DIPLOMARBEIT

Titel der Diplomarbeit

„The role of antibodies against *S. pneumoniae* vaccine antigens in neutralizing colonization and invasion of human epithelial cells“

Verfasser

Michael Ehlers

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag.rer.nat.)

Wien, 2011

Studienkennzahl lt. Studienblatt: A 490
Studienrichtung lt. Studienblatt: Diplomstudium Molekulare Biologie
Betreuerin / Betreuer: Univ. Prof. Dr. Alexander von Gabain
Acknowledgements

At first I would like to thank Prof. Dr. Alexander von Gabain for providing me the opportunity to work at Intercell, his interest throughout the time and the strong support.

I also want to thank Dr. Sanja Selak and PhD. Andrea Fritzer whose knowledge, feedback, reflection and support was very important and valuable for this work.

I’m really grateful for all the friendly support I had from my colleagues Barbara Maierhofer, Christine Triska, Mario Aistleitner, Oliver Stein, Petra Schlick and Zehra Visram.

My deepest gratitude goes to my family, for all the support and possibilities they have created for me.
Table of content

1 Abstract .................................................................................................................. 8
2 Zusammenfassung .................................................................................................. 9
3 Introduction ......................................................................................................... 11
  3.1 Disease and epidemiology of Streptococcus pneumoniae .................................. 11
  3.2 Pathogenesis of S. pneumoniae ......................................................................... 12
  3.3 Host immune response to S. pneumoniae .......................................................... 13
    3.3.1 Innate immune response to S. pneumoniae ................................................. 13
    3.3.2 Adaptive immune response to S. pneumoniae .......................................... 14
  3.4 Virulence factors of S. pneumoniae .................................................................. 15
    3.4.1 Polysaccharide capsule of S. pneumoniae .................................................. 15
    3.4.2 Pneumococcal surface protein A (PspA) ..................................................... 17
    3.4.3 Choline binding protein A (CbpA/PspC) ..................................................... 17
    3.4.4 Pneumolysin ............................................................................................. 18
  3.5 Treatment and prevention of S. pneumoniae infections ..................................... 19
    3.5.1 Current vaccine approaches and protective antigens ................................. 19
    3.5.2 Intercell’s vaccine approach and possible protective antigens .................... 20
4 Aim of the study .................................................................................................. 23
5 Materials and methods ....................................................................................... 24
  5.1 Materials .......................................................................................................... 24
    5.1.1 Laboratory equipment .................................................................................. 24
    5.1.2 Additional equipment .................................................................................. 25
    5.1.3 Chemicals and reagents ............................................................................. 26
    5.1.4 Buffers ........................................................................................................ 29
    5.1.5 Antibodies .................................................................................................. 31
  5.2 Methods ............................................................................................................. 32
    5.2.1 Bacterial strains .......................................................................................... 32
      5.2.1.1 Cultivation of S. pneumoniae strains ..................................................... 32
      5.2.1.2 Preparation of S. pneumoniae lysates ............................................... 32
      5.2.1.3 Quellung reaction of S. pneumoniae capsule ..................................... 33
5.2.2 General methods of molecular biology ................................................................. 33
  5.2.2.1 SDS-Polyacrylamide Gel Electrophoresis ......................................................... 33
  5.2.2.2 Western blot analysis ......................................................................................... 33
5.2.3 \textit{In vitro} assays .................................................................................................. 34
  5.2.3.1 Adhesion/ invasion assay .................................................................................. 34
  5.2.3.2 Neutralization assay ........................................................................................ 34
  5.2.3.3 Cultivation of eukaryotic cell lines for \textit{in vitro} assays ......................................... 35
5.2.4 Immunofluorescence microscopy of \textit{S. pneumoniae} using antigen specific antibodies
and fluorescent marker .................................................................................................. 35
  5.2.4.1 Staining of \textit{S. pneumoniae} by antigen specific antibodies .............................. 35
  5.2.4.2 Preparation of Detroit 562 cells for immunofluorescence analysis ..................... 36
  5.2.4.3 Fluorescein isothiocyanate isomer 1 (FITC) labeling of \textit{S. pneumoniae} ................ 36
  5.2.4.4 Staining of intra- and extracellular bacteria ........................................................ 36
5.2.5 Surface staining of \textit{S. pneumoniae} grown in chemically defined medium using antigen
specific antibodies analyzed by Flow cytometry ............................................................ 37
5.2.6 Generation of mono- and polyclonal antibodies against \textit{S. pneumoniae} antigens .... 37
  5.2.6.1 Generation of monoclonal antibodies .................................................................. 37
  5.2.6.2 Generation of polyclonal antibodies against ...................................................... 38
6 Results ......................................................................................................................... 39
6.1 Adhesion and neutralization experiments using Detroit 562 cell line ......................... 39
  6.1.1 \textit{Streptococcus pneumoniae} strain 4DS2341-94 shows increased adhesion to and
invasion of nasopharyngeal epithelial cells \textit{in vitro} ....................................................... 39
  6.1.2 Lysate specific sera reduce pneumococcal adhesion to Detroit 562 cells ............... 41
  6.1.3 IC47 specific antibodies do not neutralize adhesion of \textit{S. pneumoniae} strain 4DS2341-
94 to Detroit 562 cells ..................................................................................................... 43
6.2 Immunofluorescence analysis to investigate surface expression of \textit{S. pneumoniae} vaccine
candidate antigens StkP, PsaA and PcsB .................................................................... 44
  6.2.1 PspA can be detected to the same extent on dead and living pneumococci using
polyclonal anti-PspA antibodies .................................................................................... 45
  6.2.2 Pneumolysin is accessible after permeabilization only ......................................... 47
  6.2.3 StkP is accessible for antigen specific antibodies on fixed bacteria only .............. 47
  6.2.4 Extra- and intracellular bacteria cannot be distinguished by co-localization studies
using the eukaryotic EEA1 and neither by labeling of pneumococci with FITC prior to
infection ................................................................................................................................. 48
6.2.5 Detection of intracellular bacteria after prolonged incubation time .......................... 52
6.3 S. pneumoniae IC47 antigen expression under defined growth conditions ......................... 54
   6.3.1 Growth of pneumococci in the presence of Desferal is similar to bacteria grown under standard conditions ........................................................................................................ 55
   6.3.2 PsaA expression was increased under iron- and manganese limiting growth conditions 56
   6.3.3 The expression of PcsB is unaffected during the growth at higher temperatures .... 58
   6.3.4 PsaA and IC47 specific sera give a positive surface staining signal on bacteria grown under iron- and manganese limiting conditions ........................................................................ 59
7 Discussion .................................................................................................................. 62
8 References ............................................................................................................... 70
9 Appendix ................................................................................................................ 77
   9.1 Abbreviations ..................................................................................................... 77
   9.2 Curriculum vitae .................................................................................................. 79
1 Abstract

*Streptococcus pneumoniae* is a gram-positive, alpha-hemolytic bacterium that causes pathogenic infections in humans leading to pneumonia, otitis media, acute sinusitis, meningitis, bacteremia and sepsis. The bacterium is found in the nasopharynx of 5-10% of healthy adults and up to 60% of children. Despite the availability of vaccines against *S. pneumoniae*, pneumococcal infections are still a burden especially for elderly and children in developing countries. Therefore, Intercell aims to develop a protein-based vaccine that is protective in all age groups.

Intercell’s ANTIGENome approach has led to the selection of two lead candidates StkP and PcsB together with PsaA (IC47 antigens). In this study we wanted to characterize the role of antibodies against these three *S. pneumoniae* vaccine antigen candidates in neutralizing colonization and invasion of human epithelial cells. IC47 antigen expression and accessibility under various *in vitro* growth conditions was investigated by using western blot analysis and surface staining experiments. It was found that antibodies targeting IC47 antigens StkP, PsaA and PcsB do not reduce pneumococcal adhesion to the human nasopharyngeal cell line Detroit 562, whereas lysate specific antibodies reduced adhesion by 50-72% compared to the corresponding preimmune serum. Immunofluorescence microscopy experiments of *S. pneumoniae* strain 4DS2341-94 in close contact to the epithelial cell line Detroit 562 suggest a crucial role of the polysaccharide capsule for antigen accessibility, since PsaA was accessible only after prolonged contact to human epithelial cells. Western blot analysis confirmed the expression of the three candidate antigens in all tested strains *in vitro*. Further, bacteria were grown under iron and manganese limiting conditions to mimic the physiological conditions found at the human mucosa. These experiments revealed an increased expression of PsaA compared to pneumococci cultivated in chemically defined complete medium. Following surface staining experiments with pneumococci grown under iron and manganese limiting conditions also indicated an increased PsaA expression. In this study we found that there was no reduced pneumococcal adhesion to human epithelial cells in the presence of IC47 antigen specific antibodies, *in vitro*. Further *in vitro* and *in vivo* experiments are required to characterize the protective potential of IC47 specific antibodies.
2 Zusammenfassung


Charakterisierung funktionaler IC47 spezifischer Antikörper sind weitere \textit{in vitro} und \textit{in vivo} Experiment notwendig.
3 Introduction

3.1 Disease and epidemiology of Streptococcus pneumoniae

*S. pneumoniae* is an extracellular, gram-positive, alpha-hemolytic, lancet-shaped human pathogen, initially discovered in 1881 by Leo Escolar and isolated first by Louis Pasteur and George Sternberg. It is one of the most important human pathogens and is the worldwide leading cause of meningitis, bacteremia, pneumonia and also approximately one million of cases of otitis media in children.

Pneumococcus is spread through the contact between people who are ill or who carry *S. pneumoniae* in their throat. Pneumococcal carriage is common among young children. The colonization-rate of infants in the industrial countries is 60% whereas in developing countries it’s even higher reaching almost 100% during the first months of life (Coles et al., 2001). Despite almost all children becoming colonized only few of these acquisitions lead to invasive disease. The incidence of *S. pneumoniae* caused invasive disease is highest in infants below the age of 2 and in elderly over 65 years of age (Garcia-Leoni et al., 1992; Gray, Converse, and Dillon, 1979).

When *S. pneumoniae* progresses in the alveoli and blood circulation system of the human body the pathogen can cause a vast number of invasive diseases (Fig. 1). Among these is bacteremia, a disease with high mortality of 25-29% which remained stable in the last four decades and is a threat especially for the elderly (Wuorimaa et al., 2002, Gillespie et al., 1989). Another invasive disease is pneumonia an inflammatory condition of the lungs and a vast problem in developing countries. It leads to the death of one million young children every year and similar numbers of deaths also occur in adults, especially elderly (Fedson and Scott, 1999). The mortality from pneumonia generally decreases with age until late adulthood. However, elderly individuals are at particular risk for pneumonia and associated mortality. Furthermore there is the danger of community-acquired meningitis an inflammatory condition of the meninges which is still associated with a substantially high mortality, ranging from 20-50% dependent on the developmental state of the country. Thirty to 60% of the survivors suffer long-term effects such as neurological deficits, hearing loss and neuropsychological impairment (Koedel et al., 2002). Pneumococcal infections may also cause non-invasive
disease such as conjunctivitis and sinusitis (Crum et al., 2004, Bogaert et al., 2004). When \textit{S. pneumoniae} invades the auditory tubes it can lead to acute otitis media, a disease with high incidence in children. During the first two years of life 62% of the children suffer from at least one episode (Wuorimaa et al., 2002).

Due to the high medical need great efforts are being made to develop effective vaccines to prevent pneumococcal disease in both industrialized and developing countries.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure1.png}
\caption{Pathogenic route of \textit{S. pneumoniae} infection (Bogaert, De Groot, and Hermans, 2004)}
\end{figure}

3.2 \textbf{Pathogenesis of \textit{S. pneumoniae}}

Establishment of the nasopharyngeal flora occurs during the first months of life and consists of many different bacteria. At least once in a life everyone will be colonized by \textit{S. pneumoniae}. Pathogens have acquired many different mechanisms to evade or delay human host defenses in order to reach sterile parts of the body where they can cause severe disease. Pneumococcal colonization of the host requires adhesion to nasopharyngeal epithelial cells and is a key step in \textit{S. pneumoniae}’s pathogenesis. During that process surface-exposed bacterial adhesins interact with extracellular matrix proteins or bind to host cell receptors. Furthermore, adhesion of \textit{S. pneumoniae} to epithelial cells is also achieved via carbohydrates (Cundell et al., 1995), surface proteins such as PsA (Anderton et al., 2007) as well as pilus-
like structures of some isolates (Barocchi et al., 2006). Studies have shown an increased pneumococcal carriage during infancy (Vives et al., 1997) emphasizing the high risk for this age group. In general pneumococcal colonization is not followed by symptomatic disease but may lead to the progression of the pathogen into the lungs and the blood causing invasive disease if the host is not able to clear such infection (Garcia-Rodrigues et al., 2002; Ghaffar et al., 1999).

Nasopharyngeal colonization, the ability to evade the early immune response and to breach the epithelial barrier are a prerequisite for invasive pneumococcal disease. Host-pathogen interactions involved in colonization further lead to the uptake of *S. pneumoniae* into and therefore the invasion of the host cells. Furthermore translocation across the mucosal barrier includes interaction of adhesins with cellular receptors such as CbpA (also referred to as PspC) binding to human polymeric immunoglobulin receptor (hpIgR) involved in IgA transcytosis (Zhang et al., 2000) or bacterial teichoic- and lipoteichoic phosphoryl-choline binding to epithelial and endothelial platelet-activating factor receptor (PAF-r) followed by receptor-mediated endocytosis (Cundell et al., 1995). Once *S. pneumoniae* reaches the sterile parts of the human body, bacteria multiply. Bacterial lysis and the release of the cytotoxin pneumolysin as well as cell wall components induce a heavy inflammation response in the infected organs leading to critical organ damage. The mechanisms involved in generation of a divergent cellular response creating different damage reactions in infected organs still remain unknown. Clinical observations describe full recovery of the pulmonary architecture after pneumonia but irreversible damage of the brain following meningitis (Thornton, Durick-Eder, and Tuomanen, 2010).

### 3.3 Host immune response to *S. pneumoniae*

The physiologic function of the human immune system is defense against infectious microbes. The immune response to a pneumococcal infection is multifarious and involves several aspects of the innate and adaptive immune system.

#### 3.3.1 Innate immune response to *S. pneumoniae*

The early non-specific immune response of the innate immune system is critical for slowing and preventing pneumococcal infections, allowing the adaptive immune system to
respond, if necessary. The main site of interaction between host and pneumococci is the respiratory tract which is lined by continuous epithelia providing a physical barrier against the pathogen. If \textit{S. pneumoniae} successfully breaches this barrier, it encounters macrophages in the subepithelial tissue. Macrophages are phagocytic cells which express germ-line encoded host molecules, called pattern recognition receptors (PRR), recognizing conserved structures on the pneumococcal surface (Krieger, 1997). One sub-class are toll-like receptors (TLR) whose activation leads to the secretion of pro-inflammatory cytokines (Ozinsky et al., 2000). For example the lipoteichoic acid (LTA) component of the pneumococcal cell wall is recognized by TLR2 (Seo, Michalek, and Nahm, 2008). It was reported that a lack of TLR2 in mice leads to an increased severity of meningitis in a meningitis mouse model due to the missing immune response initiated by TLR2 (Echchannaoui et al., 2002). Another macrophage expressed receptor is C-type SIGN-related 1 (SIGN-R1) binding to capsular pneumococcal polysaccharides activating the classical complement pathway, thus initiating phagocytosis of \textit{S. pneumoniae} (Kang et al., 2004). Furthermore it was shown that mice lacking SIGN-R1 have an increased susceptibility to systemic and pulmonary infections (Lanoue et al., 2004). Pneumococci that are able to evade these defense mechanisms may enter the blood stream where they encounter complement proteins, plasma components of the innate immune system (Davis, Hurt, and Bollet, 1963). Furthermore, if these complement proteins get activated by the pneumococcal surface their cleavage products stimulate inflammation and coat the pneumococci enhancing their phagocytosis by macrophages.

### 3.3.2 Adaptive immune response to \textit{S. pneumoniae}

The adaptive immune system consists of the humoral immune response and cell-mediated immunity composed of highly specialized cells which recognize and remember specific bacterial structures. In fact, the response of the adaptive immune system against \textit{S. pneumoniae} mainly involves the production of specific antibodies against capsular polysaccharides and surface proteins. After 6 months of life mucosal immunity is already present due to the acquisition of anti-capsular IgG and IgA antibodies (Simell et al., 2001). These specific IgG antibodies exert different effector functions, mainly opsonization of pneumococci for enhanced phagocytosis and activation of the classical complement pathway that further leads to local inflammation, recruitment of phagocytes and the attachment of antigens to the phagocytes (Vitharsson et al., 1994). Moreover antigen-specific antibodies can
neutralize adhesins and prevent pneumococcal adhesion and invasion. For example, it was reported that anti-CbpA sera could induce decreased pneumococcal adhesion and invasion to eukaryotic host cells (Zhang et al., 2000).

In addition to the natural acquisition of specific antibodies, the cellular immune response also contributes to the protection against pneumococcal infections. Cell-mediated immunity is mediated by T-lymphocytes and their products, for example IFN-γ a potent macrophage activator (Abbas et al., 2007). Furthermore recent studies have shown that pneumococcal specific CD$^+$ and Th17 cells might also contribute to reduced duration of carriage and prevent colonization (Malley et al., 2005; Moffitt et al., 2011; Schmid et al., 2011). Intranasal immunization experiments using purified pneumococcal proteins PsaA and CbpA indicated a CD$^+$ T-cell dependent, antibody-independent protection further emphasizing the role of the cellular immune response for protection against pneumococcal colonization (Basset et al., 2007).

In conclusion, for a good protection against pneumococcal infections both innate and adaptive immunity are required, including the PRRs, complement system as well as antigen-specific antibodies for an enhanced opsonization and clearance of the pathogen.

### 3.4 Virulence factors of *S. pneumoniae*

Virulence factors are molecules which are expressed and secreted by *S. pneumoniae* and required for pneumococcal adhesion, invasion, inhibition of the host immune response and nourishment with nutrients.

#### 3.4.1 Polysaccharide capsule of *S. pneumoniae*

*S. pneumoniae’s* outermost layer consists of polysaccharides forming a 200-400 nm thick capsule (Skov Sorensen et al., 1988). The biochemical and antigenic properties of the capsule are the basis for differentiation of the existing $>90$ serotypes. With the exception of a few serotypes, the capsule is covalently attached to the thick peptidoglycan layer (Cartee, Forsee, and Yother, 2005).
The pneumococcal capsule is important for the pathogenicity of the pathogen. Despite the fact that also non-encapsulated strains can be associated with pneumococcal infection such as conjunctivitis (Crum et al., 2004), most clinical isolates found in sterile sites of the body are encapsulated. Only encapsulated pneumococci can safely transit from the luminal mucus of the entrance site to the epithelial surface, which represents the initial step of colonization. The regulation of capsule thickness in different host niches is essential to ensure the pathogen’s survival at different locations in the human body. These virulent pneumococci can occur in two different colony variants either as transparent- or opaque-phase distinguished by low or high amounts of capsule production, respectively. Differences in capsule thickness seem to affect pneumococcal adhesion and invasion substantially. While the phenotype producing a thicker capsule was shown to be more virulent in systemic infections, the transparent-phase phenotype is more efficient in colonizing the mucosal surfaces of the nasopharynx (Nelson et al., 2007; Weiser et al., 1994; Weiser et al., 1996). Interestingly, the comparison of serotype distribution revealed that some serotypes have an increased invasive potential (serotype 1, 4, 5, 7F, 8, 9V, 14) whereas others can be associated with colonization of the upper airways (serotype 3, 6A, 7, 10, 11, 19F, 23F) (Kellner et al., 1998; Kronenberg et al., 2006; Robinson et al., 2001; Saha et al., 2003).

Besides the essential role of the capsule in colonization and invasion of human nasopharyngeal epithelial cells it is also important for the evasion of the human immune system. The capsule serves as major virulence factor by preventing the pathogen from entrapment in mucus (Nelson et al., 2007) and complement mediated opsonophagocytosis. *S. pneumoniae* has acquired several mechanisms preventing opsonophagocytosis by impairing IgG, iC3b and CRB binding to the bacterial surface (Musher, 1992), decreased cleavage of C3b and also reduced phagocytosis by complement- and Fcγ-receptors (Hyams et al., 2010). Furthermore, under physiological conditions highly charged capsular polysaccharides prevent phagocytosis by human phagocytes (Lee, Banks, and Li, 1991). Differences in capsule thickness affect adhesion and invasion substantially. The feature of regulating the CPS production in different host niches is essential to ensure the pathogen’s survival at different locations in the human body.

All in all, the capsule of *S. pneumoniae* is an important virulence factor contributing to the successful survival of the pathogen within the host.
3.4.2 Pneumococcal surface protein A (PspA)

PspA is serologically variable and highly immunogenic in humans and mice. The molecular weight depends on the highly variable N-terminal sequence ranging from 66-100 kDa (Crain et al., 1990; Jedrzejas, Lamani, and Becker, 2001). Based on the sequence it can be grouped into three families and furthermore into six different clades. PspA consists of three structural domains: a charged α-helical domain, a proline-rich region and a C-terminal choline-binding domain which anchors the protein to membrane-associated lipoteichoic acids on the pneumococcal surface (Yother and Briles, 1992). It serves as an important virulence factor, since it is able to interfere with complement opsonization (Tu et al., 1999) and to bind lactoferrin (Hakansson et al., 2001), facilitating iron acquisition. In animal models active immunization with PspA leads to a good immune response and the production of antibodies against PspA which are able to provide protection against colonization, invasive disease and otitis media (White et al., 1999; Wu et al., 1997a).

Taken together, pneumococcal surface protein A is an important virulence factor involved in iron acquisition and immune evasion.

3.4.3 Choline binding protein A (CbpA/PspC)

Another pneumococcal surface protein is CbpA found in 73% of all isolates (Kaetzel, 2001). The protein is involved in immune evasion. The highly conserved 12-amino acid motif within the N-terminus binds to Factor H (Lu, Ma, and Zhang, 2006), a negative regulator of the antibody-independent alternative complement pathway. It was shown that the binding of CbpA to Factor H is also involved in a two step uptake of \emph{S. pneumoniae} into epithelial cells (Agarwal et al., 2010). This finding is in accordance with the up-regulation of CbpA upon contact to epithelial cells (Orihuela et al., 2004b). Moreover, it is required for translocation to the lower respiratory tract (Orihuela et al., 2004a). Furthermore, CbpA binds to human polymeric immunoglobulin receptor (hpIgR) via a conserved hexapeptide motif (Hammerschmidt et al., 2000) enhancing pneumococcal adhesion and invasion to hpIgR-expressing cells (Zhang et al., 2000) taking advantage of the retro-graade transport back to the basolateral epithelial surface (Fig. 2). In addition, it has been shown that CbpA deficient
strains are 100-fold less efficient in colonizing the nasopharynx of infant rats (Rosenow et al., 1997).

CbpA is another virulence factor of *S. pneumoniae* that has various functions such as immune suppression as well as pneumococcal adhesion and invasion.

![Figure 2: Schematic view of *S. pneumoniae* transcytosis via the hplgR pathway. Free or polymeric IgA-bound plgR is proteolytically cleaved at the apical site of the epithelium. Leading to the release of SC or S-IgA. When uncleaved hplgR is internalized at the apical surface and transcytosed to the basolateral surface *S. pneumoniae* takes advantage by binding to hplgR. (Kaetzel, 2001).](image)

### 3.4.4 Pneumolysin

Another virulence factor is the pore forming cytotoxin pneumolysin which is produced by all known clinical isolates (Jefferies et al., 2010). Upon secretion into the extracellular space it causes inflammation and damage of endothelial and epithelial cells, facilitating intrapulmonary growth of *S. pneumoniae* and invasion into the blood (Rubins et al., 1996). Moreover, the cytotoxin is also released by pneumococcal autolysis. In addition, pneumolysin is involved in immune evasion of *S. pneumoniae*. Its secretion might divert the immune system by activating the classical pathway specific to the toxin reducing complement protein levels in close vicinity of the pneumococcal cell (Mitchell et al., 1991). Furthermore, it was shown that gene deletion of pneumolysin leads to an increased opsonophagocytosis by the classical pathway (Yuste et al., 2005).
Both pneumolysin’s complement and hemolytic activities contribute to the pathogenesis of \textit{S. pneumoniae}.

3.5 Treatment and prevention of \textit{S. pneumoniae} infections

As already mentioned before \textit{S. pneumoniae} remains the most common cause of bacteremia, bacterial meningitis, community acquired pneumonia, otitis media and is responsible for the death of more than one million people per year worldwide. The incidence of invasive disease is highest in children below the age of two and in elderly >65 years of age (Wuorimaa and Kayhty, 2002). A number of pneumococcal infections can be treated by the administration of antibiotics. However, increasing rates of multi-drug resistant strains are becoming an additional threat (Flamaing, Verhaegen, and Peetermans, 2002; Ruhe and Hasbun, 2003; Shouval et al., 2010). Because of the high medical need, great efforts are being made to develop vaccines which can effectively prevent pneumococcal disease in industrial and developing countries. None of the current clinically used vaccines does provide full protection in children and elderly.

3.5.1 Current vaccine approaches and protective antigens

There are two different types of vaccines in clinical use: pneumococcal polysaccharide vaccine and conjugated polysaccharide vaccines. In the year 1977 Merek’s 23-valent polysaccharide vaccine (Pneumovax23), providing protection against 23 different serotypes was licensed. The 23-valent vaccine approved for adults is efficacious against bacteremia and meningitis in the elderly population, but not against pneumonia, the most prevalent disease in this age group (Jackson et al., 2003; Mangtani, Cutts, and Hall, 2003). Moreover, capsular polysaccharides are not immunogenic enough in young children (Sankilampi et al., 1996) and antibody levels rapidly decrease few months after immunization (Koskela et al., 1986). In 2000, the federal drug administration (FDA) licensed the conjugated 7-valent vaccine Prevnar7 that is highly effective in preventing invasive disease in young children and also has a significant impact on otitis media (Black et al., 2000; Black et al., 2001). Furthermore, the conjugated CPS-vaccine induces both, a B- and T-cell dependent immune response, providing mucosal immunity, hence conferring protection against invasive disease (Pletz et al., 2008). In
The conjugated 13-valent vaccine Prevnar13 was licensed in the US covering 13 of the most prevalent serotypes.

The advantage of conjugated vaccines over the pure polysaccharide pneumococcal vaccines is their high efficiency in preventing invasive disease as well as proved immunogenicity in infants and reduced nasopharyngeal carriage of vaccine covered serotypes (Hsu et al., 2009; Mbelle et al., 1999). Furthermore, all pneumococcal vaccines are directed against *S. pneumoniae*’s capsular polysaccharides (CPS) which are essential for the bacteria’s virulence and survival within its host. Nevertheless, the production of high-valency polysaccharide vaccines is expensive and only covers a minority of all 91 serotypes known to date. Besides that, conjugate-PS vaccines are difficult to manufacture, require multiple injections (Sigurdardottir et al., 1997) and do not induce a specific Th-cell memory (van Essen et al., 2000). Another problem we are facing is vaccination induced serotype replacement resulting in an increased prevalence of pneumococcal infections caused by non-vaccine covered serotypes (Ansaldi et al., 2011; Singleton et al., 2007). In addition, clinical studies have shown that current conjugated pneumococcal vaccines do not protect against pneumonia the most prevalent pneumococcal disease in the elderly aged >65 (Egger et al., 2009).

In conclusion, currently available pneumococcal vaccines only partially cover *S. pneumoniae* serotypes and the observed serotype replacement demonstrates the limitations of polysaccharide based pneumococcal vaccines. They were shown to be efficacious against meningitis and bacteremia but not against pneumonia especially in children and elderly. Thus, alternative pneumococcal vaccine approaches are needed. One promising approach is the use of recombinant protein-based vaccines in order to provide cross-protection against different serotypes.

### 3.5.2 Intercell’s vaccine approach and possible protective antigens

Intercell’s approach for a new-generation *S. pneumoniae* vaccine is based on recombinant proteins. There are several criteria which have to be fulfilled by an antigen in order to serve as a good vaccine candidate. The antigen must be immunogenic, should be conserved in all/most serotypes as well as expressed and surface exposed to be accessible for
functional antibodies. Moreover, to avoid cross reactivity there should be no homology to any human proteins.

A protein based vaccine might provide broad protection against pneumococcal infections caused by all *S. pneumoniae* serotypes, effective in children and elderly. Intercell’s approach, the ANTIGENome technology (Meinke et al., 2005) was applied in order to find antigens which are conserved among pneumococcal strains and serotypes. The use of this technology has lead to the selection of 2 led candidates SP1732 (StkP) and SP2216 (PcsB) (Giefing et al., 2008). SP1732 and SP2216 are part of IC47 together with SP1650 (PsaA) which had already previously been tested in a human phase I study.

**SP1732 - Serine/threonine protein kinase (StkP)**

SP1732 is a serine/threonine protein kinase with a molecular weight of 100 kDa and is highly conserved among various *S. pneumoniae* serotypes (Giefing et al., 2008). Compared to many other microorganisms, *S. pneumoniae* and other streptococci contain only a single gene encoding a Ser/Thr protein kinase (Novakova et al., 2010). There are many essential functions of prokaryotic STPKs targeting various cellular functions such as signaling, transport and metabolic processes, biofilm formation and also virulence in streptococci (Echenique et al., 2004). The N-terminal part of StkP comprising the kinase domain, co-localizes with FtsZ and is involved in cell-division. The C-terminal PASTA domains are surface exposed in exponential growth-phase (Yeats, Finn, and Bateman, 2002) and reported to sense unlinked Peptidogylcan (Giefing et al., 2010; Maestro et al., 2011). Giefing and colleagues showed that deletion of the StkP encoding gene led to a decreased virulence in mice. For Intercell’s IC47 vaccine the N-terminal located eukaryotic-type serine threonine kinase domain was excluded, since it shows about 35% identity to a human protein. Instead, the 314-amino acid C-terminal fragment of StkP was used as candidate antigen.
SP2216 - Protein required for cell separation in group B streptococci (PcsB)

PcsB is a peptidoglycan hydrolase and has a molecular weight of approximately 43 kDa consisting of 392 amino acids. During exponential growth the N-terminal part is surface exposed, whereas the C-terminal part is not (Giefing et al., 2008). In addition, the protein is located in the plasma-membrane and secreted into the growth environment. PcsB gene deletion experiments revealed its important role in cell wall metabolism, cell division as well as pneumococcal survival *in vitro* and *in vivo* (Giefing et al. 2008). Another interesting aspect is the up-regulation under certain stress-conditions such as increased temperature or osmolarity (Mills, Marquart, and McDaniel, 2007). Based on Intercell’s ANTIGENome approach, PcsB was one of the most immunogenic proteins in humans. Natural antibodies against PcsB are developed in childhood and disease (Giefing et al., 2008).

SP1650 - Pneumococcal surface adhesin A (PsaA)

PsaA was not identified by Intercell’s ANTIGENome technology. SP1650 is a 37 kDa surface protein expressed in all 90 serotypes and has been reported to mediate pneumococcal binding to nasopharyngeal Detroit-562 cells via the trans-membrane glycoprotein E-Cadherin (Anderton et al., 2007). Furthermore, it was found to be a member of an ATP-binding cassette transporter complex which is essential for the uptake of manganese, zinc and ferrous ions (Dintilhac et al., 1997; Russell et al., 1990). Micronutrients like divalent cations are essential and required for growth and survival of bacteria in various environmental niches especially in those within higher organisms. Recent studies revealed the central role of manganese in the regulation of stress response, physiology and metabolism in *S. pneumoniae* (Ogunniyi et al., 2010). Interestingly, intranasal immunization with a mixture of PspA and PsaA has been shown to prevent nasopharyngeal carriage in mice (Briles et al., 2000).
4 Aim of the study

*Streptococcus pneumoniae* vaccines licensed to date are all based on capsular polysaccharides, manufactured either pure or conjugated to an immunogenic carrier protein. Besides the good protection against the included serotypes there are quite some disadvantageous aspects which do favor the development of new generation vaccines using recombinant proteins. Intercell’s ANTIGENome technology has identified several potential protein candidates. After a multistep process these candidates were further circumscribed down to PcsB, StkP and PsaA (Giefing et al., 2008).

The aim of this study was to support the development of protein based vaccine against *S. pneumoniae* which is protective in infants and elderly. The biological functions of antibodies are versatile. Here wanted to investigate the role of antibodies specific for IC47 antigens in preventing pneumococcal colonization of the nasopharynx, thus providing protection against invasive disease caused by the pathogen. Further we wanted to study accessibility and expression of PsaA, StkP and PcsB under various *in vitro* growth conditions using Western blot analysis, immunofluorescence microscopy and flow cytometry.
5 Materials and methods

5.1 Materials

5.1.1 Laboratory equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer and Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blockheater (shaker)</td>
<td>MR3001 K8 Heidolph, Germany</td>
</tr>
<tr>
<td>Centrifuges</td>
<td>HERAEUS Megafuge 2.0, Kendro Lab. Products, England</td>
</tr>
<tr>
<td></td>
<td>HERAEUS Biofuge Fresco, Kendro Lab. Products, England</td>
</tr>
<tr>
<td></td>
<td>HERAEUS Multifuge 3S-R, Kendro Lab. Products, England</td>
</tr>
<tr>
<td></td>
<td>HERAEUS Multifuge 4KR, Kendro Lab. Products, England</td>
</tr>
<tr>
<td>Cytomics FC500</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>GLF Rocking Shaker 3011</td>
<td>Gesellschaft für Labortechnik, Germany</td>
</tr>
<tr>
<td>iBlot dry blotting device</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Incubator</td>
<td>HERA Cell 150, Kendro Lab. Products, England</td>
</tr>
<tr>
<td></td>
<td>HERA Cell, Kendro Lab. Products, England</td>
</tr>
<tr>
<td>Laminar flow</td>
<td>HERA Safe Laminar Flow KS12, Kendro Lab.</td>
</tr>
<tr>
<td></td>
<td>Products, England</td>
</tr>
<tr>
<td>Laser scanning microscope</td>
<td>LSM 510 META</td>
</tr>
<tr>
<td>Microscope</td>
<td>Axioskop2 plus; Axio color cam, Zeiss, Germany</td>
</tr>
<tr>
<td>OPTIMAX X-ray Film processor</td>
<td>Modell 1170-1-0000 Protec Medizintechnik</td>
</tr>
<tr>
<td>pH-meter</td>
<td>WTW inoLab pH720</td>
</tr>
<tr>
<td>Pipette boy Cell Mate II</td>
<td>Matrix Technology Corporation, England</td>
</tr>
<tr>
<td>Powersupply Power Pac</td>
<td>Bio-Rad, USA</td>
</tr>
</tbody>
</table>
Scales

PLJ4000-2M Kern & Sohn GmbH, Germany
Sartorius CP224S, Germany
XS6002S METTLER TOLEDO, Germany
Sonoplus HD2070, UW2070 BANDELIN, Germany
Synergy 2 Multi-Mode ELISA reader BioTek, Germany
Vortex Genie-2 G-560E New York, USA

5.1.2 Additional equipment

A549 cells ATCC
BCA™ Protein Assay Kit Thermo Scientific
Sheep blood agar plates Biomerieux
Calibration solution pH 4, 7, 10 HANNA instruments, Romania
Centrifugal device Amicon 10K Millipore
Cover glass No.1 18x18mm VWR international
Cover glasses, diameter 13mm No.1 Thermo Scientific
Detroit 562 cells ATCC
ECL detection Kit GE Healthcare
EMEM Sigma
F12K Medium GIBCO
iBlot Gel Transfer stacks (nitrocellulose) Invitrogen
Microscope slides VWR international
Neubauer counting chamber Labor Optik
Non-reducing sample loading buffer Thermo Scientific
Nunclon multidish 24-well plate NUNC
Poly Prep™ Slides Sigma
Precision Plus Protein dual color standard BioRad
Prolonged Gold anti-fade w/ DAPI
SDS-page PAGEr Gold Precast gels
SPIN-X 0.22 μM
Steriflip 0.22 μM pore-size
Stericup 0.22 μM pore-size

5.1.3 **Chemicals and reagents**

2-Mercaptoethanol
Adenine hydrochloride
Aceton
Albumin Bovine Serum Fraction V
β-NAD
Biotin
Calcium chloride-hexahydrate
Choline chloride
Copper(II) sulfate, pentahydrate
Cyanocobalamin
D-pantothenic acid
D-(+)-Glucose
Ethanol
Fetal Bovine Serum
FITC isomer 1
Folic acid
Gentamycin
Glacial acetic acid
Glycine
Guanine hydrochloride  Sigma
HBSS  GIBCO
Hydrochloric acid conc. 37%  Fluka
Iron (II) sulfate, heptahydrate  Sigma-Aldrich
Iron (III) nitrate, nonahydrate  Sigma-Aldrich
Kanamycin sulfate  Sigma-Aldrich
L-Alanine  Fluka
L-Arginine  Sigma-Aldrich
L-Aspartic acid  Fluka
L-Cysteine  Fluka
L-Glutamic acid  Fluka
L-Glutamine  Fluka
L-Histidine hydrochloride  Sigma
L-Isoleucine  Sigma-Aldrich
L-Leucine  Fluka
L-Lysine hydrochloride  Fluka
L-Methionine  Fluka
L-Phenylalanine  Fluka
L-Proline  Fluka
L-Serine  Fluka
L-Threonine  Fluka
L-Tryptophan  Sigma-Aldrich
L-Tyrosine  Sigma
L-Valine  Fluka
Lysozyme  Sigma-Aldrich
Manganese sulfate  Sigma
Manganese sulfate, monohydrate  Sigma
Mutanolysin  Sigma-Aldrich
Niacinamide  Sigma
Non-essential amino acids  Sigma
p-aminobenzoic acid  Sigma
PBS  GIBCO
Ponceau S solution  Sigma
Potassium phosphate dibasic, tri-hydrate  Sigma-Aldrich
Potassium phosphate monobasic, anhydrous  Sigma
Pyridoxal-hydrochloride  Sigma
Pyridoxamine di-hydrochloride  Fluke
Pyridoxine  Sigma-Aldrich
Riboflavin  Sigma
Saponin  Sigma-Aldrich
Skim milk powder  Fluka
Sodium acetate, tri-hydrate  Sigma
Sodium bicarbonate  Fluka
Sodium Dodecyl Sulfate  GIBCO
Sodium hydroxide  Sigma-Aldrich
Sodium phosphate dibasic, anhydrous  Sigma
Sodium phosphate monobasic, mono-hyd.  Sigma
Sodium pyruvate  GIBCO
Thiamine hydrochloride  Sigma
Trypsine 0.25% EDTA  GIBCO
Tween 20  Sigma-Aldrich
Triton X-100  Fluka
Uracil Sigma

5.1.4 Buffers

PBS-T PBS/0.1% Tween-20
running buffer (10x) 25 mM Tris-HCl 192 mM glycine
SDS-running buffer (1x) 1x running buffer/0.1% SDS

Chemically defined medium (CDM) for growth of *S. pneumoniae* (Reconstituted CDM referred to van de Rijn and Kessler, 1980)

For the preparation of 500 ml chemically defined medium, add the following components to 360 ml dH₂O while mixing vigorously, dissolve each component completely before adding the next one.

- 0.25 ml Iron (II) sulfate heptahydrate (2,000x) – add only to complete medium
- 0.05 ml Iron (III) nitrate nonahydrate (10,000x) – add only to complete medium
- 0.1g potassium phosphate dibasic, anhydrous
- 0.5g potassium phosphate monobasic, anhydrous
- 1 ml MgSO₄ (500x)
- 0.5 ml MnSO₄ (1000x) – add only to complete medium
- 2.5 ml of each amino acid (200x), omit Cystein and Asparagin
- 0.08 ml folic acid (500x)
- 1 ml biotin (500x)
- 0.1 ml vitamins (5000x) niacinamide, calcium pantothenate, PABA, pyridoxal, pyridoxine, pyridoxamine, thiamine, cyanocobalamin
- 10 ml riboflavin (50x)
- 2.5 ml guanine HCl (200x)
- 2.5 ml adenine sulfate (200x)
- 5 ml uracil (100x)
- 0.5 ml CaCl₂ (1000x)
- 2.248g sodium acetate, tri-hydrate
- 3.675g sodium phosphate dibasic, anhydrous
- 1.597g sodium phosphate monobasic, monohydrate
- Adjust pH slowly with good mixing to 6.93 using 1N NaOH
- 5.0g D-glucose
- 0.5g choline chloride
- 1.25g sodium bicarbonate
- Bring volume to 500 ml, final pH is approx. 7.05
- Filter sterilize media and store at 4°C, use within 3 weeks
- Before use add Cysteine (100x; 10 µl/ml CDM), β-NAD (4,000x; 0.25 µl/ml CDM) and Copper sulphate (10,000x; 0.6 µl/ml CDM)

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Mass dissolved in dH₂O unless noted otherwise</th>
<th>Comment (A =autoclave, F=filter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanin (200x)</td>
<td>2 g/100 ml</td>
<td>A</td>
</tr>
<tr>
<td>Arginin (200x)</td>
<td>2 g/100 ml</td>
<td>A</td>
</tr>
<tr>
<td>Aspartic acid (200x)</td>
<td>2 g/100 ml 0.2N HCl</td>
<td>F</td>
</tr>
<tr>
<td>Glutamic acid (200x)</td>
<td>2 g/100 ml 0.2N HCl</td>
<td>F</td>
</tr>
<tr>
<td>Glutamine (200x)</td>
<td>4 g/100 ml 1N HCl</td>
<td>F</td>
</tr>
<tr>
<td>Glycine (200x)</td>
<td>2 g/100 ml</td>
<td>A</td>
</tr>
<tr>
<td>Histidine (200x)</td>
<td>2 g/100 ml</td>
<td>A</td>
</tr>
<tr>
<td>Isoleucine (200x)</td>
<td>2 g/100 ml</td>
<td>A; heat to dissolve</td>
</tr>
<tr>
<td>Leucine (200x)</td>
<td>2 g/100 ml</td>
<td>A; heat to dissolve</td>
</tr>
<tr>
<td>Lysine (200x)</td>
<td>1.84 g/100 ml</td>
<td>A</td>
</tr>
<tr>
<td>Methionine (200x)</td>
<td>2 g/100 ml</td>
<td>A</td>
</tr>
<tr>
<td>Phenylalanine (200x)</td>
<td>2 g/100 ml 0.01N HCl</td>
<td>A; heat to dissolve</td>
</tr>
<tr>
<td>Proline (200x)</td>
<td>2 g/100 ml</td>
<td>A</td>
</tr>
<tr>
<td>Serine (200x)</td>
<td>2 g/100 ml</td>
<td>A</td>
</tr>
<tr>
<td>Threonine (200x)</td>
<td>4 g/100 ml</td>
<td>A</td>
</tr>
<tr>
<td>Tryptophan (200x)</td>
<td>2 g/100 ml 0.1 NaOH</td>
<td>F</td>
</tr>
<tr>
<td>Tyrosine (200x)</td>
<td>2.884 g/100 ml 1N NaOH</td>
<td>F</td>
</tr>
<tr>
<td>Valine (200x)</td>
<td>2 g/100 ml</td>
<td>A</td>
</tr>
<tr>
<td>Cysteine (200x)</td>
<td>0.75 g/10 ml</td>
<td>F</td>
</tr>
<tr>
<td>Guanine HCl (200x)</td>
<td>0.4 g/100 ml 1N HCl</td>
<td>F</td>
</tr>
<tr>
<td>Adenine-sulfate (200x)</td>
<td>0.4 g/100 ml 1N HCl</td>
<td>F</td>
</tr>
<tr>
<td>Uracil (100x)</td>
<td>0.2 g/100 ml</td>
<td>A; heat to dissolve</td>
</tr>
<tr>
<td>Biotin (500x)</td>
<td>10 mg/100 ml</td>
<td>F; with heating</td>
</tr>
<tr>
<td>Folic acid (500x)</td>
<td>5 mg/ml 1N NaOH</td>
<td>F; light sensitive</td>
</tr>
<tr>
<td>Niacinamide (5,000x)</td>
<td>5 mg/ml</td>
<td>F</td>
</tr>
<tr>
<td>Substance</td>
<td>Concentration</td>
<td>Notes</td>
</tr>
<tr>
<td>-----------</td>
<td>---------------</td>
<td>-------</td>
</tr>
<tr>
<td>d-pantothenic acid, 0.5Ca (5,000x)</td>
<td>10 mg/ml</td>
<td>F</td>
</tr>
<tr>
<td>Pyridoxal HCl (5,000x)</td>
<td>5 mg/ml</td>
<td>F; light sensitive</td>
</tr>
<tr>
<td>Pyridoxamine 2HCl (5,000x)</td>
<td>5 mg/ml</td>
<td>F; light sensitive</td>
</tr>
<tr>
<td>Pyridoxine (5,000x)</td>
<td>5 mg/ml</td>
<td>F; light sensitive</td>
</tr>
<tr>
<td>Riboflavin (50x)</td>
<td>10 mg</td>
<td>80 ml dH₂O + 0.12 ml glacial acetic acid, warm and bring volume to 100 ml</td>
</tr>
<tr>
<td>Thiamine HCl (5,000x)</td>
<td>5 mg/ml</td>
<td>F; light sensitive</td>
</tr>
<tr>
<td>Cyanocobalamin (5,000x)</td>
<td>5 mg/10 ml</td>
<td>F; light sensitive</td>
</tr>
<tr>
<td>p-aminobenzoic acid (5,000x)</td>
<td>5 mg/5 ml</td>
<td>F</td>
</tr>
<tr>
<td>B-NAD (4,000x)</td>
<td>10 mg/ml</td>
<td>F; prepare fresh</td>
</tr>
<tr>
<td>Calcium chloride (1000x)</td>
<td>50.6 mg/10 ml</td>
<td>F</td>
</tr>
<tr>
<td>Magnesium sulfate (500x)</td>
<td>1.7995 g/10 ml</td>
<td>F</td>
</tr>
<tr>
<td>Manganese sulfate, mono-hydrate (1000x)</td>
<td>50 mg/10 ml</td>
<td>F</td>
</tr>
<tr>
<td>Copper(II) sulfate, penta-hydrate (10,000x)</td>
<td>1 mg/ml</td>
<td>F</td>
</tr>
<tr>
<td>Iron(II)sulfate, hepta-hydrate (2,000x)</td>
<td>10 mg/ml</td>
<td>F</td>
</tr>
<tr>
<td>Iron(III)nitrate, nona-hydrate (10,000x)</td>
<td>10 mg/ml</td>
<td>F</td>
</tr>
</tbody>
</table>

Table 1: Preparation of amino acid, nucleotide, vitamin and metal stocks for chemically defined medium

5.1.5 Antibodies

Dilutions used in Immunofluorescence and Western Blot experiments are indicated in brackets.

Monoclonal mouse-anti PLY-4 (1 µg/100 µl) Abcam
Polyclonal rabbit anti-EEA1 (1:5,000) Abcam
Polyclonal rabbit anti-LAMP1 (1:500) Abcam
Omni-rabbit serum (1:1000-1:5,000) SSI
Goat F(ab’)2 Fragment anti-mouse IgG (H+L)-PE (1:100) Beckman Coulter
Goat F(ab’)2 Fragment anti-rabbit IgG (H+L)-PE (1:100) Beckman Coulter
Alexa Fluor 488 goat anti-human IgG (H+L) (1:2,000) Invitrogen
Alexa Fluor 488 goat anti-mouse IgG (H+L) (1:2,000) Invitrogen
Alexa Fluor 488 goat anti-rabbit IgG (H+L) (1:2,000) Invitrogen
Alexa Fluor 594 goat anti-mouse IgG (H+L) (1:2,000) Invitrogen
Secondary goat anti-rabbit IgG-HRP (1:40,000) Southern Biotech
5.2 Methods

5.2.1 Bacterial strains

*S. pneumoniae* strains PJ1324 (serotype 6B) and TIGR4 (serotype 4) were provided by Brigitta Henriques-Normark (Swedish Institute for Infectious Disease Control) and strain 4DS2341-94 (serotype 4) was provided by E. Ades (Centers for Disease Control and Prevention, Atlanta GA).

5.2.1.1 Cultivation of *S. pneumoniae* strains

Standard growth conditions for used for cultivation of TIGR4 and 4DS2341-94. Cultures were grown from 200 µl single-use glycerol stocks (OD$_{620}$=0.3, stored at -80°C) in 10 ml pre-warmed Todd-Hewitt Broth (THB, Becton Dickinson) supplemented with 0.5% yeast extract (THY) at 37°C and 5% CO$_2$ atmosphere. Strain PJ1324 was grown overnight in THY in different dilutions (1:500; 1:25,000, 1:1,250,000) and on the next day the culture from the last turbid tube was diluted 1:20 in pre-warmed THY. For the growth in chemically defined medium (CDM) all strains were grown under standard conditions in THY overnight, washed with PBS, resuspended in the CDM as described under 5.1.4 and incubated at 37°C with 5% CO$_2$ atmosphere. For every experiment, bacteria were allowed to grow until the early exponential phase was reached (OD$_{620}$=0.3)

5.2.1.2 Preparation of *S. pneumoniae* lysates

Bacteria were grown under standard growth conditions and were centrifuged with 1000 g for 10 minutes. Bacterial pellets were harvested, washed once with PBS and frozen overnight at -80°C. The next day, the pellets were thawed at room temperature and resuspended in PBS using 1-2 times of the pellet volume. Sonication was performed 9x 30seconds/5cycles/100% (BANDELIN-Sonoplus HD2070) on ice. Protease inhibitors were added and lysates were stored at -20°C. BCA protein assay kit was used to determine the protein concentration of the lysates. The procedure was carried out according to the manufacturer’s instructions.
5.2.1.3 Quellung reaction of \textit{S. pneumoniae} capsule

Omni serum and 0.3\% methylen blue were placed on a glass slide. A single colony from blood agar plate was added and covered by a cover slip. After 10 minutes of incubation at room temperature bacteria were analyzed under a light microscope at 1000x magnification.

5.2.2 General methods of molecular biology

5.2.2.1 SDS-Polyacrylamide Gel Electrophoresis

The proteins were separated according to their size using 8-16\% gradient PAGEr Gold Precast Gels. They were clamped in a chamber produced by BioRad which was filled with reducing SDS-running buffer. The Lane Marker non-reducing sample buffer was mixed with the lysates in a ratio of 1:4, boiled for 5 minutes at 95\^\circ C and separated on the gel. As molecular weight marker served the precision plus protein dual color standard from BioRad. Proteins were separated at 110 V 1.8 hours.

5.2.2.2 Western blot analysis

Proteins were separated by polyacrylamid gel electrophoresis (PAGE) as described in 5.2.2.1 and transferred onto a nitrocellulose membrane via the dry-blotting transfer system (iBlot, Invitrogen). The transferred proteins were visualized using Ponceau S solution. Blots were washed with \( \text{dH}_2\text{O} \) and blocked with 5\% milk powder in PBS-T at room temperature for one hour in order to prevent unspecific binding of antibodies. The membranes were washed three times for 5 minutes with PBS-T before the incubation with primary antibodies being either monoclonal mouse antibodies (0.5 \( \mu \text{g/ml} \)) or polyclonal rabbit hyper-immune sera diluted 1:5,000 in PBS-T, 5\% milk for one hour at room temperature. The membranes were washed three times for 15 minutes with PBS-T and incubated with horse-radish-peroxidase (HRP) conjugated secondary anti-mouse IgG (1:20,000) or anti-rabbit IgG (1:40,000) in PBS-T 5\% milk for one hour at room temperature. Subsequently membranes were washed at least three times for 30 minutes with PBS-T. For detection the ECL detection kit (ECL Detection reagents Amersham, GE healthcare) was used according to manufacturer's instructions. The membranes were wrapped in a transparent plastic foil and fixed in an x-ray film cassette. Membranes were exposed to ECL Hyperfilms for 5, 10, 30, 60 and 300 seconds.
5.2.3 In vitro assays

5.2.3.1 Adhesion/ invasion assay

Pneumococcal adherence and invasion assays with epithelial cells were performed in 24-well plates. Confluent Detroit 562 cells were washed and trypsiniized as described under 5.2.3.3. After centrifugation cells were re-suspended and a small aliquot was taken and stained by trypan-blue before counting in a Neubauer chamber. Detroit 562 cells were diluted to 5x10^5 cells/ml and 1 ml cell suspension was transferred into each well followed by overnight incubation at 37°C, 5% CO₂. The next day confluent epithelial cells were washed twice with HBSS + 10 mM glucose and were infected with 10^4-10^6 CFU of S. pneumoniae which were grown under standard conditions. Right before the infection bacteria were serially diluted and plated on blood-agar plates before infection to determine exact starting CFU, (T0). Detroit 562 cells and bacteria were incubated for 2 hours at 37°C, 5% CO₂ without antibiotics. Subsequently cells were rinsed at least 3 times with PBS to remove unbound bacteria. At this point, adhesive and invasive bacteria were either stained for immunofluorescence microscopy or recovered by saponin-mediated lysis of Detroit 562 cells. The lysis was carried out for 5 minutes at room temperature with 1% Saponin. The total number of attached and invasive bacteria was evaluated by serial dilutions of bacteria plated on sheep-blood agar plates. To kill adherent, extracellular pneumococci, host cells were incubated with DMEM and 1000 Units Penicillin/mg Streptomycin at 4°C for 1 hour. Intracellular bacteria were released by saponin-mediated lysis and monitored by plating.

5.2.3.2 Neutralization assay

The neutralization assay is a modified protocol of the adhesion/invasion assay. Eukaryotic cells and bacteria were seeded and grown as described under 5.2.1.1 and 5.2.3.1. Before the infection bacteria were incubated at dilutions 1:500; 1:1000 of murine anti-PsaA, anti-PspA, anti-IC47 and for anti-lysate hyperimmune sera for 30 minutes at room temperature. Bacterial growth in the presence of serum during pre-opsonization was monitored by plating serial dilutions on blood agar plates (T30) in addition to the starting CFU (T0). After 2 hours of infection Detroit 562 cells were rinsed several times with PBS to remove unbound bacteria. Adherent and invasive bacteria were released by saponin-mediated lysis and monitored after plating serial dilutions on blood agar plates.
5.2.3.3 Cultivation of eukaryotic cell lines for in vitro assays

The human nasopharyngeal epithelial cell line Detroit 562 (American Type Culture Collection) was cultivated in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 mM sodium pyruvate, 100 mM non-essential amino acids, 10 mg/ml gentamycin and 50 µM β-mercaptoethanol in T-175 cm² flasks at 37°C and 5% CO₂ atmosphere. The cells reached confluence within three to four days and were split at a ratio of 1:5. For splitting the cells the medium was removed first, cells were washed twice with 20 ml Hank’s buffer salt solution (HBSS) containing 10 mM glucose, trypsinized with 10 ml pre-warmed 0.25% trypsin-EDTA and incubated for 7 minutes at 37°C, 5% CO₂. Cells were washed with 50 ml EMEM. After centrifugation for 7 minutes at 200 g the pellet was re-suspended in 10 ml EMEM and diluted 1:5 in a new T-175 cm² cell culture flask containing 50 ml fresh EMEM-medium.

Human lung alveolar epithelial cells A549 (ATCC, type II pneumocytes) were cultured in F12K medium, supplemented with 10 U/ml Penicillin G, 0.01 mg/ml streptomycin and 10% heat-inactivated FBS. Confluent cells were split in a 1:10 ratio using the same protocol as mentioned above but F12K medium.

5.2.4 Immunofluorescence microscopy of S. pneumoniae using antigen specific antibodies and fluorescent marker

5.2.4.1 Staining of S. pneumoniae by antigen specific antibodies

For the staining of bacteria a protocol from Harry et al. 1995 was adapted. S. pneumoniae was grown under standard growth conditions. Pneumococci were washed in PBS and fixed in 3% paraformaldehyde, 30 mM sodium phosphate for 15 minutes at room temperature and 45 minutes on ice. After being washed three times in PBS with 100 mM glycine and once in PBS, the pellet was resuspended in 50 mM glucose, 20 mM Tris-HCl pH 7.5, 10 mM EDTA, 5x10⁷ CFU/100 µl cells were immediately transferred onto poly L-lysine coated Poly Prep slides and allowed to settle for at least 15 minutes. Slides were washed in PBS, dipped in PBS 0.1% TritonX-100 for 5 minutes, washed again in PBS and blocked overnight with PBS + 2% BSA. Bacteria were incubated with either 2 µg/100 µl murine monoclonal antibodies (anti-PspA, anti-PcsB, anti-PsaA, anti-StkP and anti-PLY4), 1:1000.
diluted rabbit polyclonal antibodies (anti-PspA) or murine polyclonal antibodies (anti-4DS2341-94 lysate) for one hour at room temperature. After washing, the samples were incubated with Alexa488/594-conjugated secondary goat anti-mouse IgG and/or Alexa488-conjugated goat anti-rabbit IgG antibodies for one hour at room temperature in the dark. Slides were then washed three times with PBS, mounted with Prolong Gold Anti-fade mounting solution containing DAPI and allowed to polymerize for at least 24 hours.

5.2.4.2 Preparation of Detroit 562 cells for immunofluorescence analysis

For immunofluorescence staining of *S. pneumoniae* in contact with nasopharyngeal epithelial cells, Detroit 562 cells were trypsinized, washed and counted as described under 5.2.3.3. For the seeding a protocol from Rennemeiner et al. 2007 was adapted. 3x10⁵ Detroit 562 cells were seeded on round glass coverslips (diameter 13 mm) in a 24-well plate. After three to four days cells were grown confluent and used for immunofluorescence staining. Before the infection of Detroit 562 monolayers with pneumococci cells were gently washed twice with HBSS + 10 mM glucose.

5.2.4.3 Fluorescein isothiocyanate isomer 1 (FITC) labeling of *S. pneumoniae*

The bacterial culture was grown under standard growth conditions. The bacteria were washed once in PBS and resuspended in freshly prepared FITC stock solution (1 mg/ml in 100 mM NaHCO₃/Na₂CO₃ buffer, pH 9.0). For CFU determination before and after the staining, bacteria were serially diluted and plated in triplicates on sheep blood-agar plates and incubated on 37°C, 5% CO₂. Bacteria were labeled for 20 minutes in the dark at room temperature. Bacteria were washed in PBS until supernatant wasn’t colored anymore and resuspended in respective medium.

5.2.4.4 Staining of intra- and extracellular bacteria

Detroit 562 cells were prepared as described under 5.2.4.1. The eukaryotic cell monolayer was infected for 2 hours at 37°C, 5% CO₂ with 10⁴-10⁷ CFU/300 µl bacteria grown under standard growth conditions. Non-adherent bacteria were removed by rinsing the host cells several times with PBS. Infected samples were fixed with 3.7% para-formaldehyde for 15 minutes at room temperature and blocked overnight with PBS + 2% BSA. Extracellular bacteria were stained with either 2µg /100 µl murine monoclonal antibodies (anti-PspA, anti-PcsB, anti-PsaA, anti-StkP and anti-PLY4), 1:1000 diluted rabbit polyclonal antibodies (anti-PspA, Omni serum) or murine polyclonal antibodies (anti-4DS2341-94 lysate) for one hour at
room temperature. Detroit 562 cells were then permeabilized with 0.1% TritonX-100 for 5 minutes. Extra- and intracellular bacteria were incubated with either 2 µg/100 µl murine monoclonal antibodies (anti-PspA, anti-PcsB, anti-PsaA, anti-StkP and anti-PLY4), 1:1000 diluted rabbit polyclonal antibodies (anti-PspA, Omni serum) or murine polyclonal antibodies (anti-4DS2341-94 lysate) for one hour at room temperature. After washing, the samples were incubated with Alexa488/594-conjugated secondary goat anti-mouse IgG and Alexa488-conjugated goat anti-rabbit IgG antibodies for one hour at room temperature in the dark. Slides were then washed three times with PBS, mounted with Prolong Gold Anti-fade mounting solution containing DAPI and allowed to polymerize for at least 24 hours.

5.2.5 Surface staining of *S. pneumoniae* grown in chemically defined medium using antigen specific antibodies analyzed by Flow cytometry

Bacteria were grown in chemically defined medium supplemented without or with iron and manganese. Bacteria were washed twice in HBSS + 2% BSA. Approximately $5 \times 10^5$ CFU in 100 µl HBSS and 2% BSA were incubated with murine anti-IC47, anti-PsaA, antilylsate sera, rabbit anti-PspA, anti-PsaA sera at 1:100, 1:300 and 1:900 dilution for one hour at 4°C. Before incubation with PE-conjugated secondary anti-mouse or anti-rabbit antibody for one hour at 4°C, bacteria were washed three times with HBSS and 2% BSA and were centrifuged at 1000 g. Afterwards incubation with secondary antibody cells were washed again three times with HBSS. Bacterial nucleic acids were stained with SYTO 60 for 10 minutes at 4°C using 0.05 µl/sample. Finally bacteria were fixed with 2% para-formaldehyde overnight and analyzed using a flow cytometer. Data were analyzed using FCS Express analysis software (CXP; Beckman Coulter).

5.2.6 Generation of mono- and polyclonal antibodies against *S. pneumoniae* antigens

5.2.6.1 Generation of monoclonal antibodies

Murine monoclonal antibodies (mAbs) were generated against the three *S. pneumoniae* vaccine antigens (SP1650, SP1732-3, SP2216-1) and for PspA (as positive control). Immunization of mice, fusion, sub-cloning and antibody purification was outsourced
to the company Abgent. The screening of hybridoma supernatants and selection was based on IgG subclass ELISA (for 2 epitopes of each antigen; preferably 2b and 2a were chosen). Surface staining on live pneumococci for their ability to recognize surface accessible/exposed epitopes, Western-Blot analysis with recombinant proteins and the sequence of the reactive epitope was mapped using synthetic peptides in ELISA.

5.2.6.2 Generation of polyclonal antibodies against

For the production of antigen specific anti-PspA, anti-PsaA, anti-PcsB and anti-StkP antibodies two 6-8 week old CD-1 mice were immunized with 50 µg recombinant protein plus 50 µl complete Freund’s adjuvant (CFA) in their flank after taking pre-immune sera. After 2 and 4 weeks mice received a booster injection the same way as before using the same dose in incomplete Freund’s adjuvant (IFA). Seven days after the second booster injection immune sera were taken.

For the production of polyclonal antibodies against *S. pneumoniae* expressed antigens, mice were immunized with lysates generated from bacteria grown under standard conditions.
6 Results

6.1 Adhesion and neutralization experiments using Detroit 562 cell line

The aim was to study adherence to and invasion of human epithelial cells by *S. pneumoniae* and to investigate which role the capsule plays during this process. Furthermore we wanted to investigate the role of potentially neutralizing antibodies targeting the IC47 antigens.

Every single pneumococcal strain is associated with a certain serotype, dependent on the biochemical properties of the capsule. Given that fact there are more than 90 different serotypes described to date. Pneumococcal colonization is prerequisite for diseases caused by *S. pneumoniae*. The upper respiratory airway is the primary colonization site in human. For this reason we used the human nasopharyngeal cell line Detroit 562, to mimic the conditions found in the human body as good as possible.

6.1.1 *Streptococcus pneumoniae* strain 4DS2341-94 shows increased adhesion to and invasion of nasopharyngeal epithelial cells *in vitro*

To study adhesion and invasion of *S. pneumoniae* to these eukaryotic cells *in vitro* we compared the adhesive and invasive potential of strains and serotypes differing in capsule thickness in order to find a strain which adheres best to Detroit 562 cells. The strain adhering best can be used for further experiments such as functional adhesion and invasion assays. To investigate these differences we cultured Detroit 562 cells with strains 4DS2341-94, CDC-66, CDC-72 of serotype 4, 33A, 35A respectively and let them adhere and invade for 2.5 hours. As shown in Fig. 3A, 4DS2341-94 strain had a greater ability to adhere and invade nasopharyngeal epithelial cells compared to CDC-72 and CDC-66. While for strain 4DS2341-94 ~10^6 extracellular bacteria and ~3x10^4 intracellular bacteria were recovered, for strain CDC-72 only ~10^3 adherent and even less ~10 invasive pneumococci were recovered. The ability of strain CDC-66 to adhere and invade Detroit 562 cells was comparable to strain CDC-72 with numbers of ~5x10^2 adherent and ~10 invasive recovered pneumococci. We have also observed that the amount of adherent bacteria was much higher than the amount of bacteria recovered from inside the cell. These observations were made for all three tested
strains. Furthermore the adhesive potential of the *S. pneumoniae* strains was in accordance with capsule thickness as shown in Fig. 3B. Light microscopy revealed a lower amount of capsule for strain 4DS2391-94 compared to CDC-66 and CDC-72.

When *S. pneumoniae* progresses to the lower respiratory tract the bacterium is exposed to epithelial cells that differ from those of the upper respiratory tract. At a later stage of this work we also tested pneumococcal adhesion to the human alveolar basal epithelial cell line A549. Lung epithelial cells were infected with $10^4$ and $10^5$ CFU of 4DS2341-94 (serotype 4) and PJ1324 (serotype 6B) a strain that also produces a high amount of capsule similar to what was observed in the quellung reaction for strains CDC-66 and CDC-72. When A549 cells were infected with $10^4$ or $10^5$ CFU then only ~1-2% of the starting CFU were recovered for PJ1324 whereas the amount of adhesive and invasive pneumococci of strain 4DS2341-94 reached almost ~70% as shown in Fig. 4. Again these results verify that pneumococci with less capsule are much more potent in adhering and invading not only nasopharyngeal epithelial but also epithelial cells of the lower respiratory tract. Since pneumococcal colonization primarily takes place at the human nasopharynx, we performed all future adhesion assays using the Detroit 562 cell line.

**Figure 3:** Infection of nasopharyngeal epithelial Detroit 562 cells with *S. pneumoniae* strains CDC-66, CDC-72 and 4DS2341-94. (A) Infection of $5 \times 10^7$ Detroit cells for 2.5h with $10^7$ CFU of *S. pneumoniae* strains 4DS2341-94, CDC-72 and CDC-66. The amount of adherent and invasive bacteria was determined by lysis of Detroit 562 cells followed by plating of bacteria. (B) Light microscopy of *S. pneumoniae* strains 4DS2341-94, CDC-62 and CDC-72 after quelling reaction using Omni serum (1000x magnification). Error bars indicate the standard deviation of three independent experiments.
41

Figure 4: Adhesion of *S. pneumoniae* 4DS2341-94 and PJ1324 to A549 lung epithelial cells. 5x10^5 A549 cells were infected for 2h with 10^4 and 10^5 CFU of strains 4DS2341-94 and PJ1324. After 2h infection the growth in EMEM medium (red) and the amount of adherent/invasive bacteria (yellow bar) were monitored. 4DS2341-94 strain has an increased ability to adhere to A549 cells compared to PJ1324.

6.1.2 Lysate specific sera reduce pneumococcal adhesion to Detroit 562 cells

In order to analyze the functional role of monoclonal and polyclonal antibodies specific for IC47 antigens, we set up a neutralization assay (Fig. 5) to monitor potential inhibitory effects of antibodies concerning adhesion. Previous experiments showed that 4DS2341-94 was a suitable strain for our neutralization assay having a high potential adhering to Detroit 562 cells due to lower amounts of capsule.

To determine the optimal CFU for infection 5x10^5 Detroit 562 cells were infected with strain 4DS2341-94 ranging from 10^3-10^6 CFU. As shown in Fig. 6 there is a consistent relative amount (45%) of bacteria adhering to Detroit 562 cells for every tested starting CFU condition. Based on this experiment further assays were continued with 5x10^3 CFU for infection.
STEP 1: Preincubation of bacteria with serum

STEP 2: Addition to Detroit 562 cells and incubation to allow adhesion

STEP 3: Washing to remove non-attached bacteria

STEP 4: Epithelial cell lysis and plating to determine the number of recovered bacteria

**Figure 5:** Illustration of neutralization assay. For the infection of ~5x10^5 Detroit 562 cells approximately 5x10^3 CFU of *S. pneumoniae* 4DS2341-94 cells are used to reach a MOI of 100. Prior to infection of Detroit 562 cells for 2 hours at 37°C, pre-opsonization with serum is allowed for 30 minutes at room temperature. After 2 hours of adhesion non-attached bacteria are washed away and epithelial cell lysis is carried out with 1% saponin in PBS for 5 minutes at room temperature. Recovered bacteria are plated in serial dilutions on blood agar plates.

**Figure 6:** Adherence of *S. pneumoniae* strain 4DS2341-94 to Detroit562 cells. The number of recovered bacteria (red) after infection of 5x10^3 Detroit cells with 10^3, 10^4, 10^5 and 10^6 CFU (blue) of 4DS2341-94 for 2 hours is shown.

To elucidate whether *S. pneumoniae* specific antibodies interfere with pneumococcal adherence to Detroit 562 cells, bacteria were pre-opsonized with polyclonal mouse antibodies specific for 4DS2341-94 lysate. The use of polyclonal lysate specific serum served as a control since many surface exposed proteins and polysaccharides should be recognized.
Figure 7: Inhibitory effect of lysate specific antibodies on adhesion of *S. pneumoniae* strain 4DS2341-94 to Detroit 562 cells. Before the infection of 5x10⁷ Detroit562 cells 5x10⁷ CFU were pre-opsonized with lysate specific hyperimmune mouse sera and the corresponding pre serum at 1:1000 dilution. (A) Shows the percentage of inhibition of adhesion to Detroit 562 cells in the presence of 1:1000 diluted pre- and hyperimmune anti-lysate sera relative to no-serum control and (B) Shows the percentage inhibition of hyperimmune sera relative to preimmune sera in three different experiments.

In all experiments the lysate specific hyperimmune serum was able to reduce pneumococcal adhesion to nasopharyngeal epithelial cells to an extent ranging from 42 to 75% relative to the no-serum control (Fig. 7A). The conducted neutralization experiments indicated a consistent reduction of adhesion to Detroit 562 cells in the presence of lysate specific hyperimmune sera compared to the corresponding preimmune serum by 50-72% (Fig. 7B).

6.1.3 **IC47 specific antibodies do not neutralize adhesion of *S. pneumoniae* strain 4DS2341-94 to Detroit 562 cells**

In order to test the functional role of antibodies against *S. pneumoniae* vaccine antigen candidates StkP, PsaA and PcsB in neutralizing adhesion of *S. pneumoniae* to Detroit 562 cells, pneumococci were pre-opsonized with polyclonal rabbit anti-PsaA and polyclonal murine anti-IC47 antibodies prior infection. As observed in previous experiments, the presence of lysate specific hyperimmune serum decreased pneumococcal adhesion to Detroit 562 cells by 52% compared to the corresponding preimmune serum (Fig. 8). PsaA specific rabbit sera did not significantly affect pneumococcal adhesion when compared to the
corresponding preimmune serum. Instead, compared to the no serum control, PsaA hyperimmune and corresponding preimmune sera increased the adhesion of 4DS2341-94 to Detroit 562 cells. Furthermore there was also no inhibitory effect observed for IC47 specific sera (data not shown).

Figure 8: Adhesion of *S. pneumoniae* serotype 4 strain 4DS2341-94 to Detroit 562 cells in presence of PsaA specific rabbit serum. Median CFU of *S. pneumoniae* strain 4DS2341-94 recovered after 2 hours of adhesion to Detroit 562 cells. In an assay volume of 300 µl 5x10^5 Detroit 562 cells were infected with ~ 2.5x10^3 CFU of 4DS2341-94. Adhesion of bacteria in absence (no serum) and presence of lysate specific hyper-immune and corresponding pre-immune serum at a 1:1000 dilution, as well as PsaA specific hyper-immune and corresponding pre-immune serum in a 1:500 and 1:1000 dilution is shown. Each condition was tested in duplicates and values have been normalized to CFU at T2.5 (CFU after 2.5 hours of growth in absence of Detroit 562 cells).

6.2 Immunofluorescence analysis to investigate surface expression of *S. pneumoniae* vaccine candidate antigens StkP, PsaA and PesB

Immunofluorescence microscopy is a powerful tool to visualize details of cell- and bacterial structures using fluorescently labeled marker such as antibodies. Since anti-PcsB, anti-StkP and anti-PsaA antibodies could not reduce pneumococcal adhesion to Detroit 562 cells we wanted to investigate surface expression and accessibility of these *S. pneumoniae* antigens in more detail by using fluorescence analysis. Therefore we tested two different staining methods: The staining of living versus PFA fixed bacteria. We also analyzed the influence of permeabilizing agents on signal intensity. Further on we investigated the surface expression and accessibility of *S. pneumoniae* candidate antigens during close contact to Detroit 562 cells.
For the differentiation of adherent and invasive bacteria we tried different methods including infection of Detroit 562 cells with FITC pre-labeled bacteria, co-localization studies using eukaryotic markers and double staining of bacteria using antigen specific antibodies and Omni serum in order to investigate antigen expression and accessibility inside the host cell.

6.2.1 PspA can be detected to the same extent on dead and living pneumococci using polyclonal anti-PspA antibodies

Pneumococcal surface protein A (PspA) is the most abundant cell-surface protein present on all pneumococcal strains (Langermann et al., 1994). We therefore used this protein as positive control for setting up the different staining procedures. First, when setting up a staining protocol we wanted to compare the staining of fixed and living bacterial cells to determine to which extent fixation might interfere with antigen-antibody interaction. As shown in Figure 9, PspA is expressed and accessible for polyclonal anti-PspA antibodies on dead and living bacteria. We could confirm that every bacterium was stained by the anti-PspA hyperimmune serum. Furthermore it was shown that the fixation of bacteria with PFA does not interfere with antigen-antibody interaction. In addition, the characteristic lancet-shape, pneumococcal appearance as diplococci is clearly visible.

Furthermore, we tested whether permeabilization of S. pneumoniae 4DS2341-94 before the staining with antigen specific antibodies does somehow influence the signal intensity. Bacteria from the early exponential phase were fixed and treated with the permeabilizing detergent Triton X-100. Pneumococcal cells were again stained with PspA specific rabbit serum and DAPI staining served as DNA control. As shown in Figure 10 almost no differences in signal intensity when comparing the PspA staining of permeabilized and non-permeabilized bacteria were observed. After permeabilizing cells the DAPI staining seemed to be stronger.
Figure 9: Immunofluorescence microscopy of *S. pneumoniae* 4DS2341-94 strain comparing the staining of dead (fixed) and living (non-fixed) bacteria. Bacteria were grown under standard growth conditions and stained with rabbit anti-PspA serum at 1:1000 dilution either before or after fixation with 3.7% PFA. Bacteria were detected with secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (at 1:2,000 dilution). Pictures were taken on a Axioskop2 plus microscope at 1000x magnification.

Figure 10: Immunofluorescence analysis of PspA on fixed permeabilized versus non-permeabilized *S. pneumoniae* strain 4DS2341-94. Bacteria grown at standard growth conditions were fixed, either permeabilized or untreated prior to the staining with 1:1000 diluted polyclonal rabbit anti-PspA serum. The detecting secondary antibody Alexa Fluor 488 goat anti-rabbit IgG antibody was diluted 1:2,000. Pictures were taken on a Axioskop2 plus microscope at 1000x magnification.
6.2.2 Pneumolysin is accessible after permeabilization only

We further investigated the effect of permeabilizing agents on the accessibility of intracellular proteins of \( S. pneumoniae \) strain 4DS2341-94. Pneumolysin is an intracellular protein which is secreted in the environment at a certain stage of infection. As shown in Figure 11 only bacteria treated with permeabilizing agents can be stained with anti-pneumolysin antibodies regardless whether treated with Saponin or Triton X-100. The staining of untreated pneumococci was negative which is in accordance with the fact that pneumolysin is not located on the bacterial surface.

![Figure 11: Immunofluorescence analysis of pneumolysin on fixed permeabilized versus non-permeabilized \( S. pneumoniae \) strain 4DS2341-94. Bacteria grown at standard growth conditions were fixed, permeabilized with either Saponin or Triton X-100 or non-permeabilized prior to the staining with mouse monoclonal anti-PLY-4 antibodies (at 1 µg/100 µl concentration). The detecting secondary antibody Alexa Fluor 488 goat anti-rabbit IgG antibody was diluted 1:2,000. Pictures were taken on a Axioskop2 plus microscope at 1000x magnification.](image)

6.2.3 StkP is accessible for antigen specific antibodies on fixed bacteria only

Since anti-PcsB, anti-StkP and anti-PsaA antibodies could not reduce pneumococcal adhesion to Detroit 562 cells in neutralization assays we wanted to investigate surface expression and accessibility of these \( S. pneumoniae \) antigens. After setting up a reliable
staining protocol, we tested murine monoclonal anti-StkP, anti-PcsB and anti-PsaA antibodies on living and fixed bacteria grown under standard growth conditions. Interestingly, monoclonal anti-StkP antibodies targeting the C-terminal region showed a weak, ring-like signal but only on fixed bacteria (Fig. 12A). As shown in Figure 12B and Figure 12C the staining of *S. pneumoniae* with monoclonal antibodies directed against PsaA and PcsB gave no positive signal on strain 4DS2341-94 either on living or dead bacteria.

### 6.2.4 Extra- and intracellular bacteria cannot be distinguished by co-localization studies using the eukaryotic EEA1 and neither by labeling of pneumococci with FITC prior to infection

The natural infection route of *S. pneumoniae* includes colonization of the upper airways, when bacteria adhere to the nasopharyngeal epithelium. To mimic the conditions found in the human body as good as possible, we wanted to localize pneumococci of strain 4DS2341-94 in close contact to Detroit 562 cells by using immunofluorescent staining. Hammerschmidt and colleagues found that *S. pneumoniae* down-regulates its capsule upon close contact to epithelial cells. Our aim was to setup a method to differentiate intracellular and extracellular bacteria in order to investigate expression and accessibility of the IC47 antigens of adherent and invasive bacteria.

In our first attempt to distinguish between intracellular and adherent pneumococcal cells of strain 4DS2341-94, bacteria were stained with fluorescein isothiocyanate isomer-1 (FITC) prior to infection of Detroit 562 cells. After infection adherent, extracellular bacteria were stained with rabbit polyclonal anti-PspA hyperimmune (HI) serum. Furthermore we monitored pneumococcal viability by plating serial dilutions before and after the FITC-staining as well as after the infection. As shown in Figure 13 the majority of FITC labeled bacteria has lost the fluorescent signal, whereas the polyclonal anti-PspA antibodies bound to the adherent bacteria. Bacterial plating revealed that the viability of FITC stained *S. pneumoniae* remained unaffected (data not shown). It turned out that for our purposes FITC labeling was not very suitable since bacteria divided rapidly during the 2 hours infection period losing their fluorescent signal as well as the reduced stability of the FITC dye.
Figure 12: Immunofluorescence analysis of PsaA, StkP and PcsB on fixed and living S. pneumoniae strain 4DS2341-94. (A) Bacteria were grown under standard growth conditions and stained with murine monoclonal anti-StkP, (B) anti-PcsB and (C) anti-PsaA antibodies (at 2 µg/100 µl concentration) either before or after fixation with 3.7% PFA. The detecting secondary antibody Alexa Fluor 488 goat anti-mouse IgG antibody was diluted 1:2,000. Pictures were taken on a Axioskop2 plus microscope at 1000x magnification.
Figure 11: Immunofluorescence analysis of FITC and PspA labeled *S. pneumoniae* strain 4DS2341-94 in contact to epithelial cells. Infection of 5x10^5 Detroit cells grown on glass cover-slips for 2 hours with 10^5 CFU FITC labeled pneumococci. After infection adherent bacteria were stained with either mouse monoclonal anti-PspA (2 µg/100 µl concentration) antibodies or rabbit polyclonal anti-PspA HI serum (1:1000). Primary antibodies were detected with either 1:2,000 diluted secondary Alexa Fluor 594 goat anti-rabbit IgG antibodies or secondary Alexa Fluor 594 goat anti-mouse IgG antibodies. Nucleic acids were stained with DAPI. Pictures were taken on a Axioskop2 plus microscope at 1000x magnification.

In another approach to identify intracellular bacteria within the host cells, we performed co-localization studies using the early endosomal marker EEA1 analyzed by confocal microscopy. Co-localization of stained bacteria with early endosomes would confirm their location within Detroit 562 cells. As shown in Figure 14A early endosomes were well dispersed within the host cell and bacteria were in close contact with the Detroit 562 cells. In order to investigate whether the yellow signal derives from intracellular pneumococci we made a single projection at 0 angle to the y-axis shown in Figure 14B. This analysis indicates that pneumococci are located right on top and not within the eukaryotic cell.

We were not able to distinguish extra- and intracellular bacteria by the two previous methods. In order to investigate IC47 antigen expression and accessibility of adherent and invasive bacteria we performed a double-staining of *S. pneumoniae* strain 4DS2341-94 by using two specific antibodies targeting different pneumococcal antigens. The principle of this method is shown in Figure 15.
**Figure 12:** Co-localization study of *S. pneumoniae* strain 4DS2341-94 using confocal microscopy. (A) 5x10^5 Detroit cells grown on glass cover-slips were infected for 2 hours with 10^5 CFU pneumococci of strain 4DS2341-94. Infected Detroit cells were permeabilized with Triton X-100, early endosomes were visualized with rabbit polyclonal anti-EEA1 antibodies (1:5,000). Bacteria were visualized with mouse monoclonal anti-PspA antibodies (4 µg/100 µl). Primary antibodies were detected with 1:2,000 diluted secondary antibody Alexa Fluor 594 goat anti-mouse IgG antibodies and 1:2,000 diluted secondary antibody Alexa Fluor 594 goat anti-rabbit IgG antibodies. (B) A projection from the marked area in Fig. 14A made from different rotations to the z-axis is shown. Pictures were taken on LSM 510 META laser scanning microscope at 630x magnification.

**Figure 15:** Schematic view of the double-staining method using antibodies directed against two different antigens. Human epithelial cells are infected with bacteria for 2 hours before all unbound bacteria are removed. First, adherent pneumococci are stained with rabbit anti-capsular polysaccharide antibodies followed by detection with goat anti-rabbit Alexa-488 conjugated antibodies (left picture). The eukaryotic cells get permeabilized by Triton-X 100, incubated with murine antigen-specific antibodies followed by detection with goat anti-mouse Alexa-594 conjugated antibodies. All extracellular bacteria are now stained in red and green, given a merged yellow signal whereas intracellular bacteria appear in red only (right picture).
For the double staining of adherent and invasive pneumococci we used Omni serum which contains antibodies against all capsular serotypes and PspA-specific antibodies. As shown in Figure 16 adherent bacteria were stained with PspA-specific antibodies appearing in red whereas intra- and extracellular bacteria were stained in green. In the merged picture pneumococci that appear in yellow are located extracellularly adhering to the Detroit 562 cells. For infection of Detroit 562 cells we used only \(10^4\) CFU and as we already knew from previous adhesion assays the amount of invading bacteria is very low, at approximately 1-2%. To conclude, the double-staining method using two different primary antibodies was the most reliable method.

Figure 13: Localization of *S. pneumoniae* strain 4DS2341-94 PspA and CPS specific Omni serum. 5x10^5 Detroit 562 cells were infected with 10^5 CFU of 4DS2341-94. Fixed samples were incubated first with mouse monoclonal anti-PspA antibodies (2 µg/100 µl) in order to stain extracellular bacteria only, followed by second staining after permeabilization with Triton X-100 using Omni serum for detection of extra- and intracellular pneumococci. Primary antibodies were detected with 1:2,000 diluted secondary antibody Alexa Fluor 488 goat anti-mouse IgG and 1:2,000 diluted secondary antibody Alexa Fluor 594 goat anti-rabbit IgG. Pictures were taken on a Axioskop2 plus microscope at 1000x magnification.

6.2.5 Detection of intracellular bacteria after prolonged incubation time

For the detection of intracellular pneumococci using confocal microscopy we had to modify the standard adhesion assay protocol. While in the standard adhesion assay nasopharyngeal epithelial cells were infected for 2 hours, the infection time used in the modified protocol is extended to 4 hours. Adherent bacteria were stained with Omni serum whereas extra- and intracellular bacteria were stained with mouse monoclonal anti-PspA, anti-StkP anti-PcsB and anti-PsaA antibodies after 2 and 4 hours of infection. As shown in Figure 17A after 2 hours of infection all bacteria were stained in green and red, thus are located on top of Detroit 562 cells. In contrast, after incubation for additional two hours some bacteria were located inside the cells (red), indicated by the white arrows in Figure 17B.
Figure 14: Immunofluorescence microscopy of strain 4DS2341-94 differentiating between adherent and invasive pneumococci. After infection of Detroit 562 cells with *S. pneumoniae* for 2 hours (A) and 4 hours (B) extracellular bacteria were stained with Omni serum (at 1:1000 dilution) followed by incubation with secondary Alexa Fluor 488-labeled anti-rabbit antibody (1:2,000 dilution, green). Intra- and extracellular bacteria were stained with mouse monoclonal anti-PspA antibodies (3 µg/100 µl) followed by staining with secondary Alexa Fluor 594-labeled anti-mouse antibody (1:2,000 dilution, red). Pictures were taken on LSM 510 META a laser scanning microscope at 630x magnification. Note: When taking the 2 hour time-point pictures the excitation laser (DAPI) was broken.
After 2 and 4 hours of infection, in agreement with the previous experiments PcsB was not detected by specific monoclonal antibodies (data not shown). When testing mouse monoclonal anti-PsaA antibodies we detected a weak signal after extended infection but not after 2 hours (Fig. 18). Furthermore, monoclonal antibodies specific for StkP also resulted in a positive signal after 2 and 4 hours incubation time, but we not able to differentiate between extra- and intracellular bacteria (data not shown). After an extended incubation time we were able to detect intracellular bacteria using anti-PspA antibodies. The prolonged contact of S. pneumoniae 4DS2341-94 to Detroit 562 cells also seems to partly affect the expression or accessibility of IC47 antigens.

**Figure 15:** Immunofluorescence microscopy of strain 4DS2341-94 differentiating between adherent and invasive pneumococci. After infection of Detroit 562 cells with S. pneumoniae for 2 and 4 hours, extracellular bacteria were stained with Omni serum (at 1:1000 dilution) followed by incubation with secondary Alexa Fluor 488-labeled anti-rabbit antibody (1:2,000 dilution, green). Intra- and extracellular bacteria were stained with mouse monoclonal anti-PsaA antibodies (3 μg/100 μl) followed by staining with secondary Alexa Fluor 594-labeled anti-mouse antibody (1:2,000 dilution, red). Pictures were taken on an Axioskop2 plus microscope at 1000x magnification.

### 6.3 S. pneumoniae IC47 antigen expression under defined growth conditions

Since PcsB could not be detected by antigen specific antibodies we wanted to investigate whether IC47 antigens are expressed at all under various growth conditions in *vitro*. To achieve this we cultivated S. pneumoniae under various *in vitro* conditions: In the
presence of an iron chelator as well as in chemically defined medium supplemented without or with iron and manganese. Antigens expression and accessibility was analyzed by Western blot and bacterial surface staining using flow cytometry.

6.3.1 Growth of pneumococci in the presence of Desferal is similar to bacteria grown under standard conditions

Iron is one of the most important micronutrients required for bacterial growth and survival in the host. In the mucosa, many nutrients and especially iron are limited. Therefore we wanted to analyze IC47 antigen expression under iron limiting conditions. To mimic this micro environmental condition bacteria were grown in iron depleted THY medium. As shown in Figure 19 bacterial growth under iron limiting conditions using 0.25 mM, 0.5 mM and 1 mM desferal was similar to the growth under standard conditions.

![Figure 16: Growth of S. pneumoniae under iron limiting conditions. Pneumococcal strain 4DS2341-94 was grown in THY medium supplemented without and with 0.25, 0.5 and 1 mM Desferal. Bacterial growth was measured at an optical density of 620 nm.](image)

For exactly defined growth conditions in terms of micronutrients we prepared a chemically defined medium (CDM) containing all important amino acids, nucleotides, vitamins, metal
ions and glucose. In the limited chemically defined medium we omitted iron and manganese (CPT). When monitoring bacterial growth to the early exponential phase in THY, CDM and CPT we observed that *S. pneumoniae* strain 4DS2341-94 in chemically defined medium grows twice as slow as in THY medium (Fig. 20). Furthermore bacteria grown in chemically defined complete medium reached an OD$_{620}$ of 0.3 about one hour earlier compared to bacteria grown in defined medium lacking iron and manganese. To conclude, *S. pneumoniae* strain 4DS2341-94 can be cultivated in chemically defined complete medium as well as under iron and manganese limiting conditions.

![Figure 20](image)

**Figure 20:** Growth of *S. pneumoniae* strain 4DS2341-94 in different growth media. Strain 4DS2341-94 o/n culture was washed and inoculated in THY, chemically defined complete medium and chemically defined medium lacking iron as well as manganese. The bacterial growth until the early exponential phase was measured at an optical density of 620 nm.

6.3.2 **PsaA expression was increased under iron- and manganese limiting growth conditions**

To investigate whether IC47 antigens are expressed or up-regulated under different *in vitro* growth conditions, bacteria were grown in THY, THY supplemented with 200 mM Desferal, chemically defined complete medium and chemically defined medium without iron and manganese. Lysates of bacteria grown under these conditions were analyzed by Western blot analysis. As shown in Fig. 21 the IC47 antigens StkP (100 kDa), PcsB (43 kDa) and PsaA (37 kDa) are expressed by strain PJ1324 and 4DS2341-94 after growing in THY
supplemented with and without Desferal, chemically defined complete medium and chemically defined medium without iron and manganese. These data also revealed a similar expression level of PcsB and StkP in strain PJ1324 and 4DS2341-94. Furthermore blots incubated with polyclonal anti-PsaA antibodies showed an increased amount of PsaA in pneumococci grown in chemically defined medium without iron and manganese.

**Figure 21**: Western blot analysis of IC47 antigen expression by *S. pneumoniae* strains PJ1324 and 4DS2341-94 in vitro. (A) Pneumococci were grown in THY, THY + desferal, chemically defined complete medium and chemically defined medium without iron and manganese. Bacterial cells were lysed, equal amounts of lysate (20 µg) were separated by SDS-PAGE, followed by immune-blotting with rabbit polyclonal anti-PcsB, anti-PsaA, anti-StkP antibodies (1:5,000 dilution) and detected with HRP-labeled anti-rabbit secondary antibody (1:40,000 dilution). As controls 0.2 µg of an unrelated and 0.2 µg of the specific recombinant protein were loaded on the gel. IC47 antigens were detected at: 37 kDa (PsaA), 43 kDa (PcsB), 100 kDa (StkP), N-PcsB (36kDa). (B) Ponceau S staining corresponding to PcsB Western blot shown in Figure 21A.

Further investigations focused on pneumococcal growth in chemically defined medium. Expression of StkP and PsaA was compared in strain PJ1324 and 4DS2341-94 using antigen-specific monoclonal antibodies. The immuno-blotting shown in Figure 22 confirmed an increased PsaA expression of pneumococci grown under iron- and manganese limiting conditions compared to bacteria grown in complete medium. In contrast, the expression level of StkP remained similar at both conditions. For the antigen detection we used the same specific antibodies used in previous immunofluorescence microscopy which confirms their ability to bind to the denatured protein.
Western blot analysis of StkP and PsaA expression in pneumococci grown under limiting conditions. PsaA was detected at 37 kDa and StkP was detected at 100 kDa. (A) Bacterial cells of strain 4DS2341-94 and (B) PJ1324 were cultivated in chemically defined medium supplemented with or without iron and manganese. Bacterial lysates were separated by SDS-PAGE, followed by immune-blotting with mouse monoclonal anti-PsaA, anti-StkP antibodies (0.5 µg/ml concentration) detected with HRP-labeled anti-mouse secondary antibody (1:20,000 dilution). As controls 0.2 µg of an unrelated and 0.2 µg of the specific recombinant protein were loaded on the gel. (C) Ponceau S staining corresponding to the Western blot shown in Fig. 22A. (D) Ponceau S staining corresponding to the Western blot shown in Fig. 22B.

6.3.3 The expression of PcsB is unaffected during the growth at higher temperatures

Based on reports of Mills and colleagues about a differential expression of PcsB in pneumococci grown under various stress conditions we wanted to investigate PcsB expression of *S. pneumoniae* grown at 37 and 40°C. The expression of PcsB was analyzed by using antigen-specific monoclonal antibodies. As shown in Figure 23, the analysis of different amounts of lysate ranging from 1.25-10 µg revealed that PcsB expression when grown at 40°C is comparable to the PcsB expression at 37°C.
6.3.4 PsaA and IC47 specific sera give a positive surface staining signal on bacteria grown under iron- and manganese limiting conditions

Since in Western blot analysis an increased PsaA expression by pneumococci grown under iron- and manganese limiting conditions was observed, we wanted to investigate whether an increased antigen expression comes along with an increased surface accessibility. Therefore, we performed surface staining experiments using antigen specific antibodies. Surface expression of IC47 antigens and PsaA only were analyzed on strain 4DS2341-94 grown under iron and manganese limiting and non-limiting conditions.

*Streptococcus pneumoniae* surface staining experiments were carried out to investigate the effect of an increased PsaA expression on antigen accessibility. Mouse and rabbit sera generated against PsaA, IC47 and strain specific lysate were tested. As shown in Figure 24A, a huge positive log-shift between preimmune and lysate specific hyperimmune serum in the FL2 channel with a median of 9.4 and 4531 was observed, respectively. No further increase in signal intensity when comparing the signal of lysate specific hyperimmune sera tested on bacteria grown under complete and limiting conditions with a median of 4531 to 4293 was detected, respectively. Rabbit hyperimmune serum against IC47 antigens showed a small positive intensity shift when growing in chemically defined medium without iron and manganese compared to the corresponding preimmune serum. While with the preimmune serum a median of 9.56 was observed, the hyperimmune serum was giving a slightly positive surface staining signal with a median of 19.28 (Fig. 24B). A shift in the same range was also observed for the murine anti-PsaA hyperimmune serum with a median of 20.54 compared to a median of 10.84 observed with the corresponding preimmune serum (Fig. 24C). There was no intensity shift seen on bacteria grown under non-limiting conditions. It’s important to notice, that not all
IC47 and PsaA sera could show such a shift in signal intensity. Control sera of PBS immunized mice were unable to stain the pneumococcal surface (Fig. 24D).

**Figure 24:** Surface staining of *S. pneumoniae* strain 4DS2341-94 grown in chemically defined medium supplemented with or without iron and manganese. Surface staining was performed with 1:100 diluted (A) murine lysate-specific, (B) rabbit IC47-specific, (C) murine PsaA-specific, (D) murine PBS control hyperimmune sera and PE-conjugated secondary antibodies. SYTO 60 was used to stain bacterial nucleic acids and served as control. The resulting signal from staining of bacteria grown in chemically defined complete and incomplete medium with preimmune sera is shown in blue and black, respectively. Signals from bacteria grown under non-limiting and limiting conditions stained with hyperimmune sera are shown in purple and red, respectively.
We compared the median values for IC47 and PsaA specific hyperimmune sera on bacteria grown under limiting and non-limiting conditions and found that only pneumococci with an increased PsaA expression showed a small shift in signal intensity.
7 Discussion

Pneumonia is the leading cause of mortality in children and also burdens many elderly people worldwide. The majority of pneumonia related deaths could be prevented by a proper vaccine. As part of a new vaccination strategy, the development of vaccines based on recombinant proteins might prevent future systemic infections and pneumococcal nasopharyngeal carriage. Vaccination leads to the production of specific antibodies which exert various effector functions such as, opsonization of pathogens for phagocytosis, neutralization of microbes and microbial toxin, prevention of bacterial adhesion to host cells, activation of the complement system and antibody-dependent cell mediated cytotoxicity. *S. pneumoniae*’s adherence to human nasopharyngeal cells is a prerequisite for colonization and invasive disease in the host.

In this work we wanted to investigate the functional role of antibodies directed against the candidate antigens in neutralizing colonization and invasion of human epithelial cells. We also characterized IC47 antigen expression and accessibility under various *in vitro* conditions.

Adhesion and neutralization assays

Adhesion and neutralization assays were performed to investigate whether vaccination with IC47 candidates, StkP, PcsB and PsaA induces an immune response leading to the production of neutralizing antibodies in order to prevent or reduce pneumococcal colonization of the upper respiratory tract. First, we have tested the ability of different *S. pneumoniae* strains and serotypes to adhere to and invade nasopharyngeal epithelial cells. Adhesion assay experiments revealed that *S. pneumoniae* strains belonging to different serotypes show differences in their ability to adhere and invade human respiratory epithelial cells. Interestingly, the literature suggests that the adhesive and invasive potential of *S. pneumoniae* is also dependent on the capsule thickness. In fact, adhesion of encapsulated and unencapsulated pneumococci of strain TIGR4 to primary tracheal epithelial cells isolated from C57BL/6 mice showed, that the transparent-phase phenotype characterized by a thinner polysaccharide capsule was more efficient in colonizing the mucosal surface of the nasopharynx compared to opaque-phase phenotype which exhibit a thicker capsule (Nelson et al., 2007). Differences in capsule thickness of the pneumococcal strains we have used were
determined by the quellung reaction (Coonrod, 1989). In this study we found that the serotype 4 strain with a thinner capsule has an increased ability to adhere to respiratory epithelial host cells compared to the more encapsulated strains belonging to the serotypes 6B, 33A and 35A. These findings are consistent with data from an animal model of colonization and bacteremia where only pneumococci with less capsule were able establish a stable state of colonization (Austrian, 1986; Weiser et al., 1994; Wu et al., 1997b). An explanation for this might be the better exposure of adhesins on less encapsulated pneumococci which are important for the interaction with host cells. On strains being more encapsulated, these virulence factors are covered when the polysaccharide capsule reaches a certain diameter. Furthermore we showed that the tested \textit{S. pneumoniae} strains have the ability to adhere to and invade Detroit 562 cells as well as type II pneumocytes (A549 cells), which is in accordance to a report by Zhang and colleagues who have tested pneumococcal adhesion to and invasion of several human cell lines.

The human upper respiratory tract is an ecological niche for many different bacteria (Burky and Smillie, 1929). It is very likely that every individual gets colonized by pathogens which in general results in an asymptomatic carriage but also can be followed by disease (Kyaw et al., 2002). The natural infection route of \textit{S. pneumoniae} always starts with colonization, the adherence to the epithelial barrier of the respiratory tract. This pathogen-host interaction involves adhesion molecules usually exposed on the pneumococcal surface which may also trigger attachment to and uptake in host cells (Hammerschmidt, 2006). We focused on one of the three proteins from IC47 vaccine, PsaA which has been suggested to play a role in adhesion. In neutralization assays using Detroit 562 cells performed by Romeo-Steiner and colleagues, adult sera against PsaA were shown to inhibit pneumococcal adhesion to epithelial cells in a dose-dependent manner. In contrast to their findings, we did not observe an inhibitory effect of rabbit anti-PsaA antibodies on pneumococcal adherence to Detroit 562 cells. A possible explanation is the usage of different serotypes. While we tested a strain of serotype 4 they used strains belonging to serotype 19F and 18C. Referring to the previously discussed differences in capsule thickness between different serotypes it is possible that the capsule thickness influences the accessibility of PsaA. On the other hand, PsaA might also only play a minor role in adhesion to host cells among all the other factors and molecules such as phosphoryl-choline, PspC, PavA and RgrA which are known to be involved in pneumococcal adhesion (Agarwal et al., 2010; Cundell et al., 1995; Pracht et al., 2005). A
hyperimmune serum directed against all three IC47 candidate antigens could not decrease pneumococcal adhesion to human epithelial cells. Currently there are no reports about StkP and PcsB being involved in pneumococcal adhesion. However the lysate specific control serum which contains antibodies against the majority of \textit{S. pneumonia} surface proteins was able to reduce adhesion ranging from 50-72%. It is also possible, that our IC47-specific antibodies do not have a neutralizing function, but might play a role in opsonization for phagocytic killing of the pathogen, since anti-PsaA antibodies showed killing of \textit{S. pneumoniae} serotype 6B in opsonophagocytic assays (Anderton et al., 2007). In addition, the presence of another antibody sub-class IgA, involved in mucosal immunity and prevention of colonization, might play an important role in preventing pneumococcal adhesion.

**Immunofluorescence analysis**

In our attempt to investigate the reason why IC47 antigen specific antibodies could not reduce pneumococcal adhesion to human epithelial cells, we used immunofluorescence analysis in order to analyze the accessibility and surface expression of our candidate antigens and PspA for antigen-specific antibodies. Monoclonal antibodies against PspA, the most abundant protein on the surface, stained fixed and living bacteria. The fluorescence signal is dispersed over the entire pneumococcal surface which is in accordance to the literature. Flow cytometry experiments using PspA specific antibodies characterized PspA as surface expressed as well as surface accessible antigen (Daniels et al., 2010).

An interesting finding was, that monoclonal antibodies against the C-terminal PASTA domain of our candidate StkP bind only fixed and not living bacteria. Previous surface staining experiments analyzed by flow cytometry indicated that the PASTA domains of StkP are located on the pneumococcal surface (Giefing et al., 2010). Our results are in accordance that the PASTA domain of StkP is surface exposed, but given the fact that only fixed bacteria could be stained it might also be an artifact, generated by the cross-linking of protein side chains or a capsular rearrangement during the para-formaldehyde fixation procedure. Another possibility is a partial permeabilization of the pneumococci during the fixation step that would allow the staining of StkP which is not located on the bacterial surface. Further, we found that monoclonal antibodies against PsaA could not bind to the surface of \textit{S. pneumoniae} strain
This result is in agreement with findings of Johnston et al., who observed no detectable fluorescence signal on pneumococci stained with anti-PsaA antibodies even at a 1:100 serum dilution. Nevertheless, the findings of a non-surface exposed PsaA antigen are in contradiction to a previously reported positive immunofluorescence staining of pneumococci with monoclonal anti-PsaA antibodies (Russell et al., 1990), testing PsaA as a diagnostic marker. The staining presented by Russell et al., included acetone washes which might have damaged the cell-wall as well as the capsular integrity. Furthermore, we observed no binding of PcsB-specific monoclonal antibodies to the pneumococcal surface. These data are consistent with previous findings by Mills and colleagues who assessed PcsB surface accessibility for specific antibodies by using flow cytometry analysis. Interestingly Giefing et al., who used the same strain setup has reported a positive surface staining signal with PcsB specific antibodies analyzed by flow cytometry.

It is well known that every clinical pneumococcal strain is covered by a polysaccharide capsule which might play a major role in regulating accessibility of surface antigens which are required for host cell interaction and antibody-mediated clearance by the immune system (Abeyta, Hardy, and Yother, 2003). Despite the intensive work, the mechanisms involved in capsule regulation are still poorly understood. The importance and properties of the pneumococcal capsule for the infection process of S. pneumoniae has been studied by electron microscopy, revealing a loss of capsular structure during pneumococcal adhesion to human epithelial cells (Hammerschmidt et al., 2005). Because of reported surface differences between transparent and opaque variants we investigated IC47 antigen expression and surface accessibility of adhering and intracellular bacteria after infection of the human epithelial cell line Detroit 562. As shown in the results extra- and intracellular bacteria cannot be distinguished using antigen-specific antibodies and FITC pre-labeled bacteria. An explanation for the rapid FITC signal loss might be the high growth rate of bacteria in the presence of FBS, a component of the eukaryotic growth medium. Also co-localization studies using confocal microscopy and staining with antibodies against the eukaryotic early endosomal marker EEA1 as well as S. pneumoniae specific antibodies did not verify the intracellular localization of bacteria limited by the resolution of the confocal microscope. Since the use of FITC pre-labeled bacteria and co-localization studies with an early-endosomal marker were not successful for identifying intracellular bacteria, we decided to perform a double staining with Omni serum which is used for detection of capsular polysaccharides and PspA-specific
mouse monoclonal antibodies. Using the double staining method, only after 4 hours of infection and not 2, we were able to observe intracellular bacteria. When using antibodies specific for IC47 antigens, no intracellular bacteria were detected. Interestingly, StkP was detected on the surface of adhering pneumococci, after 2 and 4 hours of infection. Since in adhesion and invasion experiments only 1% of the bacteria were recovered from inside the epithelial cells, it might be possible that the amount of invasive pneumococci is too low and therefore no intracellular bacteria could be detected. Further immunofluorescence experiments should be performed with an increased multiplicity of infection. PcsB-specific antibodies could not bind to the pneumococcal surface which is contradictory to a report where the protein was found to be located in the plasma membrane and detectable on the surface of unencapsulated S. pneumoniae derivates R6 and JD908 (Mills, Marquart, and McDaniel, 2007). The positive surface staining signal observed in Mills study might be explained by the lack of the pneumococcal polysaccharide capsule, not covering the peptidoglycan hydrolase PcsB while the capsule of the 4DS2341-94 strain we have used might hinder the binding of specific antibodies to their antigen even in close contact with human epithelial cells. Interestingly, anti-PsaA antibodies could not bind to pneumococci after 2 hours but after 4 hours of infection although the signal was very weak and not observed on every single pneumococcal cell. An explanation for this observation could be the up-regulation of PsaA in pneumococci adhering to nasopharyngeal cells. In vivo expression studies of S. pneumoniae genes using whole-genome microarrays showed a 8-fold increased PsaA expression in pneumococci with contact to epithelial cells (Orihuela et al., 2004b). The positive staining signal indicates that PsaA is located on the pneumococcal surface, which is consistent with reports of its function as pneumococcal adhesin and the linkage to the cytoplasmic membrane (Anderton et al., 2007; Jenkinson, 1994; Rajam et al., 2008).

The data presented by Hammerschmidt et al. who observed an almost complete loss of the capsular structure after 2 hours of infection could not be confirmed since antibodies specific for PcsB could not bind to the pneumococcal surface. These different observations might be explained by the use of another pneumococcal strain, A66 which also belongs to a different serotype. Furthermore, the larynx carcinoma cell line HEp-2 was used in Hammerschmidt’s study while we used the nasopharyngeal cell line Detroit 562 which might express a different set of host receptors and surface molecules.
Flow cytometry and western blot analysis

The last part of this diploma thesis focused on the expression of IC47 antigens at various *in vitro* growth conditions. Based on Intercell’s ANTIGENome technology it’s known that all three vaccine candidates are expressed *in vivo*. Western blot analysis revealed that pneumococci grown under standard *in vitro* growth conditions express all three *S. pneumoniae* candidate antigens, which is consistent with the literature (Giefing et al., 2008; Johnston et al., 2004). As already mentioned, PsaA one of our vaccine candidates is reported to be a putative pneumococcal adhesin (Anderton et al., 2007) besides that it’s also part of an ATP-binding cassette important for the transport of multi-valent cations such as iron and manganese (Dintilhac et al., 1997). Bacteria in the human body have to face various host microenvironments with a different nutrient supply. It was found that the physiological iron concentration on the mucosal surface is $10^{-18}$ M (Bullen, 1981) due to iron sequestering proteins such as lactoferrin, however bacteria normally need an iron concentration of 0.4-1 µM in order to grow properly (Joseph W. Lengler). We have monitored the growth of *S. pneumoniae* strains 4DS2341-94, PJ1324 and TIGR4 in THY and chemically defined medium in the presence and absence of iron and manganese. We observed a much slower growth in the defined medium compared to the standard growth conditions. A lag in growth for 15 hours when growing under iron and manganese limiting conditions as described in literature was not observed (Johnston et al., 2004). An explanation for that might be again the use of different *S. pneumoniae* strains, since it is difficult to obtain an identical growth pattern by different isolates (Desa et al., 2008). In a quantitative analysis of *S. pneumoniae* response to *in vitro* iron restriction analyzed by 2-Dimensional Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometers (2-D LC ESI MS/MS) an increased transcription of the PsaA encoding gene was observed (Nanduri et al., 2008). In this study, we observed the same effect on the protein level, whereas the expression of the other two candidates PcsB and StkP remained unaffected. Expression analysis by western blot was consistent with surface staining experiments. Rabbit anti-IC47 hyperimmune sera and mouse anti-PsaA hyperimmune sera showed slightly increased signal intensity on pneumococci grown under iron and manganese limiting conditions compared to pneumococci grown under standard growth conditions. The median represents the middle value of a set of intensities whereas the mean is the arithmetic average of the entire data set. The median and the corresponding mean should be within the same range. Relatively high mean values for the IC47 and PsaA samples arise from a 10%
sub-population which was identified as *S. pneumoniae* due to correct side-scatter and a positive signal in the FL4 channel (Cyto^60^ DNA stain). This small population might just express more IC47/PsaA antigen since unspecific binding of the detecting secondary antibody was ruled out. Due to the lack of time, specificity of the signal was not confirmed by blocking studies using recombinant proteins and would still be necessary.

The surface accessibility of PsaA is somehow contrary to crystal structure analysis that predicts a protein size and conformation which should be unable to extend beyond the polysaccharide capsule (Lawrence et al., 1998; Tomasz, 1981). On the other hand, reports about the binding of PsaA-coated fluorespheres to E-Cadherin transfected fibroblasts would suggest antigen surface expression and surface accessibility (Anderton et al., 2007). Another fact that supports the surface accessibility of the IC47 candidates is the natural acquisition of antibodies against PsaA, PcsB and StkP in all age groups (Schmid et al., 2011).

As previously mentioned, we found that the pneumococcal antigen PsaA is up-regulated under iron and manganese limiting conditions which are similar to the mucosal microenvironment found in the human body. Cytokine response to pneumococcal surface proteins or cell-wall components and the release of pneumolysin can lead to local inflammation. Besides redness and swelling an increased temperature is one of the major inflammatory characteristics. Mills et al., found a differential expression of PcsB in response to different kinds of stress such as high osmolarity or higher temperatures. We cultivated *S. pneumoniae* at 40°C but could not observe any alteration in PcsB expression compared to the expression level of pneumococci grown at 37°C. Mills and colleagues used 42°C whereas in our experiments bacteria were grown at 40°C only. We haven’t checked higher temperatures because that wouldn’t be adequate to the physiological conditions found in the human body except of high fever, which is a very critical condition anyway.

In summary, we have analyzed the inhibitory function of IC47 specific antibodies on pneumococcal adhesion to human nasopharyngeal cells. Our data was generated in vitro were we could not observe a reduced adhesion of *S. pneumoniae* to human epithelial cells, in the presence of StkP-, PcsB and PsaA-specific antibodies. Antibodies specific for IC47 antigens might also fulfill other functions such complement activation or opsonization of *S. pneumoniae* for phagocytic killing. In vivo conditions, present in the human body and during the infection cannot be completely obtained in vitro. The bacterial gene expression highly
regulated in order to respond to different milieus found in the human body. Nevertheless, the neutralization assay should be further evaluated by using pneumococci grown under iron and manganese limiting conditions testing PsaA-specific post-vaccination sera. An additional application is the use of unencapsulated derivates in order to investigate the role of the polysaccharide capsule in antigen accessibility. However, more studies are required to characterize the functional role of antibodies directed against IC47 antigens.
8 References


http://www.cdc.gov/vaccines/vpd-vac/pneumo/in-short-both.htm

http://www.who.int/vaccines/en/pneumococcus.shtml

Biology of the prokaryotes - Joseph W. Lengeler, Gerhart Drews and Hans Günter Schlegel
# Appendix

## 9.1 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
</tr>
<tr>
<td>CAP</td>
<td>Community-acquired pneumonia</td>
</tr>
<tr>
<td>CD&lt;sup&gt;4+&lt;/sup&gt;</td>
<td>Cluster of differentiation 4 positive t-cell</td>
</tr>
<tr>
<td>CDM</td>
<td>Chemically defined medium</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund’s adjuvant</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CPS</td>
<td>Capsule polysaccharide</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’-6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early endosome antigen 1</td>
</tr>
<tr>
<td>EMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Federal Drug Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GSK</td>
<td>Glaxo Smith Kline</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s buffered salt solution</td>
</tr>
<tr>
<td>IFA</td>
<td>Incomplete Freund’s adjuvant</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>kDA</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamid gel electrophoresis</td>
</tr>
<tr>
<td>PATH</td>
<td>Program for Appropriate Technology in Health</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate buffered saline + Tween-20</td>
</tr>
<tr>
<td>PCV</td>
<td>Pneumococcal conjugate-vaccine</td>
</tr>
<tr>
<td>PLY-4</td>
<td>Pneumolysin domain 4</td>
</tr>
<tr>
<td>RCF</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SSI</td>
<td>Serum Staten Institute</td>
</tr>
<tr>
<td>Th17</td>
<td>T helper 17 cells</td>
</tr>
<tr>
<td>THB</td>
<td>Todd-Hewitt broth</td>
</tr>
<tr>
<td>THY</td>
<td>THB + 0.5% yeast extract</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
</tbody>
</table>
9.2 Curriculum vitae

PERSONAL DETAILS

Name Michael Ehlers
Date and place of birth 21th of November 1983, Dresden
Citizenship Germany

EDUCATION

2008-2011 University of Vienna
Second academic phase in molecular biology (equivalent to masters)
2005-2008 University of Vienna
First academic phase in molecular biology (equivalent to a Bachelor)
1994-2002 Friedrich-Schiller Gymnasium Bautzen, Abitur
Grammar school
1992-1994 Frédéric-Joliot Curie Schule, Bautzen
Elementary school
1990-1992 Albert-Einstein Schule Bautzen
Elementary school

WORK EXPERIENCE

May 2010 – February 2011 Intercell AG, Vienna
Practical work for the diploma thesis in the group of Sanja Selak
Department of Serology and Immune assays
January – March 2010 Max F. Perutz Laboratories, Vienna
Internship in the group of Manuela Baccarini
October – November 2009 Intercell AG, Vienna
Internship in the department for Serology and Immune assays
August 2009 Medical University of Vienna, Vienna
Internship in the group of Bernd Binder
SKILLS

Spoken languages – German and English

Computer skills