; genes up-regulated at least 2 fold are highlighted in red; n.a.: not applicable

ORF	Gene product	T4	6B
SP2063	LysM domain protein	-2.9	-1.49
SP0615	beta-lactam resistance factor	-2.56	-1.21
SP1499	bacterocin transport accessory protein	-2.47	-2.67
SP0095	rhodanese domain-containing protein	-2.53	-1.35
SP0266	glucoseamine-fructose-6-phosphate aminotransferase, glmS	-4.65	-3.28
SP0018	Competence protein (comW)	-1.35	-4.57
SP1655	phosphoglycerate mutase	-7.69	-1.75
SP0098	hypothetical protein	-3.23	-2.27
SP0099	hypothetical protein	-3.28	-3.28
SP0574	hypothetical protein	-2.13	-2.44
SP0185	magnesium transporter, CorA family	-2.86	-1.89
SP0626	branched-chain amino acid transport system II carrier protein	-5	-2.5
SP1869	iron-compound ABC transporter, permease protein	-2.86	-33.33
SP1870	iron-compound ABC transporter, permease protein	-7.69	-1.59
SP1871	iron-compound ABC transporter, ATP-binding protein	-8.33	-14.29
SP1872	iron-compound ABC transporter, iron-compound-binding protein	-5.56	-8.7
SP1588	pyridine nucleotide-disulfide-oxidoreductase, class I	-1.69	-2.56
SP2216	secreted 45kD protein	-6.67	-1.26
SP0253	glycerol dehidrogenase	2.33	1.34
SP0157	hypothetical protein	2.23	2.04
SP0461	transcriptional regulator, putative	23.86	6.89
SP0467	cell wall surface anchor family protein	160.77	5.9
SP0467	sortase, putative	38.9	5.17
SP0468	sortase, putative	n.a.	4.13
SP0516	heathock protein GrpE	6.61	5.15
SP0517	dnaK protein	n.a.	2.95
SP0233	ribosomal protein L36	7.9	2.94
SP1472	oxidoreductase, putative	4.58	11.05

Table 2: Changes in relative transcript amounts caused by the lack of StkP, with different effect of the deletion in the two strains. Values represent fold changes in the Δ*stkP* strains relative to the wild-type strains. Genes down-regulated at least 2 fold in the Δ*stkP* strain are shown in green, genes up-regulated at least 2 fold are highlighted in red.

ORF	Gene product	T4	6B
SP0107	LysM domain protein	-3.16	2.85
SP0149	lipoprotein	-3.17	3.43
SP0641	serine protease, subtilase family		-11.11
SP1081	UDP-N-acetylglucoseamine deacetylase A		2.82
SP1479	peptidoglycan N-acetylglucosamine deacetylase A		5.41
SP1883	dextran glucosidase DexS, putative	-3.92	24.4
SP0409	conserved hypothetical protein	-2.94	
SP0627	conserved hypothetical protein	-2.13	
SP1630	hypothetical protein	-1.32	
SP0281	aminopeptidase C	-2.7	
SP0701	orotidine 5´-phosphate decarboxylase		-2.82
SP1249	guanosine 5´-monophosphate oxidoreductase	-2.56	
SP1884	trehalose PTS system, IIABC components	-5.41	17.14
SP0148	ABC transporter, substrate binding protein	-3.64	5.36
SP0620	amino acid ABC transporter, amino acid-binding protein	2.78	
SP1578	methyltransferase, putative	-1.52	4.6
SP2072	glutamine amidotransferase, class-I	-5	
SP1409	oxygen-independent coproporphyrogen III	2.23	
SP0462	cell wall surface anchor family protein	2.1	
SP0463	cell wall surface anchor family protein	368.25	
SP1758	glycosyl transferase, group1	12.87	
SP0313	glutathione peroxidase	2.3	-2.9
SP1415	glucoseamine-6-phosphate isomerase	2.5	
SP2056	N-acetylglucoseamine-6-phosphate deacetylase	2.16	-2.9
SP1263	DNA topoisomerase I	5.25	
SP1161	acetoin dehydrogenase complex, E3 component	2.93	-2.13
SP2168	fucose operon repressor, putative	5.55	-2.53
SP1025	hypothetical protein	3.8	-8.33
SP1802	hypothetical protein, similar to FtsK/S polIIE family protein	2.4	
SP1429	peptidase, U32 family	1.3	
SP1179	ribonucleoside-diphophate reductase 2	2.46	-2.15
SP1648	manganese ABC transporter, ATP-binding protein	1.19	-2.5
SP1649	manganese ABC transporter, permease protein	1.78	
SP1840	ABC transporter, ATP-binding/permease protein	2.38	
SP2022	PTS sytem, II C component	3.95	

Differences of the gene expression in the PcsB knock-out strain compared to the corresponding wild-type serotype. PcsB has been suggested to have a critical role in cell division. PcsB deletion mutant strains displayed cluster forming phenotype, altered septum formation and retarded *in vitro* growth (Giefing-Kroll, Jelencsics et al. 2011; Barendt, Land et al. 2009) with an apparent differences in protein abundance in the culture supernatant of

wild-type and mutants, the most obvious increase being associated with enolase. In order to learn more about the role of PcsB in the pneumococcus, wild-type and PcsB knock-out strains were compered by microarray analysis. RNA isolation, and quality control, the microarrays and verifying the result by RT-qPCR was done in the same way as it was with SP1732. The control gene was FtsZ as well, after it was showed that the expression level of it is unaltered in the *pcsB* mutant strain compared to the wild-type.

The main difference we found was the greatly increased transcript levels for two LysM domain proteins SP0107 and SP2063, which play role in cell wall metabolism. A 14.2 ± 1.97 and 14.4 ± 3.38 fold increase in TIGR4 and a 26.4 ± 11.62 and 33.6 ± 7.98 fold increase in the 6B strain was observed, respectively (based on three independent biological replica of quantitative real-time PCR analysis). This strong up regulation in the expression might represent a compensatory mechanism for the bacterium lacking the PcsB protein.

There are few genes that are affected only to a lower extent (2-10 folds). The rrg pilus islet (SP0461, SP0462, SP0463, SP0464, SP0465, SP0466, SP0467, and SP0468) was found to be up regulated, furthermore a phosphate transport regulatory protein (SP2088), the stress protein GrpE and the10-kDa chaperonin (SP0516, SP1907) and an ATP-dependent Clp protease (SP0338). Translation initiation factor IF-1 (SP0232), transcriptional regulator GntR (SP1714), and three ABC transporter proteins (SP1715, SP1869, and SP1872) were found down regulated.

These results suggest that there is a very selective change in global transcription as a consequence of the lack of PcsB (Table 3).

Table 3: Changes in relative transcript amounts caused by the lack of PcsB. Values represent fold changes in the Δ*pcsB* strains relative to the wild-type strains. Genes down-regulated at least 2 fold in the Δ*pcsB* strain are shown in dark green, genes up-regulated at least 2 fold are highlighted in red. (n.d., not determined)

ORF	Gene product	T4	6B
SP0107	LysM domain protein	14.2	26.4
SP2063	LysM domain protein	14.8	33.6
SP0461	transcriptional regulator RlrA	2.0	11.0
SP0462	LPxTG protein RrgA	3.4	4.8
SP0463	LPxTG protein RrgB	3.8	n.d.
SP0464	LPxTG protein RrgC	4.3	8.3
SP0467	sortase, SrtC	1.4	4.4
SP0468	sortase, SrtDe	2.5	5.1
SP0338	ATP-dependent Clp protease, ATP binding	13.8	2.6
SP0516	heat shock protein GrpE	10.0	2.7
SP1907	chaperonin, 10 kDa	2.8	2.6
SP2026	alcohol dehydrogenase, iron-containing	2.3	2.2
SP2088	phosphate transport system regulatory protein	7.3	18.9
SP0232	translation initiation factor IF-1	-3.7	-2.1
SP1714	transcriptional regulator, GntR family	-3.3	-3.0
SP1715	ABC transporter, ATP-binding protein	-2.5	-2.8
SP1869	iron-compound ABC transporter, permease	-7.4	-2.7
SP1872	iron-compound ABC transporter, iron-compound binding	-5.9	-2.8

Characterization of the three potential vaccine candidates

In order to select back-up candidates for the pneumo-vaccine project, we re-analyzed the data *in vitro* validation data obtained. The following criteria were addressed in these assays: (a) immunogenicity in humans, (b) surface exposure and/or secretion, (c) capability of inducing bactericidal antibodies, and (d) sequence conservation. In addition further selection was based on intellectual property claims (Giefing, Meinke et al. 2008). The identification of the ANTIGENome of pneumococcus revealed large number of antigens which were further analyzed by several *in vitro* assays. Immune reactivity of synthetic peptides was determined by ELISA with 20 individual human sera comprising

four IG screening pools. For further characterization generation of epitope-specific immune sera was generated in mice, using total bacterial lysates prepared from *E. coli* clones displaying immunogenic pneumococcal epitopes. Surface staining was done on encapsulated TIGR4 cells by flow cytometry analysis, and positive samples were further tested in an *in vitro* opsonophagocytic killing (OPK) assay using serotype 4 and 6B strains. To select conserved antigens gene distribution study was made by PCR using gene-specific primers from 50 different clinical strains (serotypes 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 12F, 14, 17F, 18C, 19A, 19F, 22F, 23F, 33F, 35A, and 35F). By reanalyzing these *in vitro* validation results, we were able to identify three additional highly conserved vaccine candidate antigens that could also serve as targets for monoclonal antibodies: SP2027, a hypothetical protein; SP2194, the ATP-binding subunit of an ATP-dependent Clp protease; and SP0609, which is an amino acid binding protein in an amino acid ATP-binding cassette transporter (Table 4).

Table 4: Summary table of antigens selected in the second round. Screens represents the number of screens in which the antigen was selected. Hits describe the total number of *E. coli* clones selected for one ORF. FC: flow cytomerty; GD: gene distribution; nd: not determined; +: not consistently positive in all assays performed; ++: consistently positive relative to control reagents.

ORF	Common name	Screens	Hits	FC	OPK	GD	Gene conservation (50 strains)
SN0609	amino acid ABC transporter, amino acid-binding	1	3	++	+	50/50	max. 7 aa changes; 1 strain 15 aa changes
SN2027	Conserved hypothetical protein	6	54	n.d.	n.d.	50/50	max. 3 aa changes
SN2194	ATP-dependent Clp protease, ATP-binding subunit	1	1	++	++	50/50	Few changes; >95% identity in 20 strains

For further characterization we tested the proteins from the second selection round for protection in sepsis and two pneumonia models with two different serotypes. Immunization with the corresponding recombinant proteins of the three antigens showed protection in the 6B sepsis model (Figure 10). In the WU-2 challenge both SP2027 and SP0609 were capable of reducing the bacterial load in the lung, similar to PspA (Figure 11).

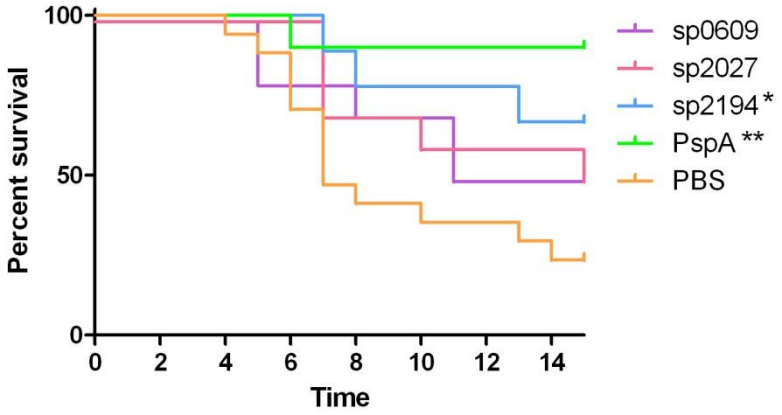

Figure 10: Protection of the three the back-up antigens against pneumococcal sepsis. Immunization of the mice with the three potential vaccine candidates was followed by intraperitoneal injection of the pneumococcal 6B strain (sepsis model). The survival of the animals was monitored for 14 days. *: p<0, 05; **: p<0, 01

In the case of the intranasal challenge with EF3030 immunization with SP2027 and SP2194 caused significant decrease in the bacterial number in the lungs, shown reproducible more effective as Prevnar (containing serotype 19 CPS) and eliminated bacteria from the lung (below the detection limit) in 50% and 60% (respectively) of the animals (Figure 12).

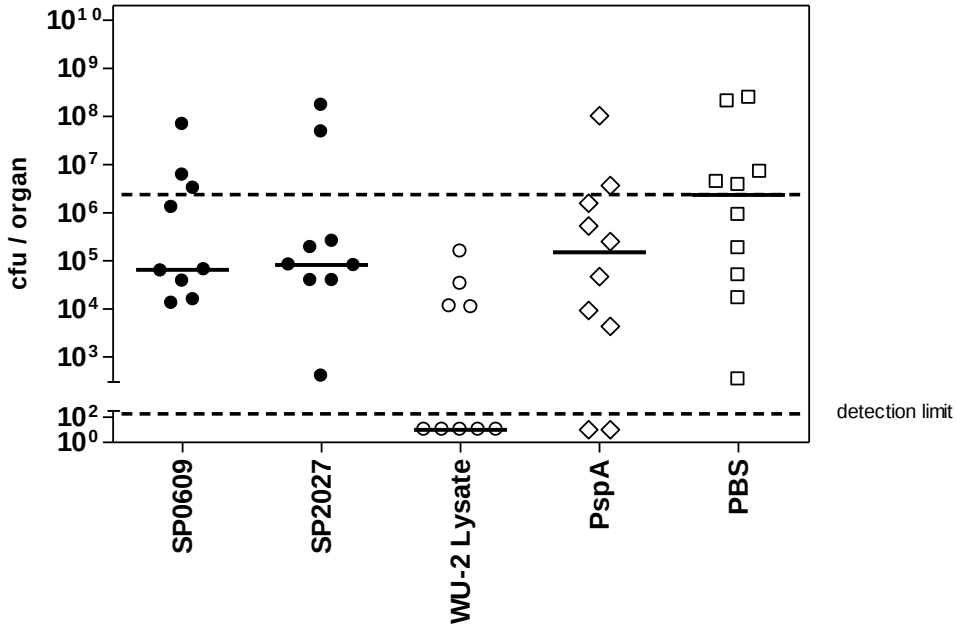

Figure 11: Pneumonia model with the WU-2 strain. The immunization with the selected antigens was followed by an intranasal challenge of pneumococcal WU-2 strain. On day 3 mice were sacrificed and the number of bacteria in the lung was determined.

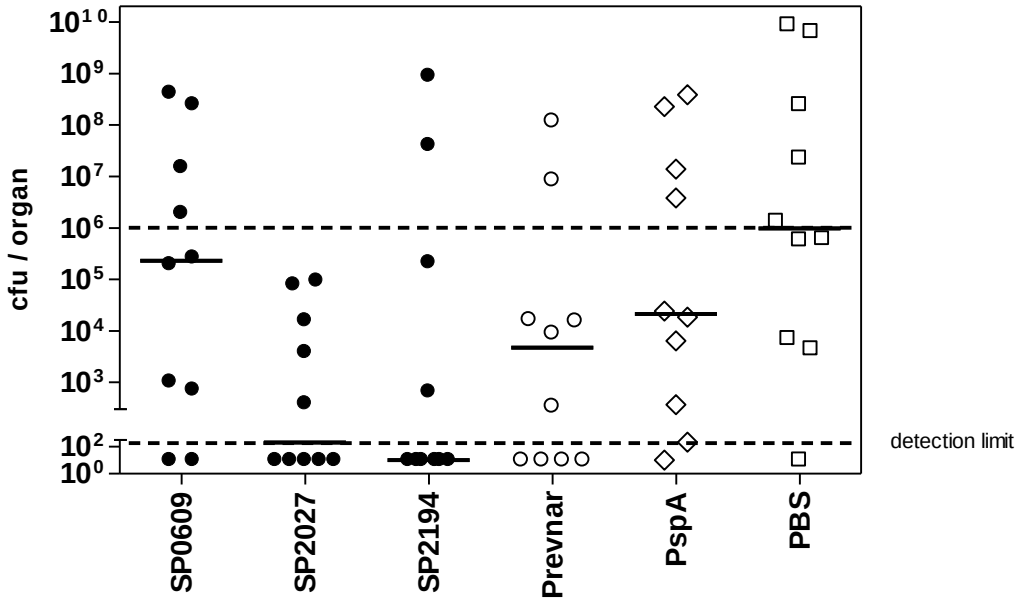

Figure 12: Pneumonia model with the EF3030 strain. After immunization with the selected antigens intranasal challenge was performed and on day 3 the lungs were removed in order to determine the number of the residing bacteria.

SP06096 (28 kDa, 765 bp) is the amino acid binding protein in an ABC-transporter. BLAST search with the SP0609 sequence revealed that it is 61.3% identic with PEB1 in *Campylobacter jejuni NCTC 11168* (alignment done on the CMR tool of JCVI), which is a protective, periplasmic binding protein component of an aspartate/glutamate ABC transporter. PEB1 is a two-domain structured antigenic factor exposed on the surface, playing role in adherence and host colonization. It is important, but not the only adhesion protein in *C. jejuni*. Inactivation of PEB1 reduced *C. jejuni* adherence to HeLa cells (50-100-fold less), and the growth rates of WT and mutant strains are similar (Pei, Burucoa et al. 1998). PEB1 is found to be protective (Du, Li et al. 2008).

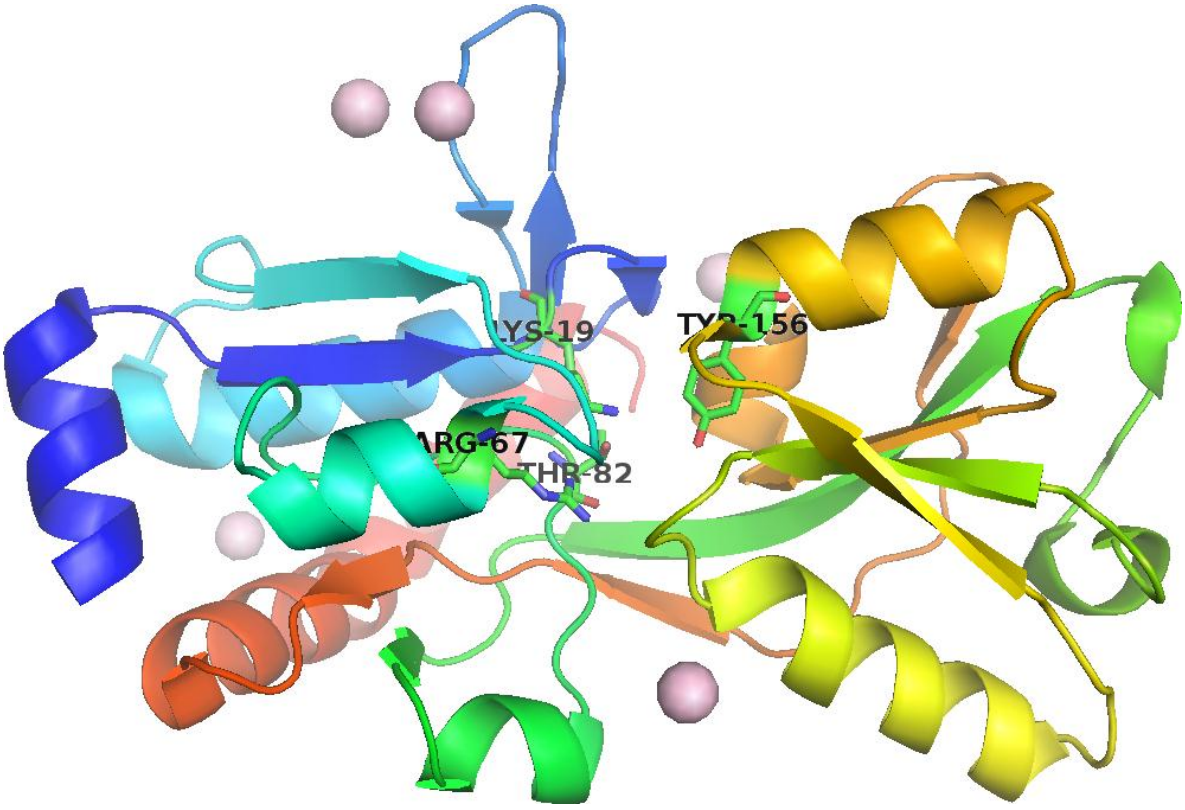

Figure 13: Putative structure of the SP0609 based on its similarity with PEB1 (*C. jejuni*). The highlighted residues are the amino acid binding units. The figure was prepared using the program Open-Source PyMOL (DeLano 2002).

PEB1 has a typical two-domain α/β structure, characteristic of periplasmic extracytoplasmic solute receptors. Arg89 and Asp174 form ion-pairing interactions with the main chain α-carboxyl and α-aminogroups, respectively, of the ligand, while Arg67, Thr82, Lys19 and Tyr156 (residues showed on Fig. 13) co-ordinate the ligand side-chain carboxyl

group. Lys19 and Arg67 line a positively charged groove, which favours binding of Asp over the neutral Asn. Both Lys19 and Tyr156 are conserved in a PEB1 homologue from the Gram-positive bacterium *Streptococcus thermophilus*, suggesting that this is also an aspartate-glutamate-binding protein. (Muller, Leon-Kempis Mdel et al. 2007).

Aligning PEB1 (accession number: Cj0921c) with SP0609 it is visible that the main ligand binding regions are basically identical in *S. pneumoniae* (regions highlighted with blue and yellow on Fig. 14, allowing us to predict that that the ligands specific for SP0609 are similarly charged as those of PEB1.

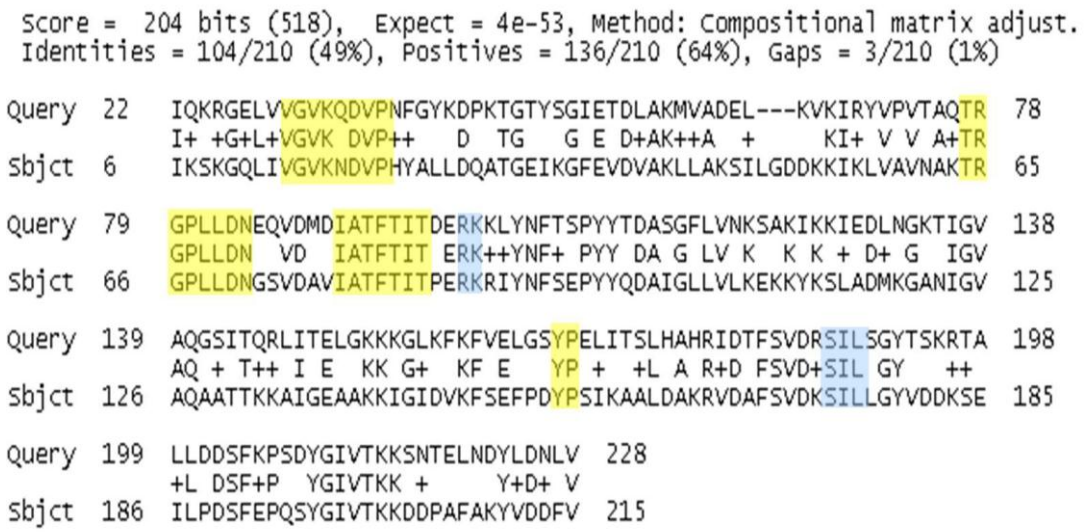

Figure 14: Amino acid alignment of the relevant fragments of SP0609 and PEB1 of *C. jejuni*. The highlighted parts are the amino acid binding areas of the PEB1, which are highly similar to the same areas of the SP0609. Yellow: co-ordinate the ligand side-chain carboxyl group; blue: ion-pairing interactions with the main chain alfa-carboxyl and alfa-aminogroups of the ligand; Query: amino acid sequence of the SP0609; Sbjct: amino acid sequence of the PEB1

SP2027 is a small (15 kDa, 411 bp) hypothetical protein. Analyzing its meta-structure we could identify the putative secondary structure elements and the transmembrane region of SP2027. The prediction of local secondary structure elements is performed by applying the next neighbor distribution functions. The local secondary structure parameter (S_i) is typically positive at residues located in α-helices, whereas residues located in extended regions display negative values. The sequence analysis provides information the residue compactness for each residue position as well, how a structural element is embedded in the context of the 3D fold (Konrat 2009), but based on this information no homologue was found thus it was not possible to predict 3D structure of the molecule (Figure 15).

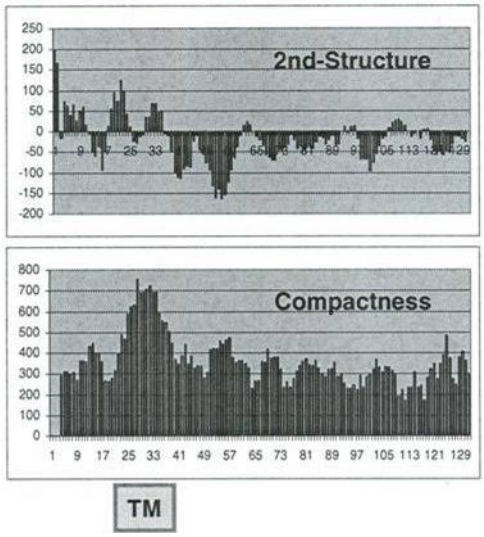

Figure 15: The SP2027 protein meta structure. Comparison was made of predicted local secondary structural features and compactness as a function of residue position. Positive secondary values are indicative of α-helical segments, continuous negative values are extended or β-strand regions. Under the value 50 are residues of loosely defined secondary structures. Large compactness values indicate residue positions typically buried in the interior of the 3D structure, whereas small values are found for residues exposed to the solvent. TM: putative transmembrane region.

SP2194 (90 kDa, 2433bp) is the ATP-binding subunit of the ClpCP (caseinolytic) protease, which is a heat-shock protein. The presence of ClpC, ClpP, and other Clp proteases in bacterial cells is fundamental for stress survival. The caseinolytic protease plays a role in thermal tolerance, control of autolysis upon stationary phase or antibiotic treatment, and chain formation of pneumococcus, although the reports are controversial and

these phenomena are still under discussion (Ibrahim, Kerr et al. 2005; Yu and Houry 2007). ClpC has major role in virulence, it contributes to the ability of the pneumococcus to grow in the lungs and blood but *in vivo* it does not affect the outcome of the disease, since the lethal event is triggered in infection before the lack of ClpC would affect the growing rate (Ibrahim, Kerr et al. 2005).

To obtain further insight into the function of the candidate antigens as targets of vaccines and monoclonal antibodies, gene deletions were generated for SP0609, SP2027 and SP2194 in *S. pneumoniae* TIGR4 (serotype 4) and PJ1324 (serotype 6B) genetic background. The respective genes were substituted with a kanamycin resistance cassette, allowing the strains to grow in medium supplied with 250 μg/ml kanamycin.

The successful deletion of the corresponding genes was confirmed by PCR and Southern blot analysis, but by Western blot we could *in vitro* not detect SP0609 or SP2027 protein using mice or rabbit immune sera specific for the respective proteins (Fig. 16). We analyzed this phenomenon by performing reverse-transcription with total RNA, and observed that these genes are transcribed indeed (Figure 17).

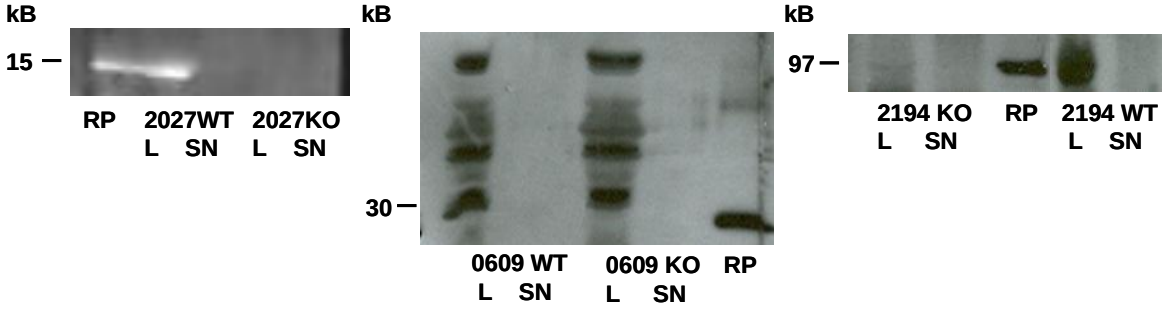

Figure 16.: Western blot analysis of expression of the SP2027, SP0609 and SP2194 genes in WT and KO strains. The second antibody is rabbit immune serum. L: lysate, SN: supernatant, RP: recombinant protein

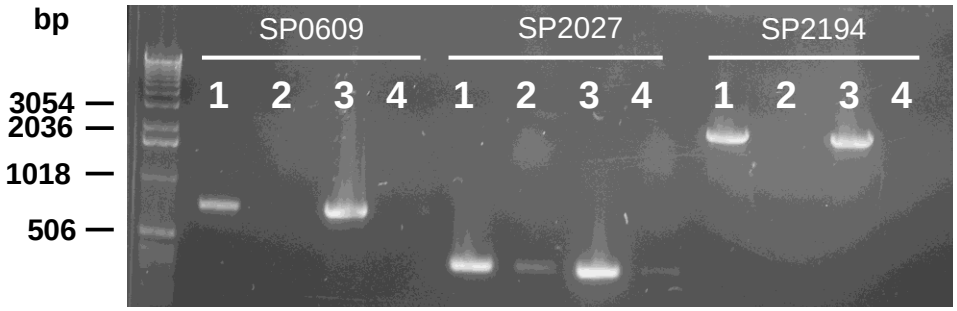

Figure 17: The genes of all the three back-up antigens are transcribed into mRNA. cDNA of T4 bacteria, generated from total RNA, was the template in the polymerase chain reaction. 1: reverse transcribed sample, 2: reverse transcription negative control, 3: positive control (T4 WT) genomic DNA as template 4: negative control (PCR made without template).

In order to clarify if SP0609 is expressed *in vitro* or if any of the bands with unspecific size are derivatives of the SP0609 we incubated anti-SP0609 monoclonal antibody with SP0609 recombinant protein in a molar ratio of 1:3 for half an hour at room temperature with moderate stirring, prior to adding it to the Western blot membrane. The results were compared to the same set of samples loaded on the gel, but without previous treatment of the first antibody. Next to the 6B WT lysate we also included a 6B strain where SP0609 is expressed from a vector (pMU-SP0609). Comparing the membranes with the normal and the blocked signals, we observed reduced signal of the band at the size of 55 kDa which is higher than the expected size (28 kDa), suggesting that SP0609 is present in a complex, for example, as a dimer (Figure 18).

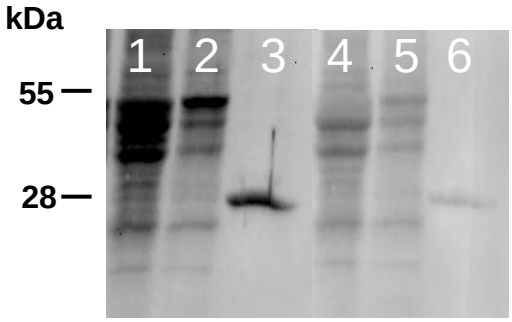

Figure 18: Western blot analysis blocking of the first antibody with recombinant protein SP0609. Line 1,2,3: Western blot using SP0609 mAbs as first antibody 1: 6B WT lysate; 2: 6B WT + pMU-SP0609; 3: recombinant protein; Line 3,4,5: First antibody was preincubated with SP0609 recombinant protein before added to the membrane 4: 6B WT lysate; 5: 6B WT+pMU-SP0609; 6: recombinant protein.

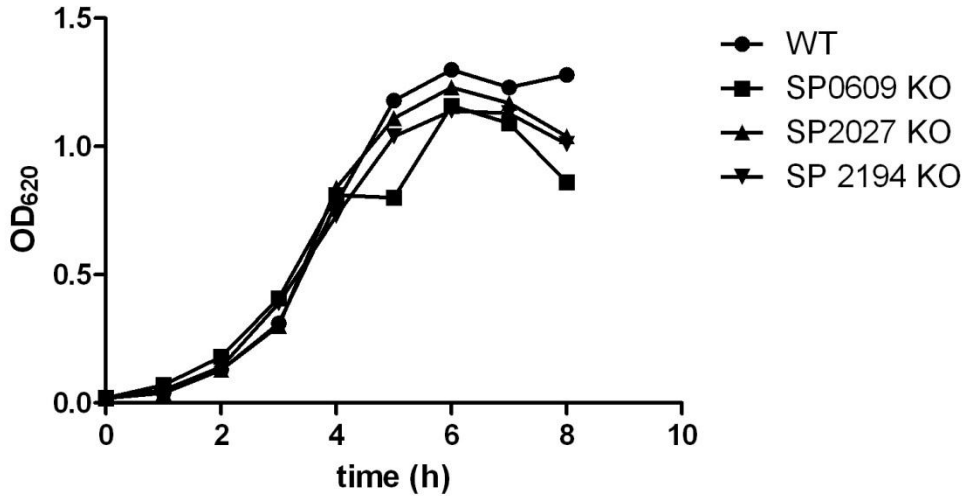

Figure 19: Growth characteristics of the KO strains compared to the WT. The cells were measured from early logarithmic to late stationary phases (determined by measurements of OD_{620}). The strains were cultivated in THY at 37°C 5% CO_2.

We did not detect differences in the *in vitro* growing characteristics of the knock-out strains compared to the wild type strain (Figure 19), which means that none of the knocked out proteins perform vital roles in the organism. Performing intranasal sepsis challenge with the T4 serotypes there were no difference observed in the case of lacking SP0609 and SP2194, but there were a reproducible loss of virulence in case of the SP20207 knock-out strain (Figure 20).

Since the majority of the data found in the literature about SP2194 is contradicting (might be explained by serotype specific behavior) and sp2027 is a hypothetical protein, we decided to mainly focus on SP0609.

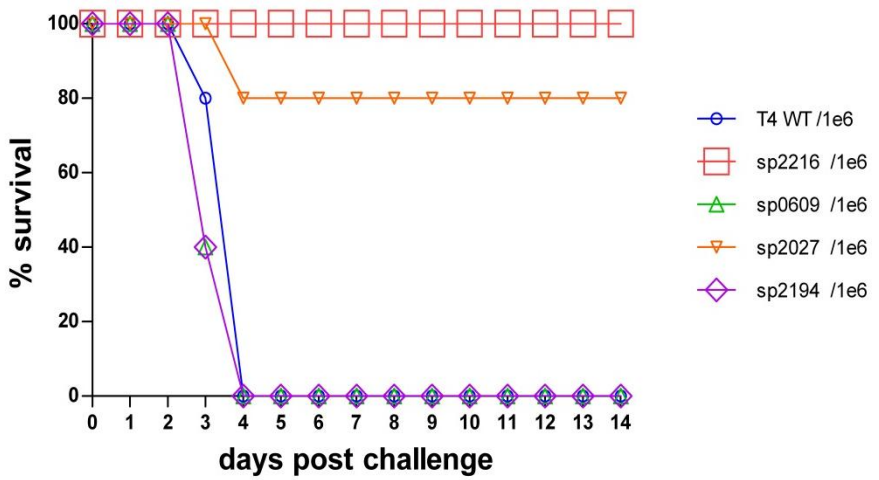

Figure 20: Challenge of C3H/HeN mice with *S. pneumoniae* strains. Intranasal sepsis model, 10^6 T4 bacteria were added to each mouse and then survival was monitored for 14 days. SP2216: ΔSP2216 strain; SP0609: ΔSP0609 strain; SP2027: Δ2027 strain; SP2194: Δ2194 strain. The experiment was repeated with similar results.

The role of SP0609 in bacterial adhesion

The bioinformatic analysis indicated that, based on similarity to PEB1 of *C. jejuni*, SP0609 could have an adhesin-like function. To investigate this, an *in vitro* adhesion assay was set up and *S. pneumoniae* TIGR4 WT and SP0609 gene deletion strains were tested for their capacity to adhere to a nasopharyngeal cell-line (i.e. Detroit 562 cells). As shown in Figure 21/A, adherence of the SP0609 deletion mutant was significantly reduced compared to wild-type thus indicating that SP0609 has indeed an adhesin-like function. Notably, growth rates were comparable between the wild-type and SP0609 gene deletion strain (Figure 21/B), thus ruling out the possibility that the observed differences in adherence result from altered bacterial growth.

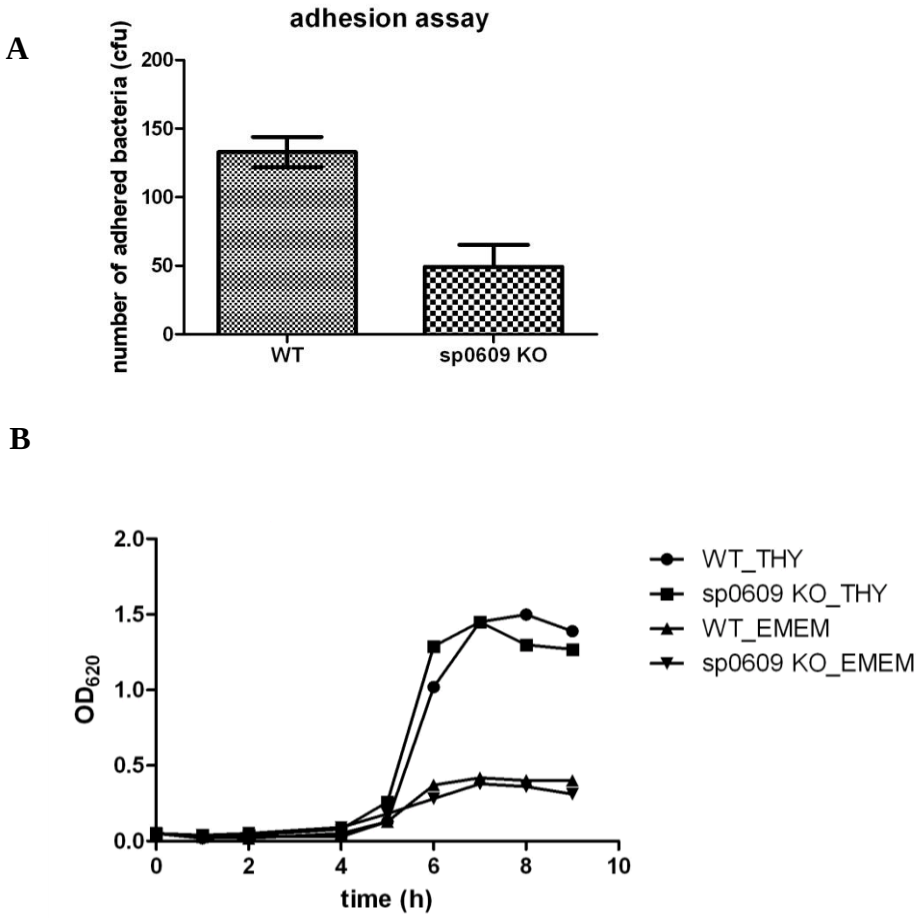

Figure 21: Exploring the adhesin-like function of SP0609, using an SP0609 deletion strain. (A) 10^7 cfu of *S. pneumoniae* TIGR4 wild-type and SP0609 gene deletion strains were incubated on a cell-monolayer of Detroit 562 cells for 2 hours. After washing, the number of adherent bacteria was determined by plating. (B) *S. pneumoniae* TIGR4 wild-type and SP0609 knock-out strain growth was assessed in both THY (Todd Hewitt broth supplemented with 0.5% yeast extract) and EMEM (Eagle´s minimal essential medium).

We investigated if monoclonal antibodies against SP0609 antigen could reduce or neutralize *S. pneumoniae* adhesion to human nasopharyngeal epithelial cells. The antibodies were generated to the mAb project (see chapter of the monoclonal antibodies), 5D is the abbreviation used on the figures for the clone 5D10G4C6, the 2F stands for 2F2B5C10 (see summary table of in the chapter of the monoclonal antibodies; Table 5), and both are murine IgG1 subtype. The inhibition of adhesion was tested with the two different anti-SP0609 monoclonal antibodies on a monolayer of nasopharyngeal cells (Detroit 562). As a negative control we used an unspecific antibody against a *Neisseria meningitides* antigen. In both cases a moderate loss of adhesion capacity of the bacteria was

detected, and since SP0609 is not affecting growth or virulence, we were also not expecting complete loss of adherence (Fig. 22/A; B). All results are normalized to the wild type strain control without added monoclonal antibody, summarizing 2-3 repeated experiments.

To investigate the specificity of these observations, we performed additional experiments to restore the adhesion of SP0609 knockout strain by extrachromosomal expression of SP0609 in the mutant strain. With fusion of SP0609 with promoter of the fucose kinase gene (fucose inducible), cloned into a vector, the SP0609 become inducible expressed; this complementation could restore the ability of adhesion to the nasopharyngeal layer (Fig. 22/C). Importantly, transformation of the knockout strain with the same plasmid but without the SP0609 gene did not affect the adhesion to human cells.

In order to verify that the inhibition of adhesion is due to the neutralizing effect of the monoclonal antibody, the gene-deleted strain was pre-incubated with mAb against SP0609. As it can be seen in Fig. 22/D, it did not change the adhesion characteristics of the knock-out strain; therefore confirming the specificity of the SP0609 antibody neutralizing effect on wild type strain. Incubation with anti-SP0609 mAb has no effect on the adhesion of knockout strain.

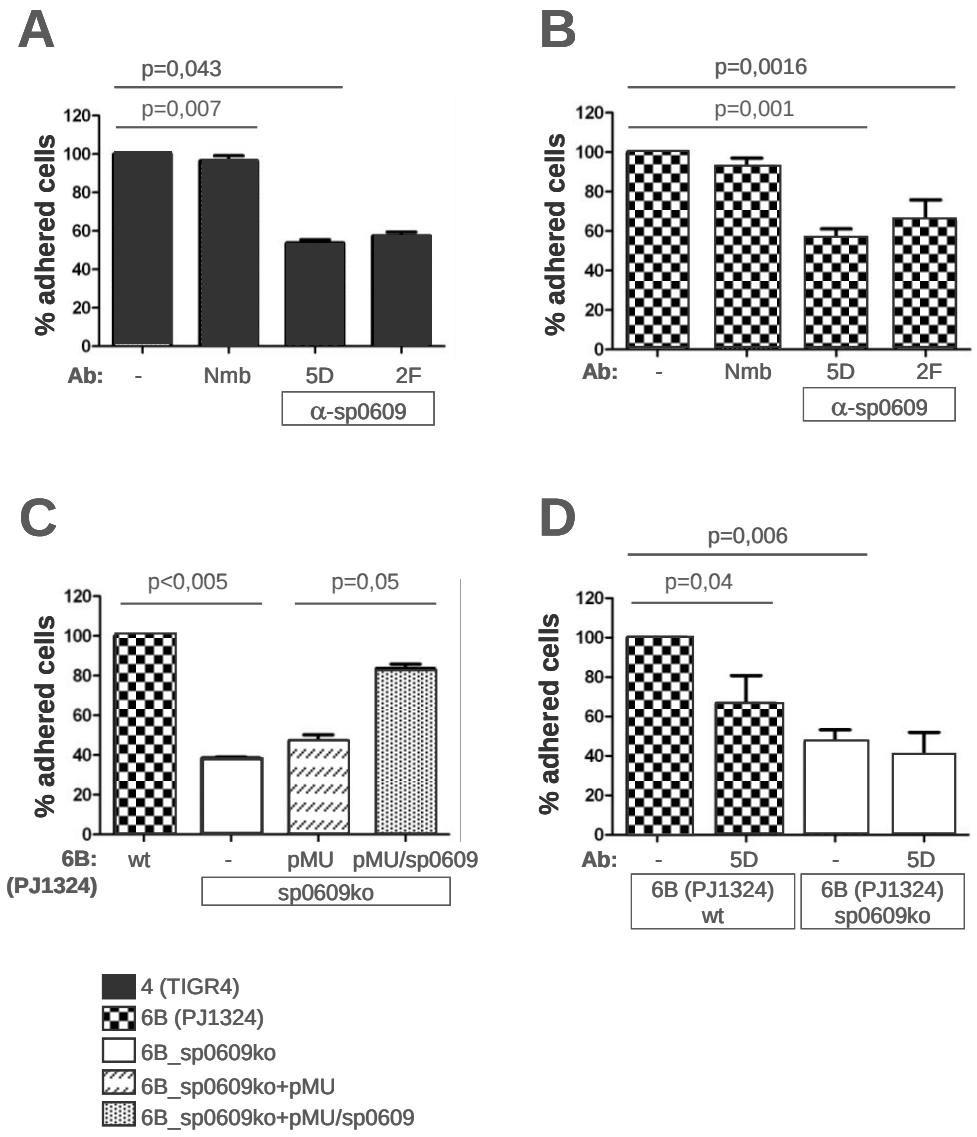

Figure 22: SP0609 is involved in *S. pneumoniae* adhesion to human nasopharyngeal cells (Detroit 562). (A) *S. pneumoniae* PJ1324 (serotype 6B) adhesion to Detroit 562 cells in the presence of mouse monoclonal IgGs against SP0609; two clones were tested: 5D10G4C6 (5D) and 2F2B5C10 (2F). Non-related, monoclonal antibody specific for *Neisseria meningitidis* serotype B antigen was used as negative control (NmB). Results are expressed as % of adherent cells recovered after 2 hr incubation with Detroit 562 cells compared to total bacterial colony forming units (cfu) added at the beginning of incubation (at time= 0 min) and normalized to the adhesion in the absence of antibodies (-). (B) The same experiment performed with *S. pneumoniae* TIGR4 (serotype 4). (C) The adhesion of *S. pneumoniae* PJ1324, serotype 6B wild type (wt) or SP0609 knock-out strain (6B_SP0609ko) without pMU vector (-), with the empty pMU vector (6B_SP0609ko+pMU) or SP0609 knock-out strain transformed with pMU vector containing SP0609 gene sequence (6B_SP0609ko+pMU/SP0609). Adhesion is expressed

as % of adherent cells normalized and compared to *S. pneumoniae* PJ1324, serotype 6B wild type (wt). (D) Adhesion of wt and ko strains with or without anti-SP0609 mAb. p values are determined by one sample location test (t-test).

Prophylactic passive immunization – proof of concept

Murine monoclonal antibodies (mAbs) were raised against the three *S. pneumoniae* vaccine antigens (SP1732-3, SP2216-1, SP1650), for the „back-up“antigens: SP0609, SP2194, SP2024 and for PspA (as positive control). Immunization, fusion, subcloning and antibody purification was out-sourced to the company Abgent. Screening of hybridoma supernatants and selection of monoclonal antibodies was done based on IgG subclass ELISA, Western blot analysis, and surface staining. IgG subclass ELISA was performed in order to choose 2 epitopes of each antigen; preferably IgG2a and IgG2b subclasses (Figure 24), which are the most similar to the human IgG1 antibodies. In case of no IgG2 was found, a murine IgG1 was taken. Western blot was done with recombinant proteins to test binding specificity of the hybridoma supernatants (Fig. 23). To test on live bacterial cells for the antibodies ability to recognize surface exposed epitopes, surface staining was performed. The sequence of the reactive epitope in the antigen was mapped using synthetic peptides in ELISA. Most of them recognize linear target sequences, exept anti-PspA 7E6D, which probably is specific for a conformational epitope. Table 5 summarizes all the selected monoclonal antibodies and the data of their *in vitro* validation.

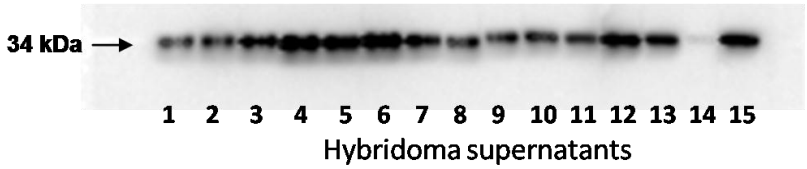

Figure 23: Selection of anti-SP1650 IgGs by specificity to the antigen. Anti-SP1650 hybridoma supernatants containing different monoclonal antibodies were analyzed in Western blot using SP0609 recombinant protein.

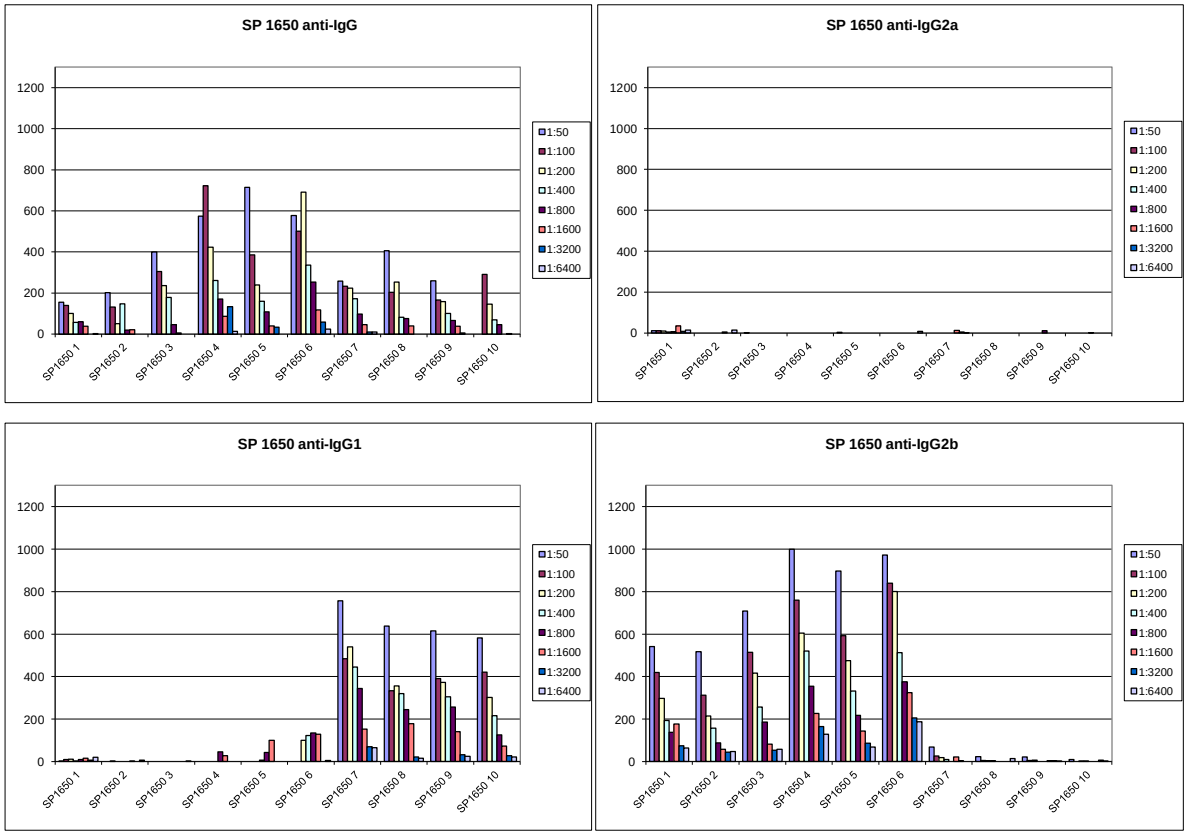

Figure 24: IgG subclass determination of the monoclonal antibodies by ELISA. The IgG subclass of interest is captured by anti-human IgG subclass-specific antibody and quantified by an enzyme-labelled anti-IgG antibody. 10 different anti-SP1650 hybridoma supernatants were compared in eight dilutions.

Table 5.: Summary table of the monoclonal antibodies. The abbreviations are used further in the text. IgG type. Western blot reactivity was tested on recombinant peptides; the surface staining was done with hybridoma supernatants on the 6B PJ1324 and TIGR4 WT strains. *:clone died, none: conformational epitope

clone name	abbreviation	antigen (mAb #)	IgG type	reactive peptide	Western blot	surface staining
71CT103.8.8*	103.8.8	SP1732-3 (1)	IgG1	n.d.	+	-
71CT103.12.6	103.12.6	SP1732-3 (2)	IgG1	n.d.	+	-
72CT59.1.1	59.1.1	SP2216 (1)	IgG1	#7	+	-
72CT12.3.4*	12.3.4	SP2216 (2)	IgG1	#2	+	-
72CT37.8.7	37.8.7	SP2216 (3)	IgG2b	n.d.	+	-
68CT92.2.3	92.2.3	SP1650 (1)	IgG1	n.d.	+	-
68CT99.7.4*	99.7.4	SP1650 (2)	IgG2b	n.d.	+	-
5D10G4C6	5D10G	SP0609 (1)	IgG1	#13	+	-
2F2B5C10	2F2B	SP0609 (2)	IgG1	#6	+	-
1D4C10D2	1D4C	SP2027 (1)	IgG2b	#3	+	-
7F7G7G5	7F7G	SP2027 (2)	IgG2b	#1	+	-
2B1C11F1	2B1C	SP2194 (1)	IgG1	#40	+	+/-
5D7D2G9	5D7D	SP2194 (2)	IgG1	#10, 11, 23, 24, 35	+	-
3G3E8E3		PspA (1)	IgG1	#8	+	+
7E6D9F4	3G3E	PspA (2)	IgG2b	none	+	+

The mAbs were tested in a series of experiments in order to explore protection (passive immunization) in mice. 6-8 week-old female C3H/HeN mice were immunized intraperitoneally with 100 µg per mouse of each mAb. Rabbit immune serum against PspA and PBS were included in each experiment as positive and negative controls, respectively. 3 h post-immunization animals were challenged intraperitoneally using 10^5 colony forming units (cfus) of *S. pneumoniae* strain 6B, and survival was monitored for 14 days.

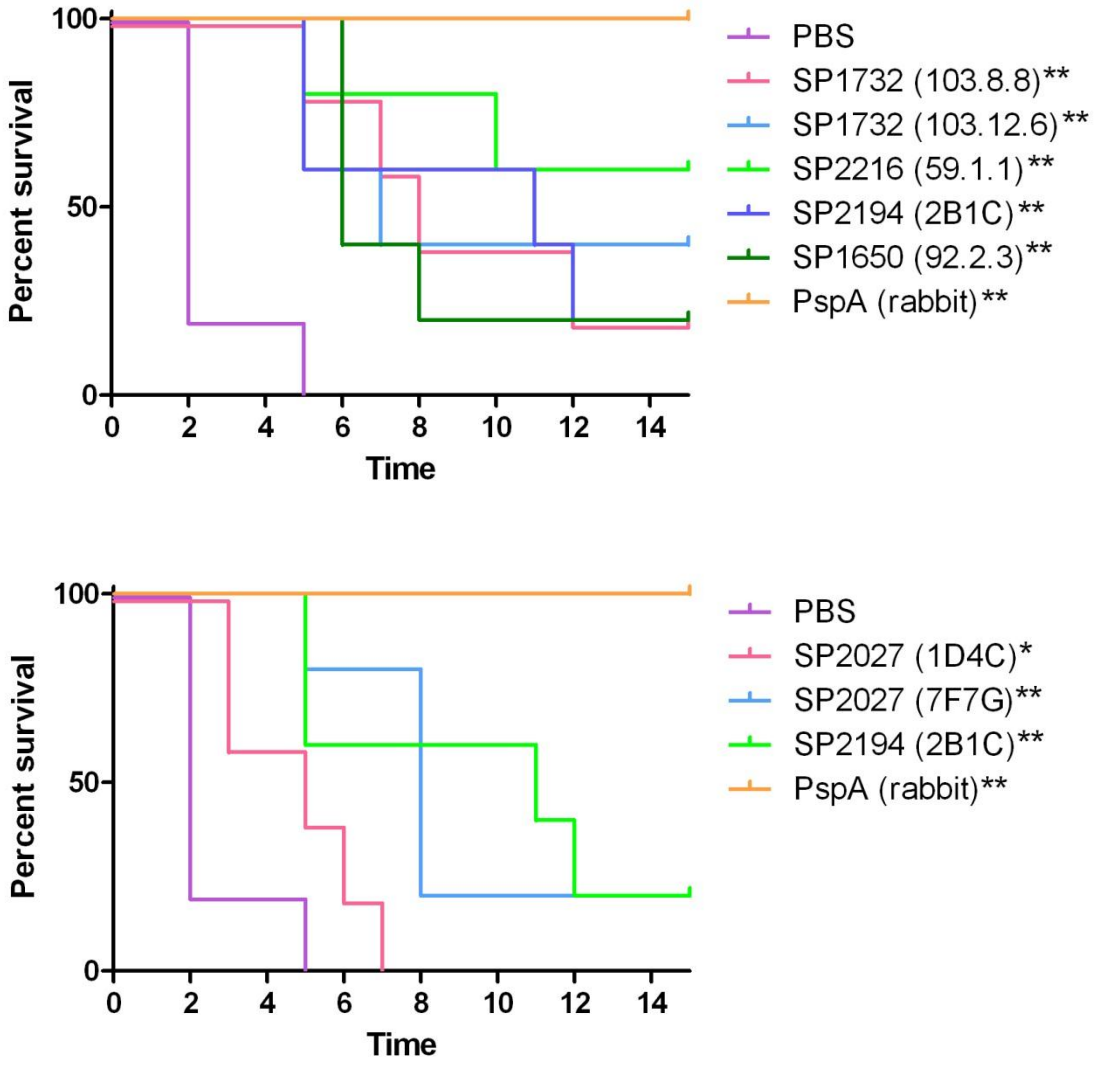

Figure 25: Prophylactic protective efficacy of mAbs against *S. pneumoniae* challenge, sample curves. The survival curves were compared in this case to the negative control group (PBS), and significance of protection was assessed by Mantel-Cox log-rank test (** $p<0.005$, * $p<0.05$). The two graphs are from the same experiment, but samples are split in order to follow easier the curves

In Figure 25, the survival curves are shown for one representative experiment. In this particular experiment, eight mAbs directed against different pneumococcal antigens were tested and the survival curves were compared to the PBS group as negative control. As it is shown in the summary table, later on unspecific antibodies were used as negative controls.

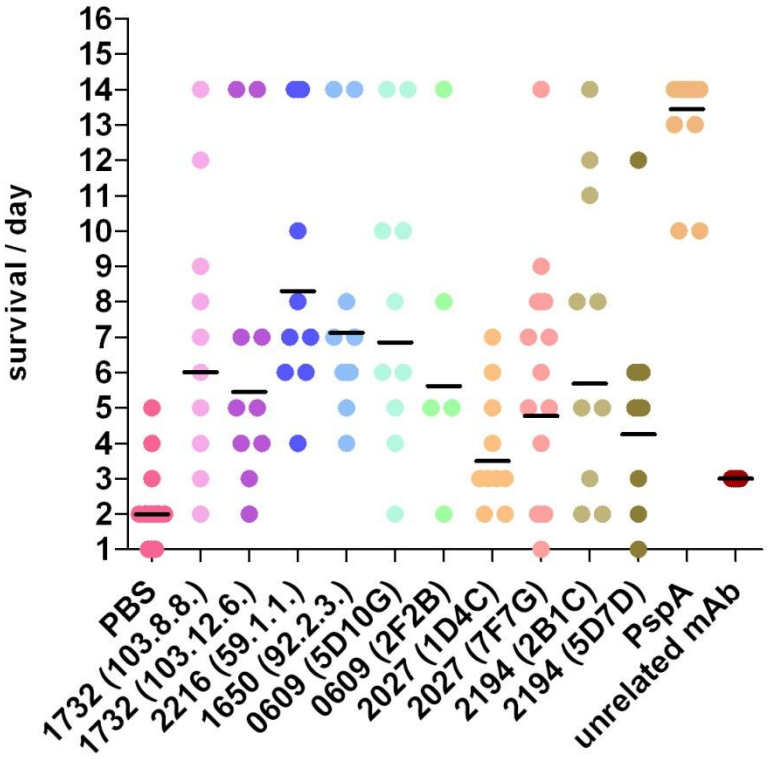

Figure 26: Prophylactic protective efficacy of monoclonal antibodies against *S. pneumoniae* challenge. The challenge was made 3 hours postimmunization. Dots are death events of mice from two experiments, dots at day 14 are mice survived; the line shows the geometric mean of data.

In total, four independent experiments were performed, and the data are shown in Fig. 26 and Table 6. In summary, mAbs directed against antigens SP2216, SP0609, SP2027 and SP2194 could protect against pneumococcal sepsis to some extent, although the degree of protection varied with the different experiments. Thus, further studies were performed to verify these data and to select most protective antibodies.

Table 6: Summary of protection data, using murine mAbs directed against antigens SP2216, SP0609, SP2027 and SP2194. The significance of protection against *S. pneumoniae* challenge was calculated by Mantel-Cox log-rank test, compared the effect of the pneumococcal monoclonal antibodies against the unrelated antibody (** $p<0.005$, * $p<0.05$, "-"$p>0.05$ (no significant protection observed)). Back-ups: SP2027, SP0609, SP2194 introduced together; n.d. indicates that the mAb was not tested in the particular experiment.

Antigen		**Exp. 1.**	**Exp. 2.**	**Exp. 3.**	**Exp. 4.**
SP1732	103.8.8	-	**		
SP1732	103.12.6		*	*	
SP2216	59.1.1	**	**		
SP1650	92.2.3	**	**		
SP0609	5D10G			**	*
SP0609	2F2B			*	
SP2027	1D4C	-	*		
SP2027	7F7G	**	**		-
SP2194	2B1C		**	-	*
SP2194	5D7D			**	
Back-ups	3 mAbs				**
PspA serum	+ control	**	**	**	**

Additional experiments performed with a different *S. pneumoniae* strain of the same serotype (PJ1324, 6B), did not show the same protection pattern: with the exception of anti-PspA 7E6D monoclonal antibody, no protection was achieved with any of the previously tested antibodies specific for SP2216, SP0609, SP2027 and SP2194 proteins (data not shown). Since the aim was to select cross-protective monoclonal antibodies equally efficient against different strains, we decided to focus more on PspA monoclonal antibody, as this antibody was consistently protective in all experiments.

Additional experiments were performed with the aim to further characterize protection against sepsis with PspA antibodies. To better reflect conditions during sepsis,

we decided to change the time between the immunization and challenge to 24 hours. This approach ensures that the bacteria and antibody meet in the blood and not in the peritoneum where instead of sepsis other infection mechanisms are involved, such as local neutralization of bacterial colonization and local inflammatory response. Even under this new conditions and the increased administration time, anti-PspA 7E6D was found to be highly protective even at the very low concentration: 10 µg/mouse (Figure 27).

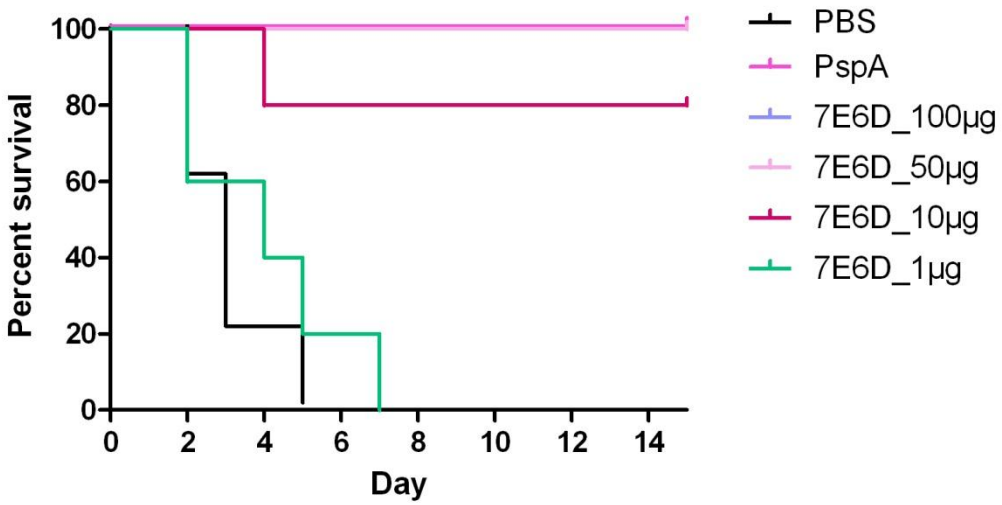

Figure 27: Survival curves of sepsis experiment after immunization with anti-PspA mAb (7E6D). Experiments were performed with five mice per group. Passive immunizations were done by intraperitoneal injection of PspA monoclonal antibody at indicated dose; PspA rabbit serum or buffer without antibodies served as control. Protection is expressed at % of surviving mice in each group compared to positive control, PspA (rabbit serum).

For further characterization, opsonophagocytic killing (OPK) activity induced by PspA mAb was tested. *S. pneumoniae* PJ1324 was incubated with the monoclonal antibodies for 30 min at room temperature, to opsonize the cells. Then HL60 cells were added and phagocytosis could occur for another 60 minutes at 37°C. Surviving bacteria were plated on blood agar, and cfu was counted on the next day. The strain PJ1324 was tested for complement sensitivity as well, and showed that it is not sensitive to it.

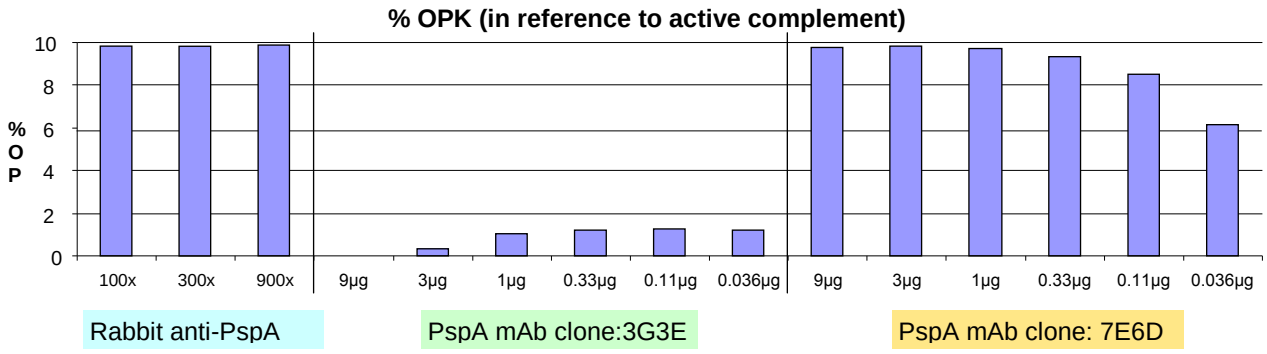

Figure 28: OPK activity of PspA specific monoclonal antibodies 3G3E8E3 and 7E6D9F4. Opsonophagocytic killing activity of HL60 cells in presence of active complement and PspA specific mAb clones 3G3E8E3 and 7E6D9F4. Percentage killing is shown in reference to active complement control. OPK activity of polyclonal rabbit anti-PspA serum at 1:100, 1:300 and 1:900 dilution and of PspA specific mAbs 3G3E8E3 and 7E6D9F4 starting at an amount of 3 µg in 3 fold dilutions down to 0.0012 µg per assay volume (150 µl).

PspA 7E6D mAb induced very strong and reproducible OPK activity, till even 1 µg of the mAb showed a comparable effect to the positive control rabbit anti-PspA poyclonal serum (Fig. 28). The IgG1 clone does not induce OPK activity even at very high concentrations (up to 60 µg/ml). This difference in the OPK activity induced by the two antibodies may be due to difference in surface accessibility of target epitopes or because of the epitope location in reference to complement inhibiting region of PspA.

To clarify this issue we performed surface staining with *S. pneumoniae* 4DS 2341-94. Bacteria (4DS 2341-94 and capsule-negative T4 strain) from the early logarithmic growth phase and incubated with the monoclonal antibodies before detection with PE-conjugated goat anti–mouse IgG antibodies. Target epitope of PspA mAb 7E6D clone appears more surface-accessible, it shows higher signal intensity compared to the 3G3E mAb (Figure 29). We have seen the same results on capsule-negative T4 as well.

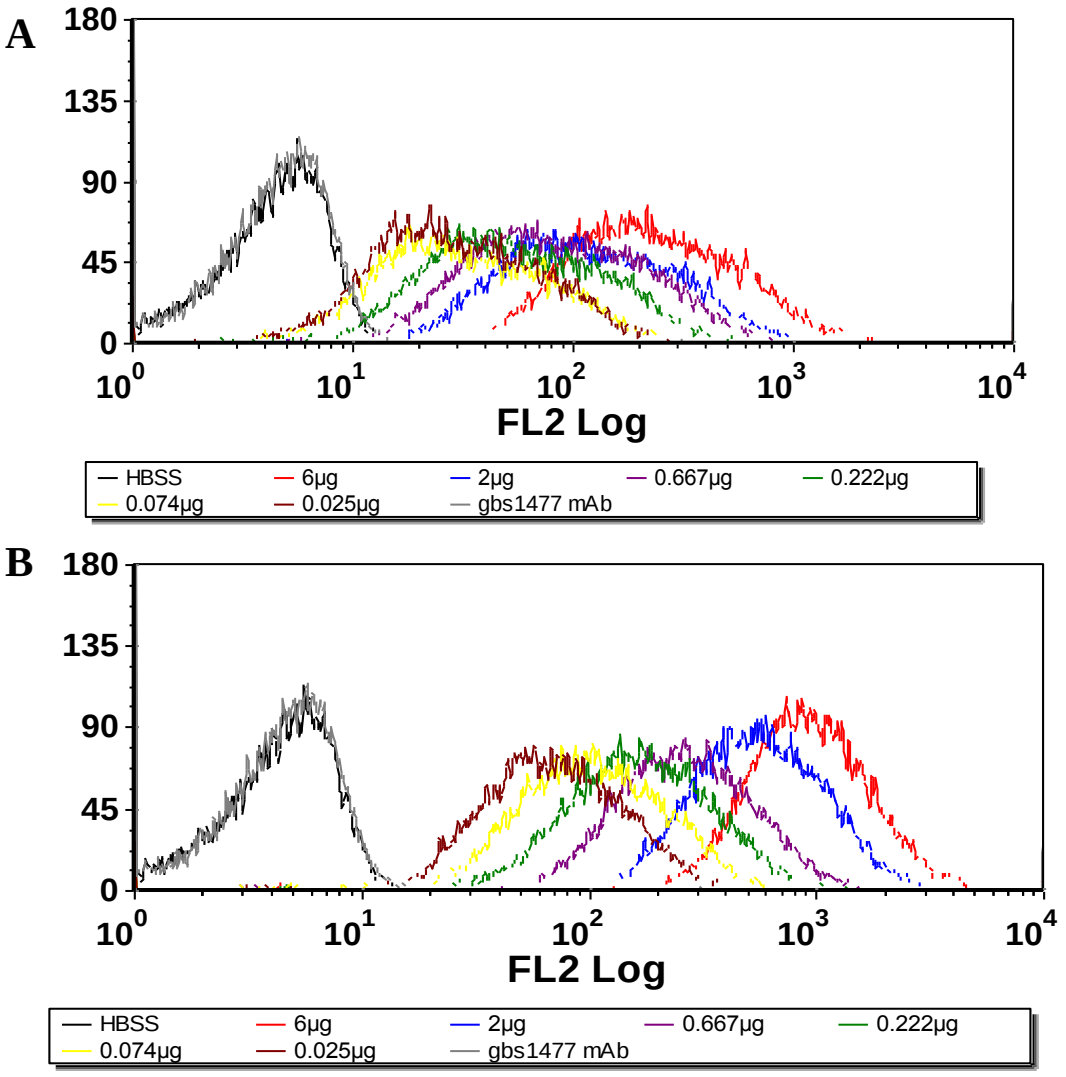

Figure 29: Surface staining of PspA using mAbs clone 7E6D9F4 and 3G3E8E3 on the serotype 4 strain 4DS2341-94. A. Detection of PspA on the pneumococcal surface using decreasing amounts of mAb clone 3G3E8E3 ranging from 6 µg to 0.025 µg per assay volume (100 µl). A gbs1477-specific mAb served as unspecific staining control. **B.** Detection of PspA on the pneumococcal surface using decreasing amounts of mAb clone 7E6D9F4 ranging from 6 µg to 0.025 µg per assay volume (100 µl). A gbs1477-specific mAb served as unspecific staining control.

Monoclonal antibody effect in murine pneumonia model (model with WU-2)

In the first experiments we observed that mice were protected to variable degree (but always significant) by each monoclonal antibody against sepsis caused by a serotype 6B strain by prophylactic administration.

However, in mouse models of pneumococcal pneumonia the same antibodies did not reduce the bacterial load in the lung significantly in case of infection with a serotype WU-2 strain (Figure 30). Because of the limitations of the amount of purified antibody and because of other consideration regarding to our WU-2 pneumonia model (difficulties in differentiating of bacteria colonizing the lungs from blood borne colonies) we didn't performed similar experiments with higher concentration of antibodies.

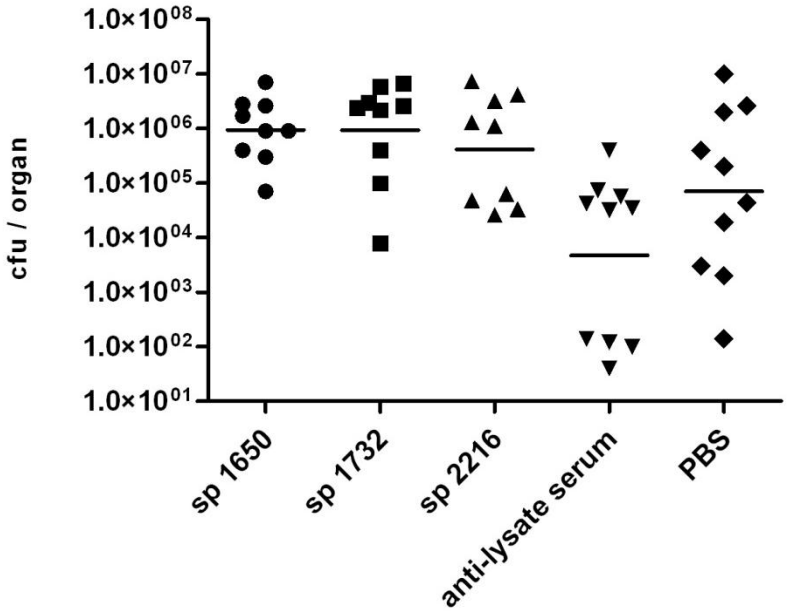

Figure 30: Number of colony forming units (cfu) found in the lungs of the animals three days after the challenge. The first 3 groups were immunized 24h before challenge with the above mentioned monoclonal antibodies. As positive control we used anti-lysate hyperimmune serum.

Co-localization of mAb and pathogen in situ by Xenogen system (pneumonia model with EF3030)

To understand more of the nature of the pneumonia model, we performed the experiments by immunization with fluorescently labelled anti-SP1732 antibody and challenged the animals with EF3030 pneumococcal strain and its luminescent derivative XEN11 (a product of Caliper LifeSciences). In this latter strain there is a luciferase gene embedded into its chromosome (Francis, Yu et al. 2001), thus enabled to produce light that can then be visualized through the tissues of a live animal using specialized imaging equipment and software designed and built by Caliper LifeSciences (Xenogen Camera System). These highly sensitive dual bioluminescence and fluorescence imaging systems allows us to use significantly fewer animals and also to follow infection and clearance *in situ*.

The first three groups were immunized with the antibody, group 1 had no challenge, group 2 was challenged with EF3030 and group3 was challenged with XEN11. Group 4 was challenged with EF3030 and group 5 with XEN11, but these last two groups did not get immunized. All groups were monitored using the Xenogen Camera System for 3 days.

Following the antibody in the first three groups we saw that within some hours it was distributed systematically (Figure 31), independent of bacterial infection, and it stayed within the three days of the experiment.

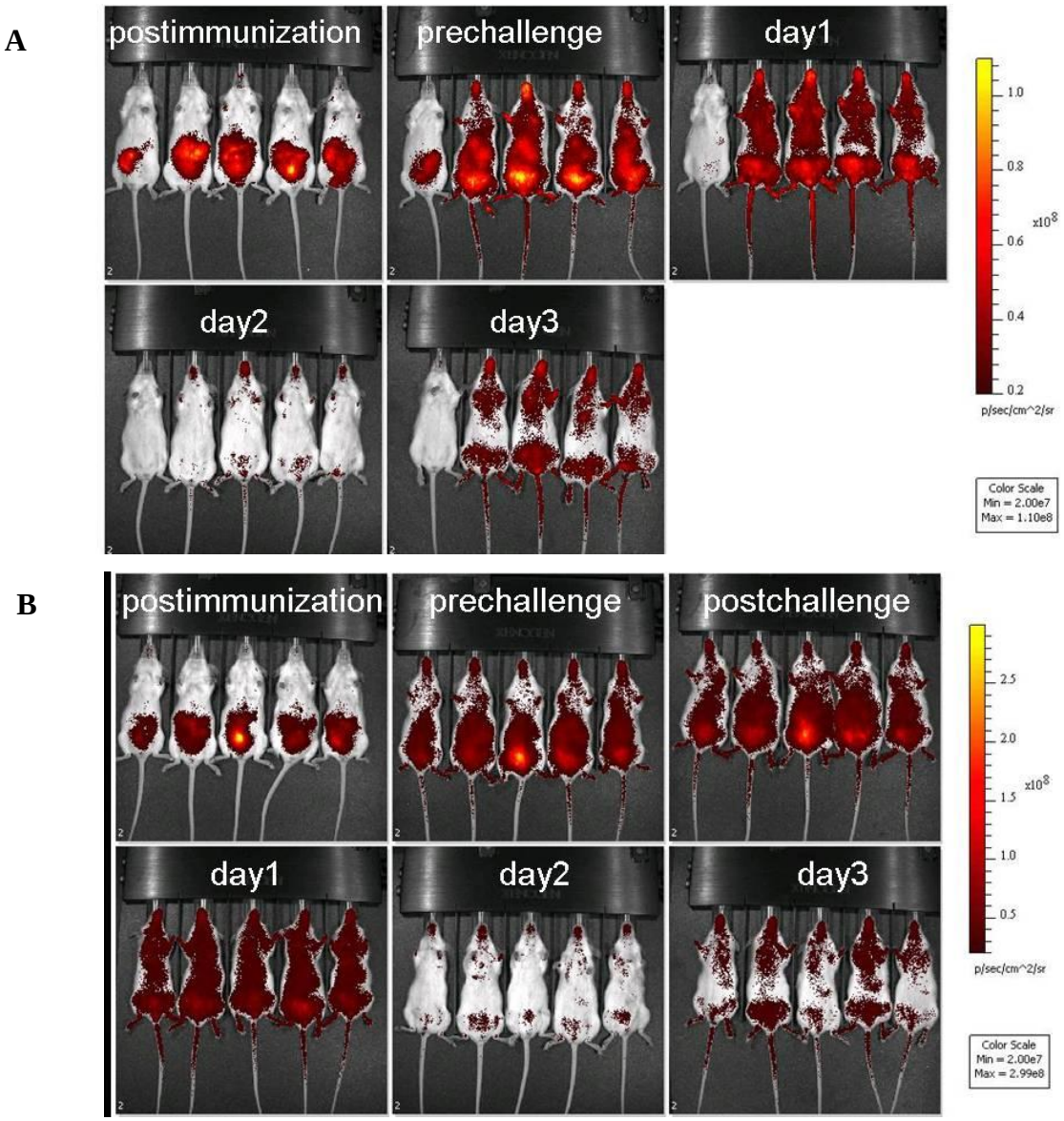

Figure 31: Fluorescence pictures of the ventral side of the animals, A) group 1 and B) group3. The fluorescent antibody was injected into the intraperitoneum, and the images were taken on a daily basis with the *in vivo* imaging camera. There is no difference in the antibody-distribution compared to the not-infected group. Note, that photos of day 2 and day 3 are not changed. The interesting phenomenon of the less signal on day 2 might due to technical error.

We were not able to see any co-localization of the antibody and the bacteria, but we did not see any protection effect of the mAb either (Figure 32). Since the pneumonia model with these settings is not accurate and reliable, first we have to find an experimental setup for the pneumonia, where our antibody candidates would elicit protection, and then investigate further the localization and monitor clearance *in situ*.

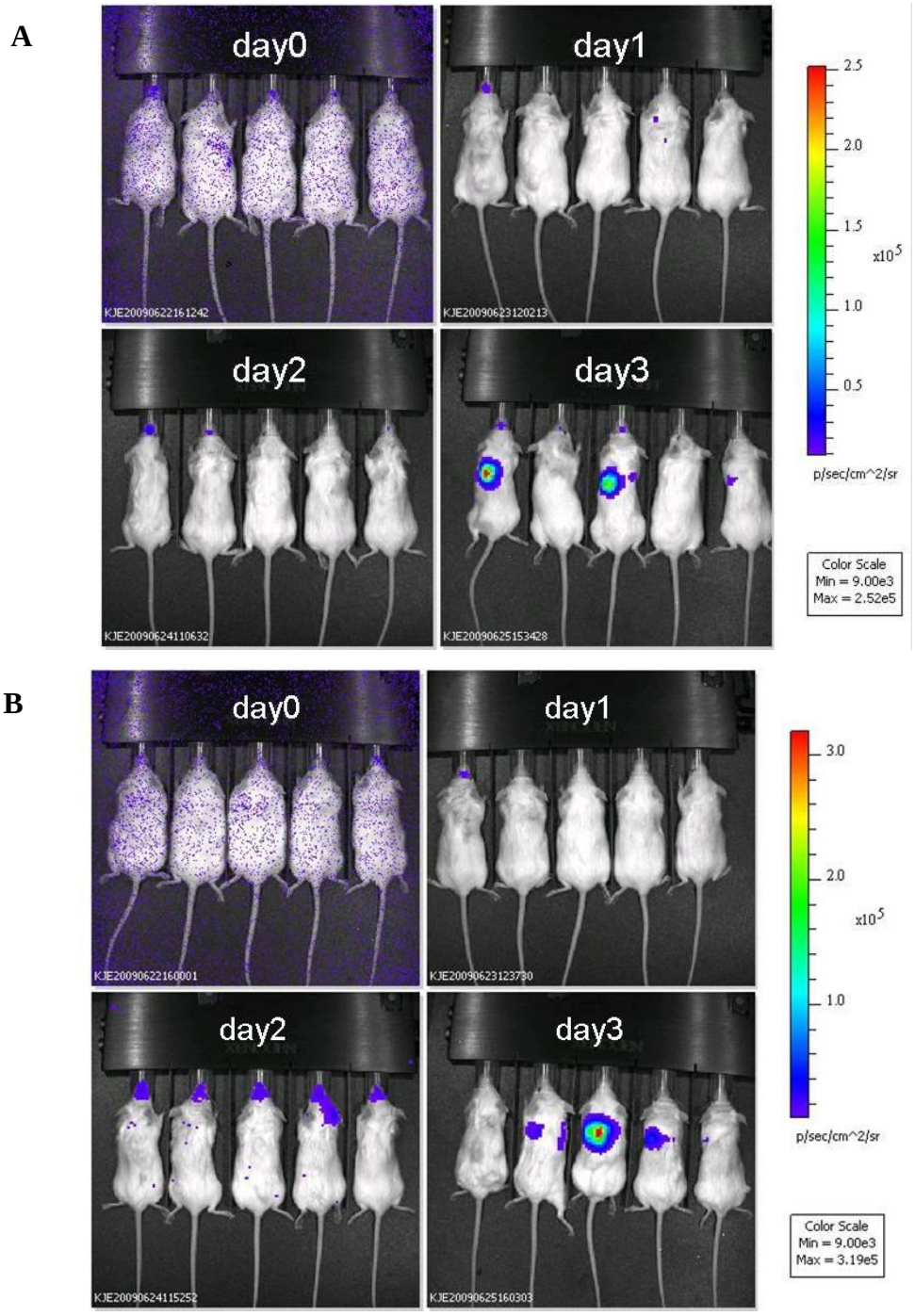

Figure 32: *In vivo* imaging for pneumonia mouse model. Mice were challenged intranasally with *S. pneumoniae* XEN11 and monitored for 3 days. The luminescent intensity reflects bacterial load (red, high bacterial load, blue: low bacterial load) d0:day0, d1: first day post challenge, d2: second day post challenge, d3: third day post challenge

A) Daily images of group 3 B) Daily images of group 5

Characterization of human monoclonal antibodies against SP2216

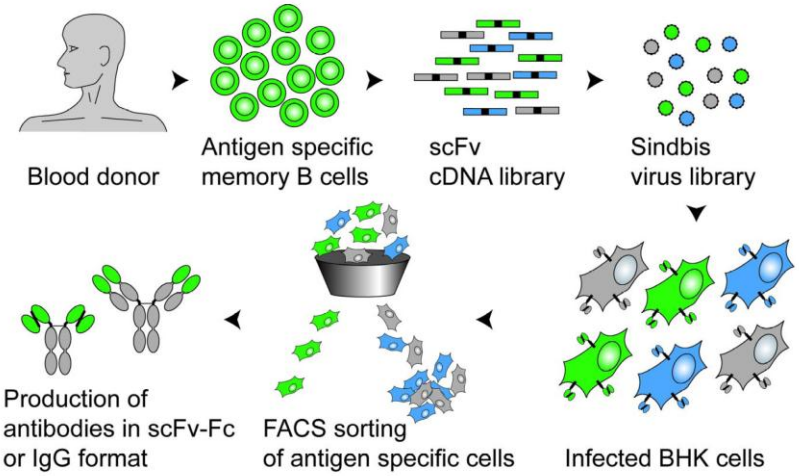

Figure 33: Isolation of human antibodies by mammalian cell display. green: high-affinity binders, blue: low-affinity binders, grey: non-binder fragments. (Beerli, Bauer et al. 2008)

Because of the initial good effect seen with the murine polyclonal sera, we decided to test the selected epitopes as human antibodies as well. The first candidate, the anti-SP2216 human monoclonal antibody was prepared using Intercell´s antibody discovery platform. Antigen-specific, isotype switched B cells are isolated from the PBMC of a human donor by FACS by using fluorescently labelled antigen as bait. RNA isolated from these specific B cells is used to generate a random combinatorial single-chain variable fragment (scFv) library. This enriched library, typically consisting of high-affinity binders, low affinity binders, and nonbinders, is then converted to a high-titer Sindbis virus expression library. Infection of BHK cells generates a pool of infected cells, each expressing at their surface one specific antibody. Single cells expressing a functional antibody are then isolated by flow cytometry and sorted onto a monolayer of BHK cells. Once the virus has spread and expression of antigen- specific antibody is verified, the variable regions are cloned and the antibody can be expressed in any desired format (Figure 33).

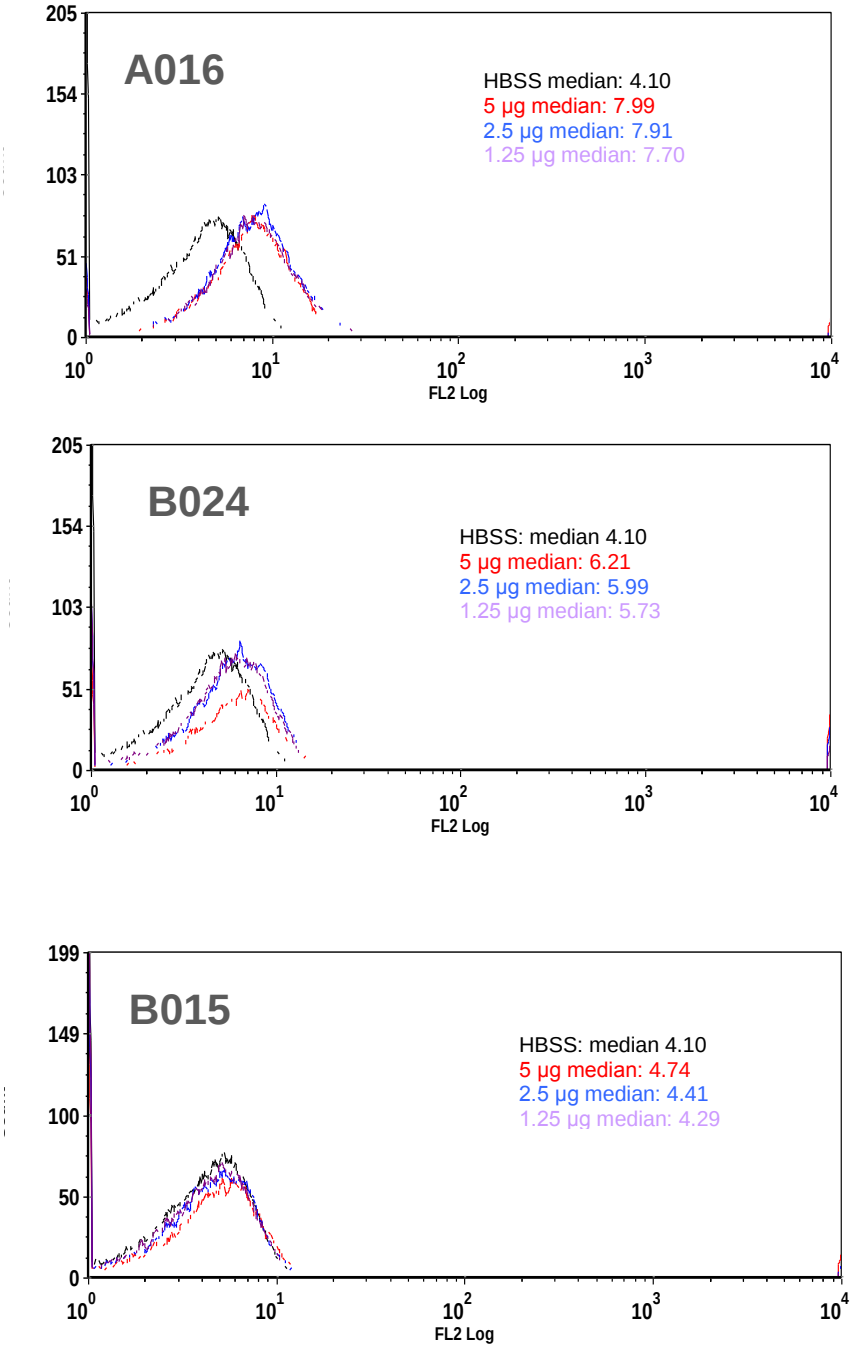

Figure 34: Surface accessibility of the antigens targeted by the human mAbs against SP2216. Surface staining of *S. pneumoniae* TIGR4 capsule negative mutant strain was done using monoclonal anti-PcsB scFvs (5-2.5-1.25 µg) as primary antibodies.

As first attempt human antibodies in the form of scFv against SP2216 were prepared, with three different epitopes, A16, B15 and B24. A16 binds the same epitope as the murine anti-SP2216 antibody clone 59.1.1 (Fig. 35). All of the scFvs were available with murine and human Fc (Fcγ2c and Fcγ1 respectively).

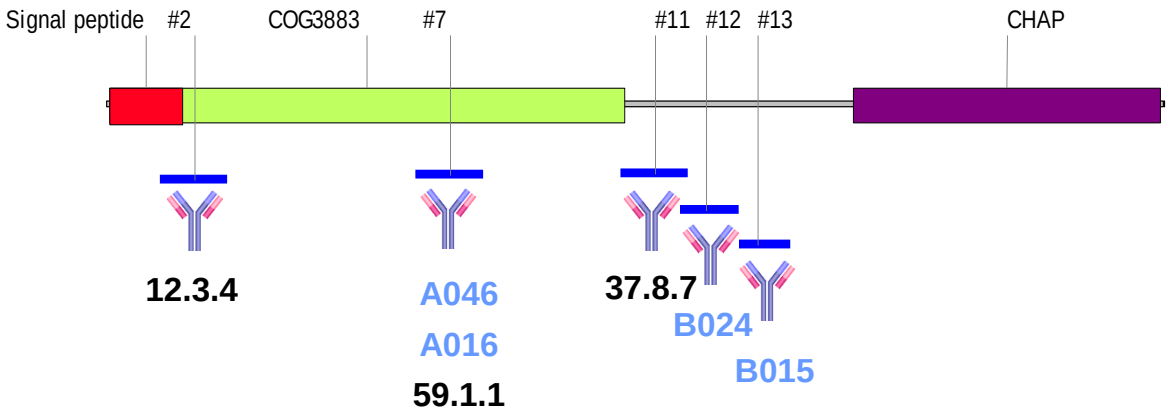

Figure 35: Epitope mapping of murine and human monoclonal antibodies against the SP2216 antigen with ELISA performed with synthetic peptides. The domain structure was predicted *via* the conserved domain database (CDD) at NCBI. The black identification numbers are the murine epitopes, the blue ones are the human epitopes.

The staining by the human antibodies was negative on encapsulated strains, but on the capsule negative T4 genotype (FP312) we found positive staining for the A016, faintly positive and negative staining for B024 and B015, respectively (Fig. 34). The signal of the murine mAb 59.1.1 staining (binding the same epitope as the A016) was also positive on capsule negative strain. Binding could be inhibited by blocking with recombinant SP2216 protein, proving binding specificity (Fig. 36).

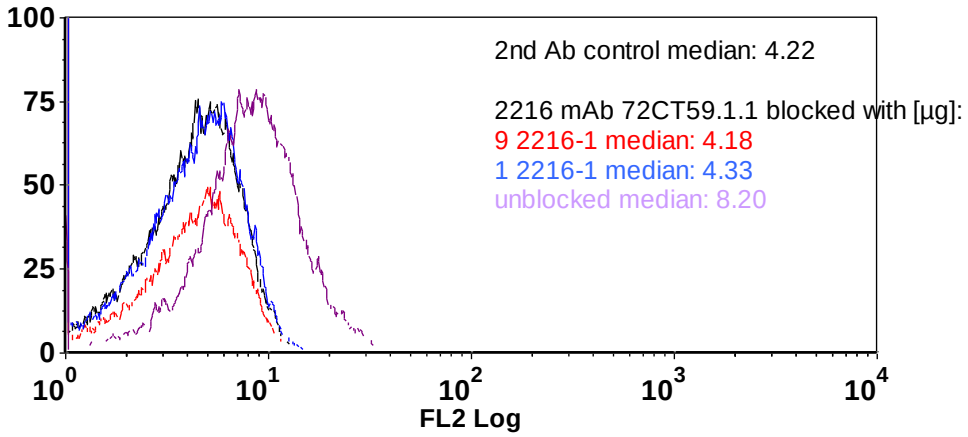

Figure36: Surface accessibility of the antigens targeted by the murine mAbs against SP2216. Surface staining of *S. pneumoniae* TIGR4 capsule negative mutant strain was done using monoclonal anti-PcsB 59.1.1 as primary antibody (1.25 µg) blocked with 9 or 1µg of recombinant SP2216 protein.

After characterizing the human scFv *in vitro*, we performed various animal challenges in order to assess their ability for protection. First, mice were immunized in our sepsis model, and 24 h after the antibody injection challenged with the strain PJ1324 (6B serotype). After 14 days of monitoring we didn't detect any protection in the immunized groups, regardless of which type of Fc, human or murine, the antibody had (Figure 37).

This could happen because of the different half-life of the human antibodies in mice because of no protection activity of the epitopes, or because they do not recognize any proteins on the surface of the PJ1324 strain. To answer this question, we performed the assay with slightly different settings. The antibodies and the bacteria was pre-mixed in the syringe, and then injected to the animals in intraperitoneal route. As positive controls we used the anti-PspA 7E6D monoclonal antibody and the anti-SP2216 59.1.1. mAb, as negative control an unspecific scFv – human Fc was injected. To be able to test the concentration of antibodies in the blood, there were two groups for each epitope, one was infected, the other not. Blood was taken from the mice one day before the challenge, 6 hours, 30 hours, four days and 1 week after the injection. After the 2 weeks of monitoring a more significant protection of the human monoclonals was seen (Figure 38).

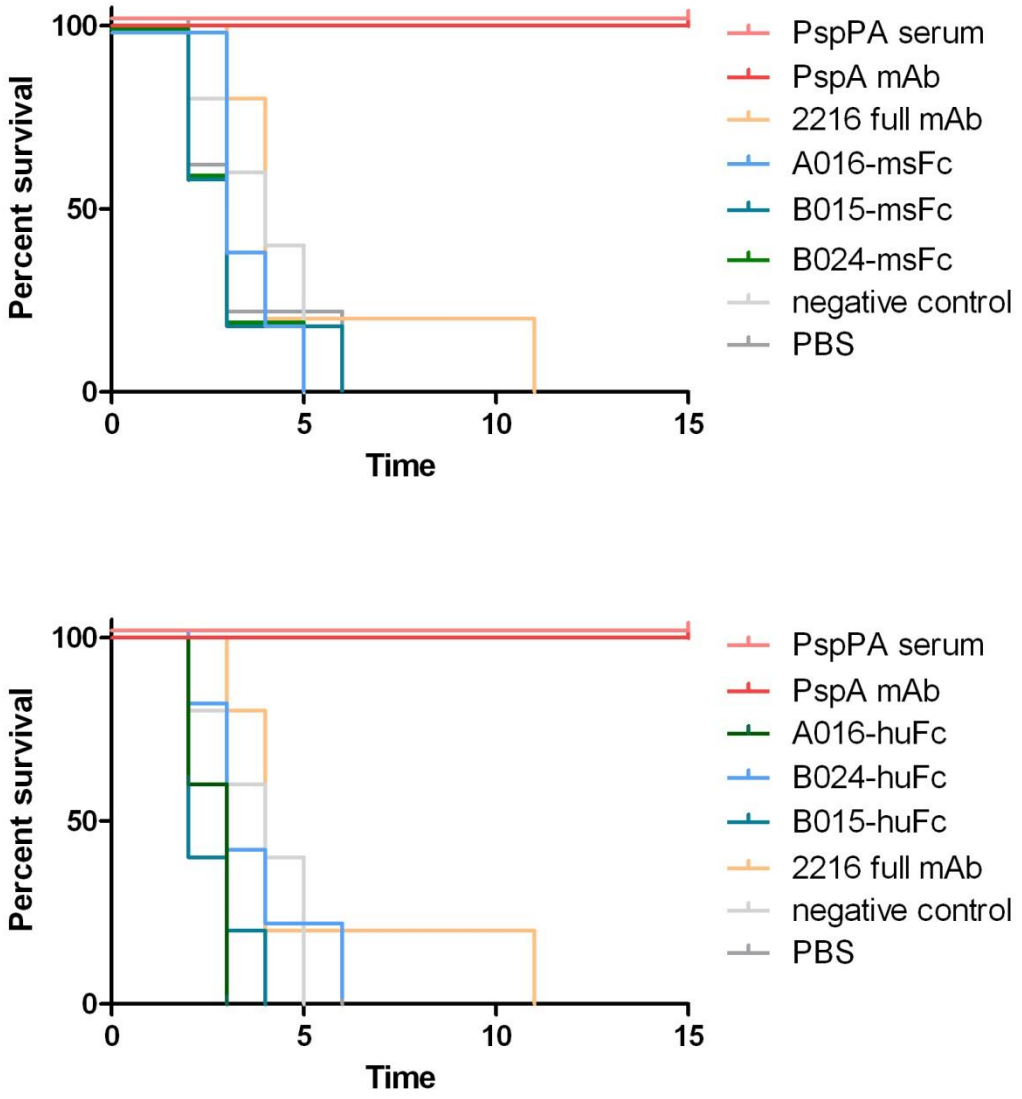

Figure 37: Protection of scFvs in a sepsis experiment. Before the challenge the mice were immunized with human monoclonal antibodies against SP2216. The immunization did not reduce virulence of *S. pneumoniae*. For an easier view the experiment is split into two graphs, the upper one shows the survival curves of the groups which got murine antibody and the controls, the lower one shows the groups which got human antibody, and the control groups as well. PspA mAb is the murine antibody 7E6D (as positive control); negative control is an unrelated scFv (D005); 2216 full mAb is the murine antibody 59.1.1.

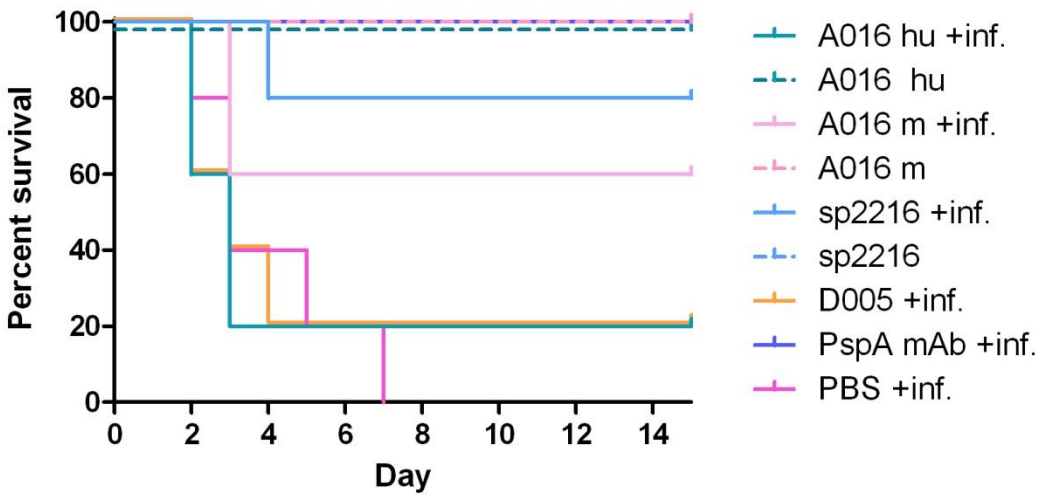

Figure 38: survival curves of mice immunized with anti-SP2216 human monoclonal antibody. The groups where +inf. is indicated were challenged as well, at the same time as the immunization. A016 hu: clone with human Fc, A016 m: clone with murine Fc SP2216: anti-SP2216 mAb, D005: unspecific mAb (negative control)

When we measured the amount of antibody in the blood with ELISA, we found that in case of the infection, although the mice were protected against pneumococcus, the amount of the human antibody rapidly decreased compared to the groups, where were no infection or to that group which got the fully murine antibody anti-SP2216 (Figure 39). This might due to the fact that the scFv-hu-Fc is a human Fcγ1, and the scFv-ms-Fc is a murine Fcγ2c, both have a high affinity to FcR binding, while the anti-SP2216 is a murine IgG1 with low affinity FcR binding. Contradicting to the possibility that the scFv-Fc is cleared together with the bacteria, we found, that the amount of the unspecific, non-bacterial anti-D005 scFv with murine Fc was also rapidly decreased till the fourth day.

Protection assay with the one syringe model (bacteria and antibody pre-mixed and injected together) was performed with the other clones as well, with similar results. Note, that the ones with murine Fc portion protects always better, though the epitope is the same, most probably because of the differences between the human and the murine immune system (Figure 40).

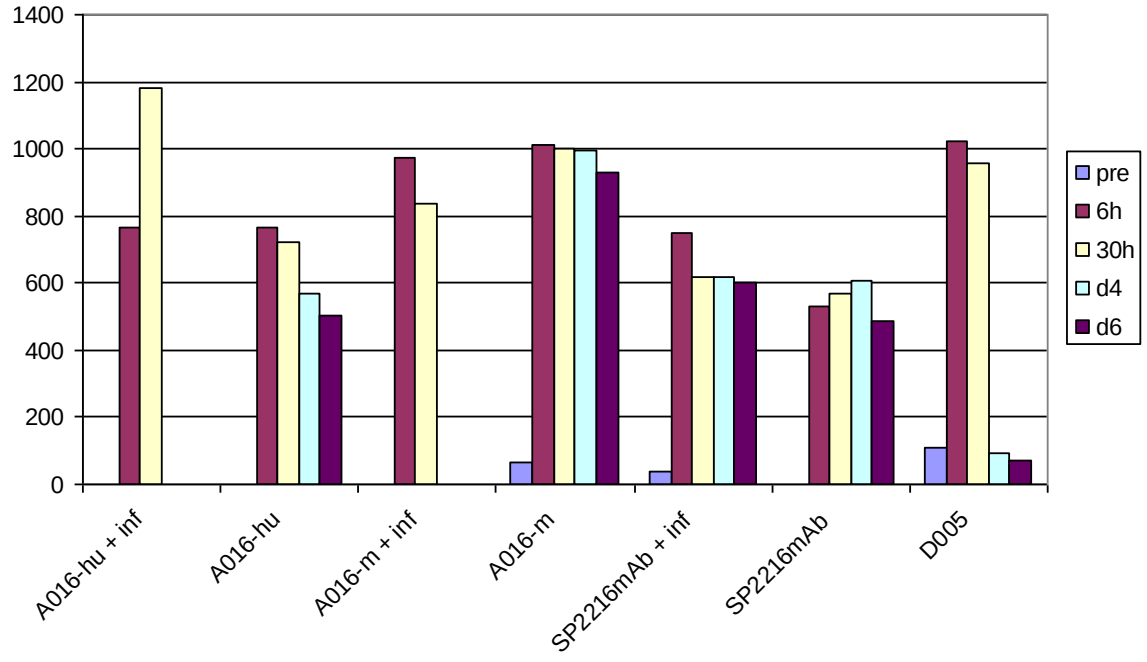

Figure 39: Serum antibody levels at different time-points. Sera from groups of five mice are pooled and analyzed by ELISA at a 1:200 serum dilution using SP2216 or M2 peptides (for the negative control D005) as coating antigens. pre: preserum, 6 h: 6 hours postchallenge, 30 h: 30 hours postchallenge, d4: four days after challenge, d6: 6 days after challenge. Serum was diluted 1:200

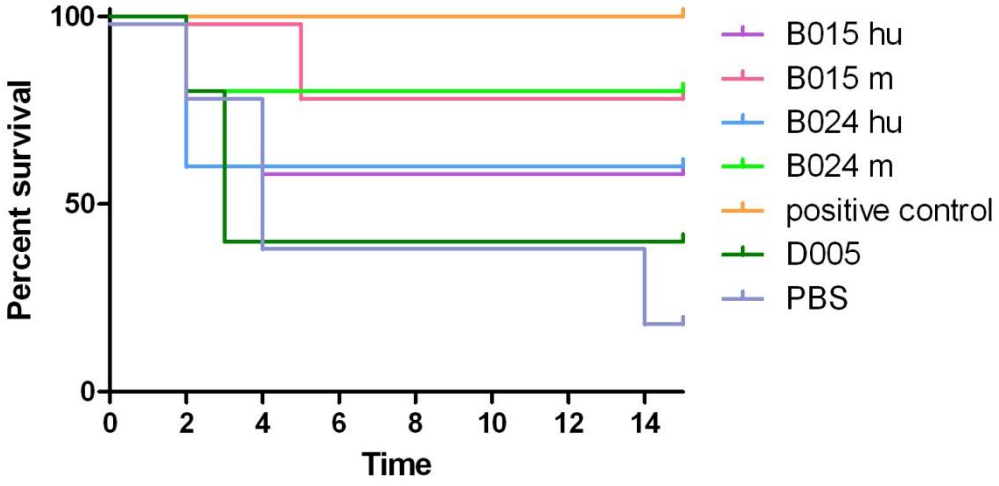

Figure 40: Protection by anti-SP2216 human monoclonal antibody in sepsis model. The groups were challenged at the same time as the immunization. B015 hu, B025 hu: clone with human Fc; B015 m, B024 hu: clone with murine Fc; positive control: anti-PspA mAb (murine); D005: unspecific mAb (negative control)

Discussion

Gene expression pattern of StkP and PcsB

StkP (annotated as SP1732 in the TIGR4 pneumococcal genome) is a eukaryotic type serine/threonine protein kinase. Based on our previous studies and also others' with gene deletion mutant strains, StkP seems to have important non-redundant functions in bacterial multiplication, virulence and competence (Echenique, Kadioglu et al. 2004; Giefing, Meinke et al. 2008). It is localized at the septal site and at the duplicated equatorial sites, co-localization with FtsZ at the constriction ring. Recombinant StkP binds to FtsZ via its kinase domain, but FtsZ assembly is not dependent on the presence of StkP. So StkP recruitment to the septal site may activate downstream pathways of the cell wall synthesis, StkP was found to phosphorylate phophoglucosamine mutase (GlmM) (Giefing, Jelencsics et al. 2010; Novakova, Saskova et al. 2005).

The resulting phenotype from deletion of stkP, the cells become more sensitive to various stress conditions. StkP has been also reported to play a regulatory role in gene transcription (Saskova, Novakova et al. 2007). In addition to the dramatic decrease in *in vitro* growth and virulence, electron microscope analysis revealed a rather elongated cell shape instead of cocci in the stkP mutant strain (Giefing, Meinke et al. 2008). StkP phophorylates a large number of response regulators and thus activate and suppress transcription.

To identify interaction partners of StkP we performed a microarray analysis. The genes, affected by the altered level of expression, play different roles in cell wall envelope, energy metabolism, transport and binding, or in the pilus assembly. This data and literature comparison revealed that StkP controls the expression of *piuBCDA*, *pcsB* and the gene of the rhodanase-like protein SP0095 independent of the genetic background and growth conditions. Genes encoding components of piuBCDA, containing the components of an iron uptake ATP binding cassette (ABC) transporter (also called a fat or pit1 operon) that is required for virulence were most severely repressed. GlmS that was downregulated in our analysis acts together with GlmM, which is substrate for StkP (Novakova, Saskova et al. 2005). GlmM plays role in the biosynthesis of UDPN-acetylglucoseamine, hence StkP was

proposed to be involved in the regulation of this cell wall biosynthetic pathway. Sensitivity of mutant strain to various stress conditions might be related to positive regulation of PcsB and LysM domain protein transcription that may cause changes in the wall composition. Gene products involved in stress response, like heat shock protein GrpE and DnaK protein (SP0516, SP0517 respectively) were up regulated. Additionally genes encoding proteins involved in detoxification and toxin resistance, like rhodanese domain-containing protein (SP0095), beta-lactam resistance factor (SP0615), and bacteriocin transport accessory protein (SP1499) were down regulated in the KO strains.

Repression of comW, which is essential for competence, supports the observation that KO strain lost its ability for DNA uptake. ComW expression is dependent on the response regulator ComE (Luo, Li et al. 2004). Our data suggest that StkP may be capable to phosphorylate ComE and consequently activate the transcription of comW.

Consequently the observed decrease in virulence of StkP deletion mutant may be attributable to several factors: 1) reduced growth rate 2) reduced cell wall fitness (GlmM, PcsB, LysM) 3) impaired iron support (PiuBCDA)

Changes in the expression level of several other genes were found to be dependent on the genetic background (Table 2). The genes encoding an extracellular pilus, like SP0461 (transcriptional regulator) and SP0464 (subunit) are upregulated. The other two structural pilus subunits SP0462 and SP0463 genes were upregulated in the TIGR4 genetic background. The sortases (SP0467-SP0468), which are responsible for pilus assembly, were also upregulated in both of the genetic background. The upregulation of the pilus locus was not described previously since the pilus locus is also only present in a limited number of strains.

StkP was previously identified as a promising vaccine candidate. It was found to be conserved among clinical isolates (>99.5% identity) and cross-protective against different pneumococcal serotypes in a murine lethal sepsis and a pneumonia models (Ref). The presence of specific antibodies in human sera suggested that StkP was expressed during pneumococcal disease and colonization. We could confirm that StkP is, although effects of this regulatory function seems to be dependent on the genetic background, a key player in the pneumococcal cell cycle by influencing the expression of several important gene products, thus its role as a vaccine candidate is strongly affirmed.

PcsB in *S. pneumoniae* and in other streptococcal species has been suggested to have a critical role in cell division. PcsB in *S. pneumoniae* was previously described to be essential in the D39 and R6 strains (Ng, Robertson et al. 2003; Ng, Kazmierczak et al. 2004; Ng, Tsui et al. 2005; Barendt, Land et al. 2009) but successful generation of *pcsB* null mutants in the TIGR4 (serotype 4) and PJ1324 (serotype 6B) genetic backgrounds suggests that PcsB is not essential for *in vitro* viability in all pneumococcal strains (Giefing, Meinke et al. 2008).

The *pcsB* deletion mutant strains displayed the same cluster forming phenotype, altered septum formation and retarded *in vitro* growth (Ng, Kazmierczak et al. 2004). Complementation of these mutants by transformation with a plasmid carrying *pcsB* for extra-chromosomal expression of the protein the growth rate, cell morphology, as well as antibiotic sensitivity of the bacteria could revert back to wild-type. The absence of PcsB resulted in apparent differences in protein abundance in the culture supernatant of wild-type and *pcsB* null mutants, the most obvious increase being associated with enolase (Giefing-Kroll, Jelencsics et al. 2011). In addition to its catalytic function in the glycolytic pathway, enolase was found to be an important virulence factor, but only present in very low amounts on the pneumococcal surface (Bergmann and Hammerschmidt 2006; Kolberg, Aase et al. 2006).

Based on the immediate proximity of *mreC* and *mreD* (sp2218 and sp2217), the pneumococcal homologues of morphogenic proteins of rod-shape bacteria (Osborn and Rothfield 2007) to the *pcsB* locus, phenotypic effects might be linked to polar effects caused by modifications in the *pcsB* locus. However, we detected no changes in the transcript levels of these genes in our *pcsB* mutant strains. Moreover, deletion of these genes was not linked to morphological changes that are characteristics for *pcsB* cells (Barendt, Land et al. 2009). The accumulation of certain proteins in the supernatant of Δ*pcsB* strains seems to be related to their anchoring mechanism: while LPXTG-anchored proteins such as SP0082 or SP0498, or choline binding proteins such as PspA were not enriched in the Δ*pcsB* strains, other proteins that are probably more loosely attached to the cell wall such as MalX and SP0107 were found at higher concentrations in the supernatant, suggesting for micro-changes in the cell wall due to the lack of PcsB (Giefing-Kroll, Jelencsics et al. 2011).

The role of PcsB in peptidoglycan metabolism is further strengthened by our observation that LysM domain containing proteins – SP0107 and SP2063 – were highly overexpressed in Δ*pcsB* strains created in two different genetic backgrounds. These genes were also identified as the most up-regulated transcripts in a PcsB under-expressing R6 strain (Barendt, Land et al. 2009). Deletion of SP0107 leads to overall thickening of the cell wall pointing towards a role in peptidoglycan hydrolysis (Vega Masigniani, 7th International Symposium on Pneumococci and Pneumococcal Diseases, Tel Aviv, Israel, March 16, 2010). Our data suggest that lack of PcsB might be partially compensated by overexpression of SP2063 and SP0107 allow growth, although at a greatly reduced rate.

Transcriptional changes were otherwise restricted to small set of genes. However, we also found increased expression of stress response genes– although different ones, namely chaperonin SP1907 and heat shock protein GrpE instead of groEL and groES – confirming an overall stressed condition of the mutants.

These results suggest that there is a very selective change in global transcription as a consequence of the lack of PcsB. The significantly increased expression of LysM domain proteins that are implicated in cell wall metabolism might represent a compensatory mechanism for the bacterium to survive the loss of function of the PcsB protein.

Characterization of the pneumococcal antigens

The second of round of selecting antigens. The development of protein based vaccines is an urgent need due to the limitations of current polysaccharide-based pneumococcal vaccines. A series of *in vitro* epitope-based validation assay were applied to preselect vaccine candidates for animal testing. In the first round, 6 proteins (PspA, PspC, StkP - SP1732, PcsB - SP2216, SP0368 and SP0667) were selected for further examination and to develop a subunit vaccine using combination of them (Giefing, Meinke et al. 2008). The reanalysis of the *in vitro* data was based on the same criteria as for the first generation of vaccine candidates. Proteins should be conserved, with serotype-independent expression, surface exposed, or secreted, and highly immunogenic. SP0609, SP2027 and SP2194 were selected to complement the SP2216, SP1732 and SP1650 antigens in a future vaccine

against *S. pneumoniae*. A major requirement for viable vaccine candidates for the prevention of pneumococcal disease is the demonstration of protective effects against several of the major disease causing serotypes. The limitation of the different animal models that try to mimic human disease can be compensated for by testing multiple *in vivo* models. In addition to the 6B serotype-induced i.p. sepsis model, the candidates were also tested by i.n. challenge of mice, with sepsis-causing serotype 1 (T4) and pneumonia-inducing serotype 3 (WU-2) or 19F (EF3030) strains. Immunization with these antigens caused protection in certain animal experiment, but their efficacy was far from the protection elicited by the original pneumococcus vaccine candidates, selected in the first round.

Since proteins are not always expressed during colonization and invasion, by further characterization of the proteins we might get information about the expression pattern of them, and these experiments might help us to understand more about the virulence of the pathogen as well. By generation of the corresponding KO strains we could learn more about the role of the selected proteins. The loss of these genes caused no particular phenotype, *in vitro* growing activity and morphology was unaltered in all the cases. Testing *in vivo* the virulence of the mutant strains a significant loss was seen in case of the SP2027 gene deletion, but not with SP0609 and SP2194, suggesting that these latter proteins are not crucial for colonization and/or invasion of the murine host. By preliminary literature research we found that the role of SP2194, the ATP-binding subunit of a Clp protease is strongly serotype dependent (Ibrahim, Kerr et al. 2005; Yu and Houry 2007). No homologue was found based on the *in silico* data of putative structure for SP2027, a conserved hypothetical protein. And thus we decided to focus on SP0609.

The role of SP0609 in adhesion. SP0609 is the amino-acid binding protein of an ABC-transporter. Alignment search of SP0609 reveals that it is highly similar to PEB1, a *C. jejuni* protein, part of an ABC-transporter, and adhesion protein. The pathogenic mechanisms of *C. jejuni* after infection of the human intestinal tract are relatively poorly understood, but involve mucosal adherence, host cell invasion and toxin production. The 28 kDa protein PEB1a, which was highly immunogenic and demonstrated by electron microscopy to be surface exposed (Kervella, Pages et al. 1993), was shown to be required for adhesion of *C. jejuni* to HeLa cells. Importantly, a *peb1a* mutant strain showed decreased adhesion in tissue culture studies, and was markedly deficient in the colonisation

of a mouse model of infection, (Pei, Burucoa et al. 1998) indicating that the PEB1a protein is an important virulence factor in *C. jejuni* (Muller, Leon-Kempis Mdel et al. 2007).

Our data suggest that SP0609 similar to PEB1 plays role in bacterial adhesion during colonization. By incubating the bacteria with anti-SP0609 monoclonal antibodies before adding them to a monolayer of nasopharyngeal cells, the adhesion of pneumococcus could be partially inhibited. The lack of the SP0609 gene resulted in not complete but significant loss of ability to adhere as well. Therefore, these results indicate that SP0609 antigen may transiently interact with the surface of human nasopharyngeal cells, thereby facilitating adhesion to the mucosal surfaces which may lead to a more successful colonization. Pneumococcal attachment to eukaryotic cells is meditated by a diverse group of surface molecules including phosphorylcholine, choline binding proteins, LPxTG proteins, lipoproteins, and non-classical adhesins (Fischetti 2006), which explains, why the lack of one is not resulting in complete loss of adhesion activity. The nasopharynx is the main reservoir for polysaccharide encapsulated bacteria, such as *S. pneumoniae*, *Haemophilus influenzae* type b and *Neisseria meningitidis*. Exposure to these organisms, as in day care centres or after close contact with infected persons, has been shown to be a risk factor for the development of invasive disease (Gray, Converse et al. 1980). Due to its role in adhesion, antibodies against SP0609 antigen may reduce intranasal colonization by *S. pneumoniae* and so reduce the invasion into the middle ear. This would indicate a potential protective effect of anti-SP0609 antibodies at the early stages of infection during the colonization of mucosal surfaces.

These data are encouraging and suggest that SP0609 antigen could be considered as a candidate antigen for protection against pneumococcus, or as a candidate antigen for a combination vaccine against otitis media.

Pneumococcal vaccine candidates as targets of monoclonal antibodies

Advantages and disadvantages of monoclonal antibody therapy. Antibody therapy is – after so many years of negligence- again in the focus of medical research as possibility against viral infections, inflammatory disorders, and certain malignancies. Serum therapy was abandoned because of toxicity associated with the administration of heterologous sera, difficulty in administration, narrow specificity, lot-to-lot variation and expense (Casadevall 1996). But the major development of molecular biological techniques in the past decades and recent advances in the technology of monoclonal antibody production allows to sort out these difficulties reducing side effects of the serum therapy, and monoclonal antibodies became a versatile, low toxic, pathogen specific solution for the emerging antibiotic resistance problem. Mabs are homogenous immunoglobulins that, by definition, recognize one epitope and have markedly higher specific activity than polyclonal preparations, which means greater therapeutic efficacy as well.

The disadvantages of the monoclonal antibody therapy include limited usefulness against mixed infections, and the need for early and precise microbiologic diagnosis, systemic administration and high cost (Casadevall 1996). Pathogen specificity could be a disadvantage in dealing with mixed infections, as infection of multiple *S. pneumoniae* serotypes was recognized as a cause for the failure of serum therapy (Bullowa 1937). For pathogens that are antigenically variable, one solution is to use antibody cocktails of agents active against the most common antigenic types. Timepoint of diagnosis is an issue as well: antibody-based serum therapy for pneumococcal pneumonia was most effective if serum was administered within 3 days of the onset of clinical symptoms (Casadevall and Scharff 1994). Because antibodies are proteins, therapy for invasive infections is likely to require systemic administration, what is a serious disadvantage in developing countries where access to medical care is limited. Furthermore once a hybridoma is made it is a constant and renewable source and all batches will be identical, but time scale is long, and manufacturing of mAbs is expensive.

Serious adverse reactions can occur with high-dose (0.5 to 2 g/kg) antibody therapy, however, with MAb preparations is unlikely to require the high doses of immunoglobulin.

Possible failures at selecting antibodies are the not universal antigen expression during bacterial growth or the immune status of targeted population (Baker 2006; Nagy, Giefing et al. 2008)

Pneumococcal targets of monoclonal antibodies. The prevalence of pneumococcal strains with high-level antibiotic resistance increased the need for new approaches. As a prophylaxis anti-pneumococcal antibodies could be used for flu patients, as a therapeutic, given adjunct to antibiotics, or as a last-resort treatment, when vaccination and antibiotics do not represent preventive or therapeutic options any longer. Although vaccine is the best form of prevention, in certain cases it cannot trigger protection, as in patients, whose immune response are weak or absent, such as elderly or immunocompromised people.

This led us to try to establish a human mAb-based immune therapy and prophylaxis against pneumococcal infectious diseases based on the already selected and characterized antigens as targets for monoclonal antibodies. The selection criteria for a protein-based vaccine antigen and for an antigen targeted by a monoclonal antibody are in many points common: it should be expressed during human disease, it should be highly conserved among the different strains and clinical isolates, accessible for functional antibodies or effector immune cells, it should play role in virulence of the pathogen and/or essential for survival (Nagy, Giefing et al. 2008). A combination of antibodies targeting several different molecules key to bacterial survival or pathogenic mechanisms is expected beneficial, just as with the vaccine antigens. Protein antigens as those, identified with the ANTIGENomes program could be effective targets for monoclonal antibodies in case of mixed infection with various serotypes.

But good vaccine antigens are not always good targets for antibodies. Antibody itself my serve as a coordinating link between the humoral and cell-mediated branches of adaptive immune system (Brady 2005), and so immunomodulation can also play important role by reducing pro-inflammatory cytokine and chemokine expression and enhancing cellular immunity eliciting antibody mediated protection (T_h2 response) in order to reduce inflammatory response, and not leading to a T_h1 or T_h17-response, which would favour inflammation. Weak surface expression, especially if its expression varies with the cell cycle can be unfavourable for a putative antibody target as well. Furthermore the

opsonophagocytic killing activity might be an important feature of the antibodies to achieve strong protection; this information cannot be gathered from the *in vivo* data.

To achieve our aim, we first generated murine monoclonal antibodies against pneumococcal protein vaccine antigen candidates, tested these murine antibodies for antibacterial functionality in *in vitro* and *in vivo* models, and then select the best epitopes to produce human antibodies. To obtain the best protection apart of selecting the epitopes, the best functioning isotype, subtype and the glycolization level of the constant regions has to be determined as well. The most advantageous is to use complete immunoglobulins that can interact with the host's immune cells. For that reason antibody human subclasses IgG1 and IgG3 that have the greatest capacity to activate complement and bind to phagocytic cells through Fc receptors are preferentially chosen (Nagy, Giefing et al. 2008).

The generation of the murine antibodies were outsourced to an external company but the selection of the mAbs was made in-house by Western blot, ELISA and surface staining. Preferentially IgG2a or IgG2b were chosen by *in vitro* assays, because these are the most similar to the human IgG1, and with these subtypes one can expect the best OPK activity. In the cases where no IgG2a or IgG2b were found we selected murine IgG1. The antibodies were tested in our sepsis experiment and showed significant protection against *S. pneumoniae* serotype 6B, but not against the clinical isolate PJ1324 which is a 6B serotype as well. Apart from the virulence we did not find any difference of the genotype, as determined by multi-locus sequence typing, in the two strains (data not shown). The only antibody, which protected the mice from sepsis in all cases, was the anti-PspA mAb clone 7E6D subclass IgG2b, even at a very low dose. The same antibody showed very high OPK activity as well. Unfortunately *S. pneumoniae* is a very diverse bacteria, and since an antibody therapy should cover as many types as possible, we have to be keen on choosing cross-reactive epitopes, which elicits protection in case of mixed pneumococcal infection as well. Furthermore PspA is a protein well accessible on the surface of *S. pneumoniae*; therefore it is easily accessible to anti-PspA monoclonal antibody, unlike other *S. pneumoniae* monoclonal antibody targets, which are not very abundant on the bacterial surface. Therefore, passive immunization is likely not the best mode of protection based on the antigens SP1650, SP1732, SP2216, SP2027, SP2194 and SP0609.

PspA is known to play a major role in the pneumococcal virulence; it binds human lactoferrin and interferes with complement deposition on the bacterial surface (Ren, Szalai

et al. 2003). PspA is relatively variable at the DNA and protein sequence levels; two major alleles have been identified (family 1 and 2) and are present on almost all the pneumococcal isolates from patients with invasive pneumococcal disease. (Hollingshead, Baril et al. 2006) Each PspA family is subdivided into several clades: PspA family 1 contains two clades (1 and 2) and PspA family 2 three clades (3, 4 and 5). PspA from WU-2 (family 1), and PspA from TIGR4 (family 2) for example are not cross-protective in sepsis experiments (unpublished data of Lukas Stulik) A combination of three antigens from family 1 (clade 2) and family 2 (clade 3 and 4) would elicit protection against the vast majority of *S. pneumoniae*

The next step in our plan, the determination of the functionality of the selected antibody is necessary, since combining different functions in a therapy means higher efficacy. Opsonophagocytic antibodies bind to surface structures of the pathogens and enhance their uptake and killing through polymorphonuclear cells and macrophages through complement fixation and binding to Fc receptors. Neutralizing antibodies can act without the assistance of host factors and cells, but their successful interference with bacterial multiplication depends on their ability to neutralize bacterial proteins that have essential functions *in vivo* survival or growth.

As it was mentioned above, in addition to the very potent anti-PspA mAb, more epitopes would be still required targeting other antigens and/or with different mode of action, in order to fight more efficient against bacterial survival or pathogenic mechanisms. Since the antigens selected for the pneumococcus-vaccine as an antibody target showed less efficacy, it would worth to re-analyze our *in vitro* validation data. All should be good candidates since they are by definition recognized by antibodies. Unfortunately *in vitro* is not possible to have stricter selection criteria, to answer the remaining questions; immune serum has to be generated, which is not possible with a big pool of proteins. Display techniques, as surface expression by *E. coli* results in high background as well. To choose more adequate targets for antibodies, re-analysing of *in vitro* data and further literature search for new results might help.

To ease the way of animal models are carried out, we performed initial experiments by bioluminescent pathogenic pneumococcus and fluorescent antibodies. This technique might help to follow disease and bacterial clearance *in vivo*, without scarifying the animals,

this reducing the number of mice used in each experiment. Preliminary results gave hope, but also many difficulties emerged. The sensitivity of the system is very high, but in the same time we have to count with the thickness of the animals; it is possible to create 3D picture, by predicting the dimension of the infection but it also may happen, that for some days the bacteria is "hiding" at lower levels. Taking pictures of the dorsal and the ventral sides of the animal might help in this problem.

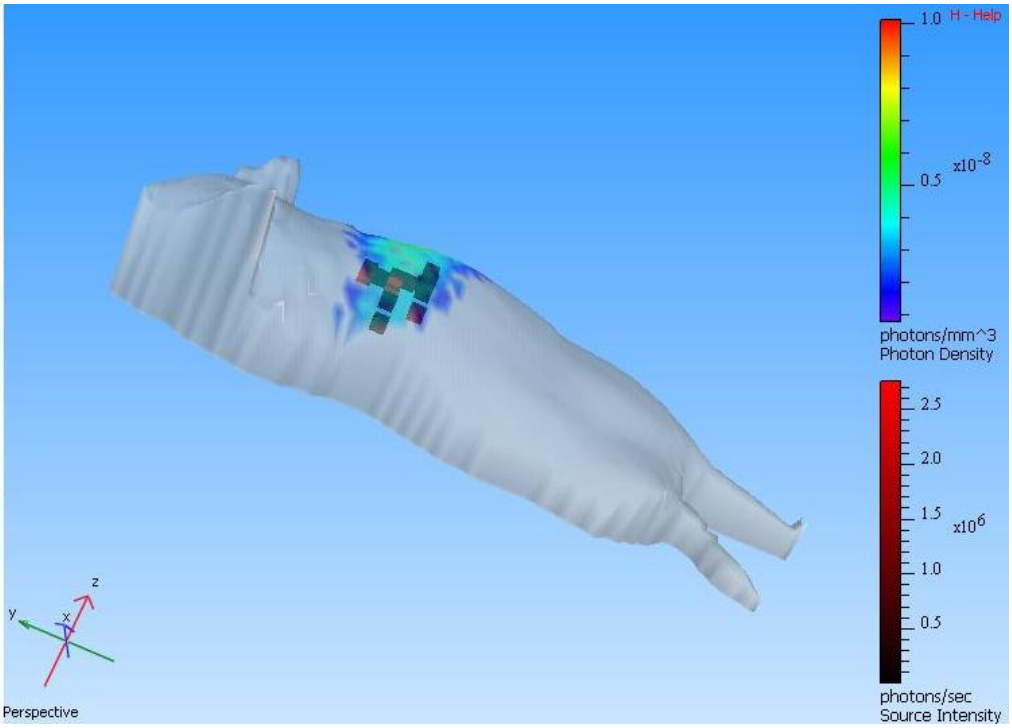

Figure 41: 3D reconstruction of pneumonia in mouse. The picture is not real 3D, assembled from photos done in different depth, but the Xenogen system is able to predict the volume of the infection.

Furthermore the luminescent strains available (in our case XEN11, which is a derivative of EF3030) are not many, and the uncontrolled transformation of the luminescent gene might cause loss of other genes (Francis, Yu et al. 2001). In the future we have to find a model with reliable virulence (has to be comparable to non-luminescent derivatives with the same genetic background) or a different transformation method in order to enter the

luminescent genes to the genome, and antibody which is protective not just in sepsis, but in pneumonia models as well.

Because of the initial success and despite of all the emerging difficulties with the murine antibodies in parallel we started the first experiments with the human antibodies. Anti-SP2216 single-chain variable fragments were generated by using of a Sindbis-based mammalian display with three different epitopes and each connected to human or murine Fc portion. However, similarly to the above mentioned problem with the fully murine antibodies, with our standard sepsis settings using the clinical isolate PJ1324 these antibodies had no protection against pneumococcus. Nevertheless, it is possible that some of these antigens are transiently exposed on the bacterial surface during a specific infection stage (e.g. during adhesion and invasion of human respiratory epithelium) or in a specific tissue environment (e.g. reduced concentration of certain minerals and growth factors favour upregulation of the antigens involved in the uptake of these nutrient sources). For this reason, we decided to find out if they could contribute to protection in any specific disease stage that could be represented by a certain *in vivo* experimental model. Instead of the 24 hours long gap between immunization and challenge, antibody and bacteria was given together. In this way we were able to show significant protection of the scFvs with both murine and human Fc portion. Although this is not the best physiological setting what we could imitate, but it gives us information if there is any sense to go towards to this direction. Obviously testing more human antibodies against other antigens is required to find the best combination of different epitopes and antibody-functions.

An interesting phenomenon was observed in the experiments with the human antibodies. Injecting them into naïve mice, their average half-life in mice is comparable to the half-life of the murine antibodies (over 6 days). But in case of infection the clearance rate of the human antibodies were significantly higher, in less than 4 days it disappeared completely from the blood. Since it happened with the non-streptococcus specific negative control as well, we cannot explain this with the higher binding affinity of the human Fc portions to Fc receptors. It might due to the fragility of the scFvs bound to an Fc. Pneumococcal bacterial degradation of immunoglobulins is known, as the IgA1 protease which cleaves human immunoglobulin Al (IgAl) into Fab and Fc fragments (Proctor and Manning 1990). In the future we have to demonstrate the stability of the scFv versus

natural antiobodies (IgGs), or, in case of easy degradation of them by the pathogens, decide for a different technology to prepare the human antibodies.

Engineering the Fc region of an antibody is crucial to achieve the best characteristics. Improving antibody binding to Fcγ receptors of the complement factors leads to improvement in their therapeutic efficacy (Kim, Park et al. 2005) and glycolisation of the Fc portion may increase the effector function. IgGs are produced as heterogeneous population of glycoforms in mammalian cells, individual IgG moleculaes vary with the respect to terminal galactose or galactose-sialic acids. They also differ with respect to the presence or absence of a fucose residue attached to the core structure of the glycan chains (Kim, Park et al. 2005). The presence of specific oligosaccharide structures was reported to affect the biological activity of the antibody by influencing the interaction with FcγRs (Jefferis, Lund et al. 1998). Modification of the carbohydrate moiety has proven to be a successful approach to enhance antibody dependent cellular cytotoxicity (ADCC) (Shields, Lai et al. 2002; Shinkawa, Nakamura et al. 2003). Glyco-engineered antibodies also performed better than their unmodified counterparts in the depletion of B-cells in a whole blood assay, where both ADCC and CDC contribute to the elimination of target cells (Ferrara, Brunker et al. 2006).

Since mice are not the natural host for *S. pneumoniae,* it is challenging to recapitulate the relevant human diseases in animals. Next to the well-established sepsis model, a functioning pneumonia model has to be set in the near future. Furthermore immunocompromised conditions or age-dependent immundeficiencies are also necessary to mimic, in order to test the antibodies in the most possible way. Combinations of different epitopes with different mode of action are also required to do. Anti-SP221 scFvs will be tested together and then in the future, when more human monoclonals will be generated against the other pneumococcal antigens, the best combination will be determined.

Producing therapeutic sera was very expensive and so, when antimicrobial chemotherapy was first introduced, enthusiasm was expressed for combining serum therapy and antimicrobial chemotherapy. Support for combined therapy came from animal studies, which suggested that combination therapy was more effective than either therapy alone against several pathogens, (Macleod 1939). However, several studies showed that combined therapy was not more effective than antimicrobial chemotherapy alone and that it

caused significantly more side effects (Dowling 1940) Now, with the new generation of antibodies, co-administration of antibiotics and monoclonals has to be considered again: it is very important to test for the synergitic effect of treatment with antibodies and antibiotics in the animal models.

Conclusion

The aim of this thesis was to characterize some well conserved pneumococcal antigens, which could be suitable for a broad protein based vaccine and/or a monoclonal antibody based therapy.

By studying the natural human immune response against pneumococcus, StkP and PcsB and three other antigens (SP0609, SP2027 and SP2194) were selected based on their high level of conservation across a variety of clinical isolates, accessibility for antibodies on the surface, induction of opsonizing antibodies and cross-protection in animal models of invasive disease, as promising targets for a novel pneumococcal vaccine.

Analysis of the gene expression pattern of the ΔstkP (SP1732) and the ΔpcsB (SP2216) pneumococcal strains showed that StkP influences a large number of response regulators, and thus affect activation and deactivation of transcription. PcsB plays a role in the peptidoglycan metabolism and only have a restricted influence on transcription as determined by analysis fo the knock-out strain.

Because of the preliminary in vitro and in vivo and in silico results of the back-up antigens SP0609, SP2027 and SP2194, we decided to focus mainly on SP0609. SP0609 is an amino-acid binding protein of an ABC-transporter and as we found out plays a role in the bacterial adhesion during colonization.

Proteins important in bacterial multiplication are also highly attractive for the development of monoclonal antibody based therapeutics. In order to develop an antibody-based therapy, we generated mouse mAbs against the five above mentioned antigens and two additional (PspA and PsaA) ones, and tested them for their potential as anti-infective agents in various in vitro and in vivo assays. From our studies just one, the anti-PspA mAb provided cross-protection against different strains. The anti-PspA mAb showed strong surface staining and opsonophagocytic killing activity.

Nowadays there are numerous reports on serotype replacement due to vaccination, reducing the effect of Prevnar in European countries such as Finland and the UK, and the absence of community immunity in countries other than the US. All this increases the awareness in the scientific and medical community that polysaccharide based vaccines will not be able to do

the job in providing continuous protection against pneumococcal disease and thus encourages the development of alternative vaccines. Also, especially the most affected populations - the elderly and very poor – still lack appropriate prevention strategies due to either the lack of efficacy of available vaccines or the high costs associated with the capsular polysaccharide conjugates. Besides the complex pneumococcal biology and epidemiology – success of a protein based vaccine will also highly depend on careful planning and design of the crucial clinical trials in order to demonstrate its superiority to polysaccharide based approaches. Despite the competition and challenging tasks we are facing - showing safety and immunogenicity of the IC47 vaccine comprising of PcsB, StkP and PsaA - reassures us to pursuit towards further clinical development in children and elderly.

The emergence of multidrug resistant bacteria is a growing challenge for the healthcare; current antibiotics are unable to keep nosocomial infections in check. The recent inclusion of monoclonal antibodies into the arsenal of novel therapies revitalizes a historical concept of using antibodies in treating and preventing bacterial infections. Adaptive immunity to extracellular bacteria, such as the S. pneumoniae, is largely conferred by antibody. Antibodies specific for both bacterial polysaccharide and protein antigens was shown to protect the host from infections with otherwise lethal pneumococcal strains. On the way of finding protective vaccine antigens by the ANTIGENome technology we discovered other proteins playing role in the virulence and metabolism of pneumoccous. These proteins might be promising targets of a monoclonal antibody therapy which still requires further in vitro and in vivo screening of the epitopes, and developing an efficient technology to engineer and produce the antibodies against them.

Abbreviations

cfu: colony forming units

CPS: capsular polysaccharide

ko: knock-out

mAb: monoclonal antibody

PcsB: secreted 45 kd protein, SP2216

PsaA: pneumococcal surface adhesin A, SP1650

PspA: pneumococcal surface protein A, SP0117

scFv: single-chain variable fragment

StkP: serine/threonine protein kinase, SP1732

wt: wild-type

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