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“Science cannot solve the ultimate mystery of nature. And that is because, in the last analysis, we ourselves are part of nature and therefore part of the mystery that we are trying to solve.”

Where Is Science Going?, Max Planck (1932)

*My family
and
Barbara*

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Abstract

Streptococcus pneumoniae is a common human pathogen that causes a variety of life-threatening invasive diseases such as pneumonia, bacteremia and meningitis. Despite the availability of licensed vaccines and antibiotic treatments, morbidity and mortality attributed to this bacterium remain significant in developing and developed countries. Due to increasing antibiotic resistance and limited efficacy of existing vaccines in at-risk populations, there is a need for new treatment strategies such as passive immunotherapy using human monoclonal antibodies (mAbs).

In this study, three conserved antigens of *S. pneumoniae* – lipoteichoic acid (LTA), pneumococcal surface protein A (PspA) and pneumolysin (PLY) – were characterized for their suitability as targets for a mAb-based anti-infective therapy.

Although isolation and purification procedures could be optimized for LTA from *Streptococcus pyogenes*, native LTA could not be extracted from *S. pneumoniae* in sufficient quantity and quality, thus limiting more in-depth studies of this antigen.

Recombinant full-length PspA and PLY as well as domains thereof were expressed, purified and subsequently proven to be highly immunogenic in naïve C3H/HeN mice. These antisera were characterized in-depth *in vitro*: in surface staining and in ELISA, antibodies were shown to recognize PspA in a clade-specific manner. Polyclonal antibodies against Family 1 PspA also reacted with other Proline-rich cell-surface proteins – presumably PspC – but not with Family 2 PspA.

Consequently these antisera were tested *in vivo* by passive transfer and subsequent lethal challenge with different *S. pneumoniae* strains in mice. The results correlated with surface staining data: anti-PspA hyperimmune sera were only effective against pneumococci expressing homologous PspA but not against those with a heterologous variant. Anti-PLY sera were not fully protective although conferring prolonged survival. Interestingly the observed protection correlated with the level of inflammatory IL-6, induced in mice.

Two *in vitro* assays exploiting the function of PLY were set up to allow a detailed characterization of selected antibodies: a *Hemolysis-Inhibition Assay* and an *hTLR4-Reporter Assay*. PLY-specific murine polyclonal and monoclonal antibodies reduced the hemolytic activity of PLY on erythrocytes and interfered with the activation of TLR4 through PLY.

Since human mAbs against PLY will be generated from B-cells based on the “*Sindbis Virus Based Mammalian Cell Surface Display*” technology, healthy human donors were identified based on their antibody titers in ELISA. In addition PMBC staining conditions that are required for the selection of antigen-specific memory B cells were optimized.

In conclusion, a deeper insight into the mode of action of PspA- and PLY-specific antibodies could be gained with this work and analytical methods that are required for the selection and validation of human mAbs were developed. This way a basis for the development of a mAb-based therapy for the prevention and treatment of life-threatening pneumococcal diseases was established.

Keywords: *Streptococcus pneumoniae*, monoclonal antibodies, LTA, PLY, PspA

Kurzfassung

Streptococcus pneumoniae ist ein weitverbreiteter Erreger verschiedenster lebensbedrohlicher Krankheiten wie Pneumonien, Bakteriämien oder Meningitis. Obgleich lizenzierte Impfstoffe und Antibiotika zur Verfügung stehen, bleibt die Morbidität und Mortalität, nicht nur in Entwicklungsländern, sondern auch in Industrieländern auf einem hohen Niveau. Aufgrund zunehmender Antibiotikaresistenzen und der suboptimalen Wirksamkeit verfügbarer Impfstoffe in Risikogruppen, steigt der Bedarf für neue Therapien, wie beispielsweise einer passiven Immunisierung mit monoklonalen Antikörpern.

In dieser Arbeit wurden drei konservierte Antigene von *S. pneumoniae* – Lipoteichonsäure (LTA), pneumococcal surface protein A (PspA) und Pneumolysin (PLY) – auf ihre Eignung als Zielmoleküle für die Entwicklung einer anti-infektiösen Antikörper-Therapie hin untersucht.

Obwohl die Isolierung und Reinigung nativer LTA für den Erreger *Streptococcus pyogenes* erfolgreich optimiert wurde, konnte LTA nicht in ausreichender Menge und Reinheit aus *S. pneumoniae* gewonnen und somit keine weiterführenden Studien mit diesem Antigen durchgeführt werden.

Verschiedenste Konstrukte von PspA und PLY wurden rekombinant exprimiert, gereinigt und nachfolgend deren immunogene Wirkung in naiven C3H/HeN Mäusen demonstriert. Die gewonnenen Antiseren wurden in Folge genauestens *in vitro* analysiert. Oberflächenfärbungen lebender Pneumokokken zeigten, dass PspA spezifische Antikörper nicht alle PspA-Varianten detektieren konnten, sondern nur solche des selben PspA-Typs. Polyklonale Antikörper gegen Typ 1 PspA reagierten auch mit anderen Prolin-reichen Oberflächenproteinen, vermutlich PspC, jedoch nicht mit PspA Typ 2.

Des Weiteren wurden die generierten Antiseren in zuvor etablierten Mausmodellen durch passiven Transfer und nachfolgender letaler Infektion mit verschiedenen *S. pneumoniae* Stämmen auf ihre Wirksamkeit getestet. Die Ergebnisse korrelierten mit den Daten der Oberflächenfärbung: Anti-PspA Hyperimmunseren induzierten eine Protektion gegen Pneumokokken mit homologen jedoch nicht heterologen PspA. Anti-PLY Seren hingegen konnten keine vollständig protektive Wirkung erzielen, bewirkten jedoch eine erhöhte Überlebensrate. Die beobachtete Protektion korrelierte mit der Stärke der durch die Infektion induzierte inflammatorischen IL-6 Reaktion in den Tieren.

Im Hinblick auf die Wirkungsweise von PLY wurden zwei *in vitro* Analysen zur Antikörper-Charakterisierung entwickelt: ein Hämolyse-Inhibitions Assay und ein hTLR4-Reporter Assay. Pneumolysin spezifische murine poly- und monoklonale Antikörper bewirkten eine Reduktion der hämolytischen Aktivität von PLY auf Erythrozyten und interferierten auch mit der Aktivierung von TLR4 durch PLY.

Humane monoklonale Antikörper sollen in weiterer Folge aus humanen B-Zellen basierend auf der „*Sindbis Virus Based Mammalian Cell Surface Display*“ Technologie generiert werden. Zu diesem Zweck wurden gesunde Spender aufgrund ihres Antigen-spezifischen Titters im ELISA identifiziert. Zudem wurden die für die B-Zell-Selektion notwendigen PBMC-Färbetechniken optimiert.

Zusammenfassend demonstriert diese Arbeit einen vertiefenden Einblick in die Wirkungsweise von PspA und PLY-spezifischen Antikörpern sowie die Entwicklung von für die Selektion und Validierung humaner monoklonaler Antikörper notwendigen Analyseverfahren. Somit konnte die Grundlage für eine Antikörper-basierende Therapie zur Prävention und Behandlung von lebensbedrohlichen Pneumokokkeninfektionen geschaffen werden.

Schlagwörter: *Streptococcus pneumoniae*, monoklonale Antikörper, LTA, PLY, PspA

Table of Contents

A - Introduction.....	10
1 Streptococcus pneumoniae	10
1.1 The Morphology of <i>S. pneumoniae</i>	10
1.2 The Pathophysiology of <i>S. pneumoniae</i>	11
1.3 Pneumococcal Infection and the Host Response in Human.....	13
2 Prevention and Treatment of <i>S. pneumoniae</i> Infections	15
2.1 State of the Art Vaccines Against Pneumococcal Infections.....	15
2.2 The History of the Prevention of Pneumococcal Infections.....	16
3 Potential Pneumococcal Antigen Targets for a mAb-Based Therapy	18
3.1 Protein Virulence Factors and Cell-Surface Proteins of <i>S. pneumoniae</i>	18
3.2 Lipoteichoic acid (LTA)	19
3.3 Pneumococcal surface protein A (PspA, SP0117)	23
3.4 Pneumolysin (PLY, SP1923)	27
4 Monoclonal Antibodies – The Golden Bullet For Fighting Infectious Diseases?.....	30
4.1 Technologies for the Generation of mAbs.....	30
4.2 The Potential of Antibody-Based Therapies	33
B - Aims of the Study.....	35
C - Materials and Methods	36
1 Bacteria.....	36
2 Eukaryotic Cell Lines	36
3 Isolation and Purification of Human Peripheral Blood Mononuclear Cells and Red Blood Cells	37
4 Generation of Recombinant Antigens	38
4.1 General DNA- and Protein- Methods.....	38
4.2 Generation of Bacterial Expression Hosts for Protein Expression.....	39
4.3 Small-Scale Expression Analysis	40
4.4 Large-Scale Expression and Purification of Recombinant Proteins	40
4.5 Coupling of Antigens to FluoSpheres®	42

5 Isolation, Purification and Quantification of LTA	42
5.1 Small-Scale Optimization using LTA from <i>S. pyogenes</i>	42
5.2 Purification of LTA from <i>S. pneumoniae</i>	43
6 Animal Studies	46
6.1 Animal Guidelines – Tierversuchsantrag	46
6.2 Generation of Hyperimmune Sera in <i>M. musculus</i>	46
6.3 Dose Finding Studies for Lethal Infection of <i>M. musculus</i> with Pneumococcal Strains	46
6.4 <i>In vivo</i> Efficacy Testing of Polyclonal Antibodies	47
7 In vitro Assays	47
7.1 Western Blot Analysis	47
7.2 Enzyme Linked Immunosorbant Assay (ELISA)	48
7.3 Surface Staining of Live Pneumococci	49
7.4 Surface Staining of Eukaryotic Cells	49
7.5 Hemolysis-Inhibition Assay	50
7.6 TLR4-Reporter Assay	51
D - Results	52
1 Generation of Recombinant Proteins	52
1.1 Sequence Information and Designing of the Recombinant Constructs	52
1.2 Cloning and Protein Expression	54
2 Generation and Screening of Mouse Hyperimmune Sera	58
3 The <i>in vivo</i> Virulence of Pneumococci in Mouse <i>via</i> Different Application Routes	59
4 Antigen Specific Assays and Experiments	61
4.1 Lipoteichoic acid (LTA)	61
4.2 Pneumococcal surface protein A (PspA, SP0117)	66
4.3 Pneumolysin (PLY, SP1923)	72
5 Titer-Screenings of Sera from Healthy Human Donors	90
E - Discussion and Conclusion	91
F - Bibliography	98

G - Index of Figures and Tables	131
1 Table of Figures.....	131
2 List of Tables.....	133
3 Table of Supplementary Figures.....	134
4 List of Supplementary Tables	135
H - Appendices	A-1
A-1 Table of Abbreviations.....	A-1
A-2 Primer Sequences	A-4
A-3 Protein Sequence Alignments of PspA from Several Pneumococcal Strains	A-4
A-4 Serum-Screenings of Human Blood Donors.....	A-6
A-5 Titer-Screenings of Mouse Hyperimmune Sera.....	A-13
A-6 <i>In vivo</i> Virulence of Different <i>S. pneumoniae</i> Strains.....	A-15
A-7 Summarized Data of the <i>in vivo</i> Efficacy Testing of Polyclonal Antibodies	A-15
A-8 Data Read-outs of the IL-6 ELISA	A-17

A - INTRODUCTION

1 STREPTOCOCCUS PNEUMONIAE

1.1 The Morphology of *S. pneumoniae*

After its first isolation, identification and cultivation from human saliva, by G.M. Sternberg and L. Pasteur in 1880, *S. pneumoniae* was identified as one of the most prominent causes of bacterial-induced deaths worldwide to date. [1-3]

S. pneumoniae, which is also called “pneumococcus”, is a Gram-positive, catalase-negative, round to lancet-shaped coccus with a diameter of 0.5 - 1.25 μm . Pneumococci mostly appear in pairs (diplococci, growing in chains, Figure 1-A), are not forming spores, are non-motile and are encapsulated by a polysaccharide capsule. [1, 4-5]

S. pneumoniae can be cultivated on blood-agar plates under aerobic atmosphere supplemented with CO_2 , forming glistening colonies with approximately 1 mm in diameter. Alpha hemolysis – the breakdown of hemoglobin by pneumolysin (PLY) – of the blood leads to a transparent halo surrounding of the growing colonies (Figure 1-B). [1, 4-5]

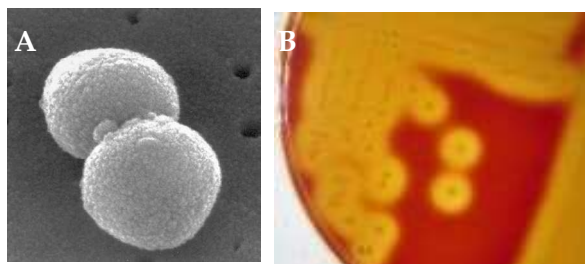


Figure 1: The Morphology of *S. pneumoniae*. A. Scanning electron microscopy image of dividing cells.^[6] B. Colonies growing on blood agar. ^[4]

As pneumococci produce autolysin (*LytA*, an amidase cleaving N-acetylmuramoyl-L-alanine in pneumococcal peptidoglycan [7]) the cells are disrupted and disintegrated causing killing of the entire culture when grown to stationary phase. The lysis due to autolysin also causes observable morphological changes of the colonies from initially plateau-type morphology to a collapse in the center after initiation of autolysis. [4, 8-9]

With the exception of some isolates associated with conjunctivitis, most clinical isolates of *S. pneumoniae* are encapsulated by an approximately 200 to 400 nm thick polysaccharide capsule. The capsular polysaccharides give rise to more than 91 known pneumococcal serotypes: these major determinants of the pathogenicity of this microorganism inhibit complement deposition and also interfere with the detection of surface antigens through

antibodies and therefore protect pneumococci from opsonophagocytosis.^[4, 10] Serotypes without a capsule are non-virulent and thus only colonize the nasopharynx but cannot transit efficiently from the luminal mucus to the epithelial surface. ^[4, 10] Each serotype typically includes a number of genetically divergent clones with different invasive disease potential. ^[11-13] Pneumococci can change their capsular thickness spontaneously, this *phase-variation* leads to a capsular switch from opaque (designed for survival in blood) to transparent (suited for colonization of the nasopharynx) colonies. ^[4, 14-16]

Clinical studies showed that *S. pneumoniae*, as a naturally competent organism, can uptake foreign DNA – *via* bacterial intra- and inter-species gene transfer and phage transduction – and incorporate homologous sequences by transformation. This allows pneumococci to switch to serotypically distinct capsular types *in vitro* and *in vivo*, or leads to resistance against antibiotics.

[7-8, 13]

1.2 The Pathophysiology of *S. pneumoniae*

Subsequent to the discovery in the late 19th century, *S. pneumoniae* was identified as causative agent of life-threatening invasive and non-invasive diseases in children (Figure 2) and elderly, in developing and developed countries. ^[1, 3-4]

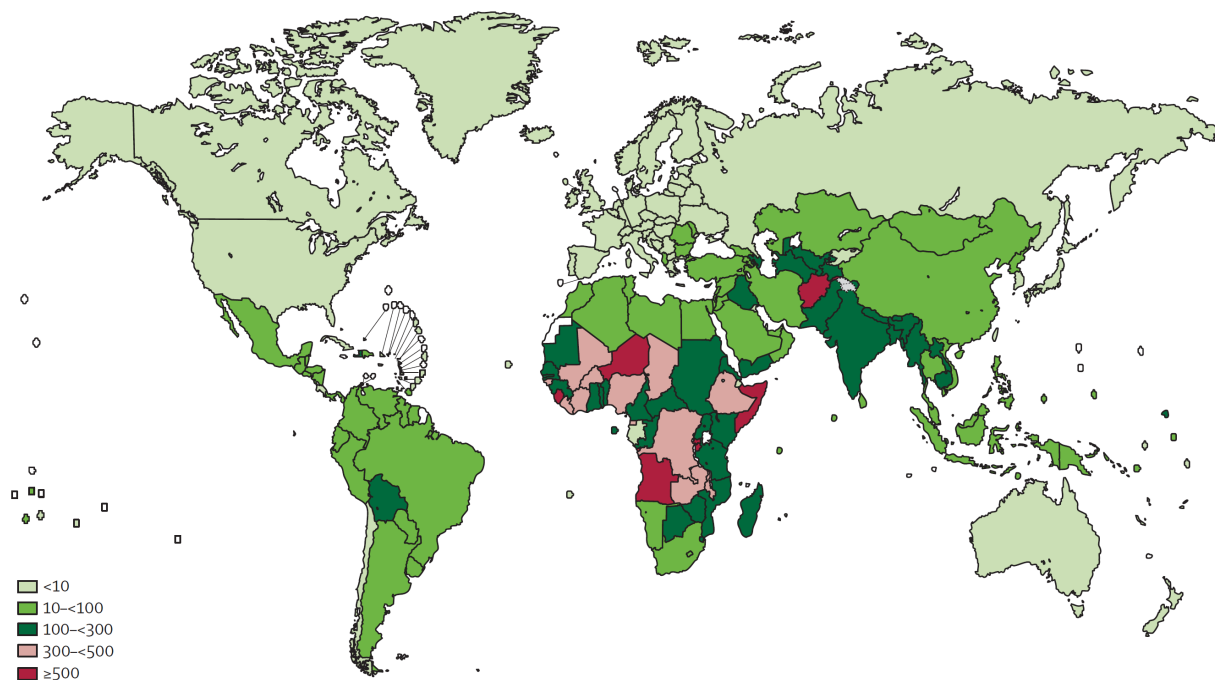


Figure 2: Pneumococcal Deaths in Children. The number of pneumococcal deaths per 100,000 children is depicted for children younger than 5 years (HIV-negative pneumococcal deaths only) published by the WHO. (modified from ^[17])

A - INTRODUCTION

Pneumococci *inter alia* induce conjunctivitis, community-acquired pneumonia (CAP), bacterial meningitis, bacteremia, otitis media, sinusitis, septic arthritis, osteomyelitis, peritonitis, tracheobronchitis, bronchitis, cellulitis, pericarditis, endocarditis and myositis. ^[3-4, 18] Bronchial pneumonia, involving the alveoli contiguous to the larger bronchioles of the bronchial tree, is most prevalent in infants, young children and aged adults whereas lobar pneumonia, involving all of a single lobe of the lungs – the entire area involved tends to become a consolidated mass – is most prevalent in younger adults. ^[4, 19]

S. pneumoniae is spread from person-to-person by direct contact and can be carried without symptoms for weeks to months before it is cleared. The mucosal and systemic immunoglobulin response is mainly directed against the serotype-specific capsular polysaccharides but also against major cell-surface proteins such as pneumococcal surface protein A (PspA), which is discussed in more detail in the following chapters. ^[7, 20-22]

As a typical extracellular bacterial pathogen, *S. pneumoniae* has to encounter mucus secretion minutes after entering the nasal cavity (exemplified in Figure 3 showing the progress of an infection from 30 minutes to 14 days post infection in mice ^[7]), adhere to host cells, replicate and escape clearance and/or phagocytosis. This is followed by manifesting infection *via* direct extension – in the upper and/or lower respiratory tract – lymphatic or hematogeneous spread – in the blood, peritoneum, cerebrospinal fluid, or joint fluid. Hereby the capsule enhances pneumococcal persistence by limiting mucus-mediated clearance. ^[4, 7, 10]

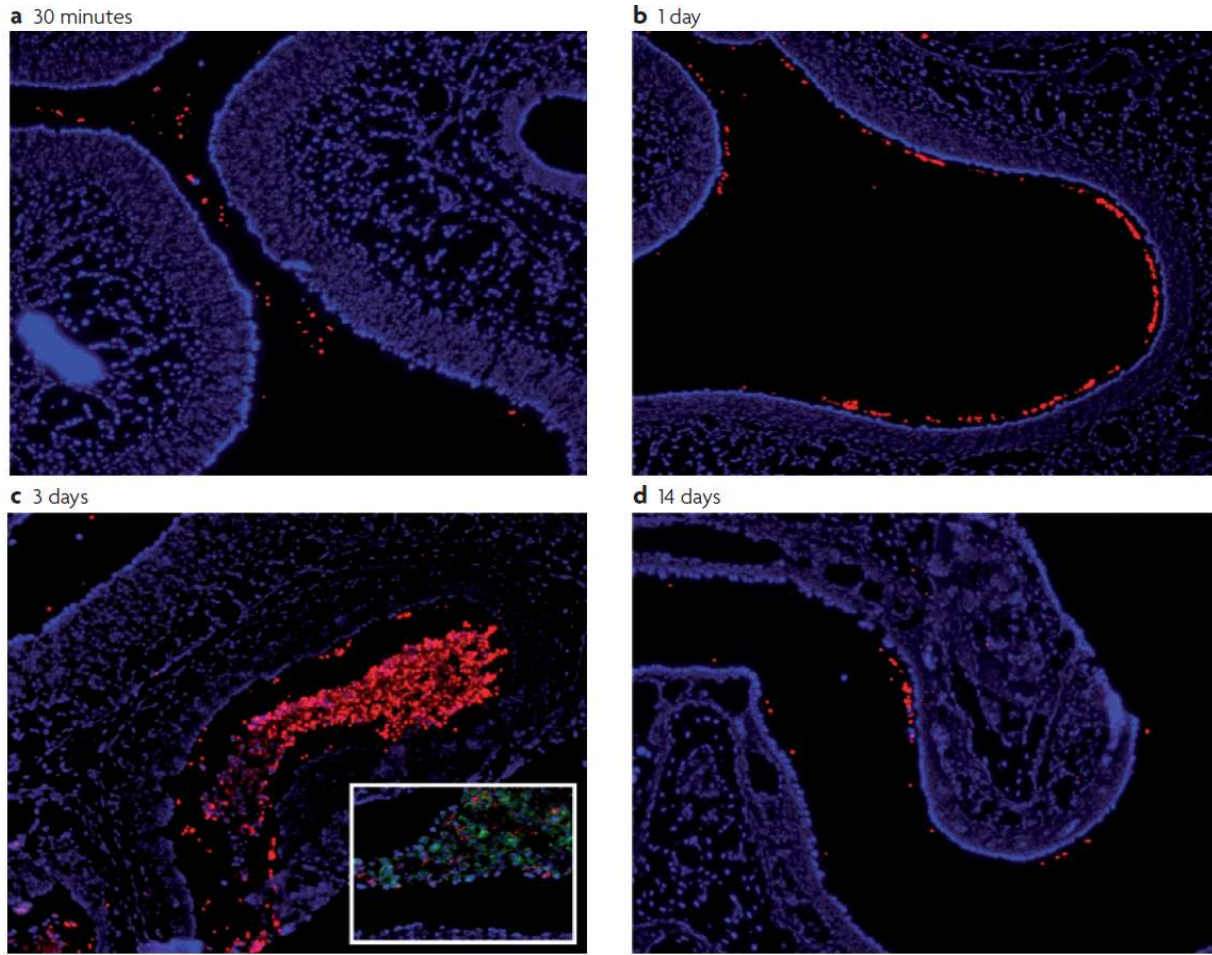


Figure 3: Nasal Colonization by *S. pneumoniae*. The bacteria (in red) are detected using serotype-specific antisera and the animal tissue (blue) is stained using DAPI. a, 30 minutes, b, 1 day, c, 3 days and d, 14 days post infection . [7]

1.3 *Pneumococcal Infection and the Host Response in Human*

Compared to any other vaccine-preventable disease the infection with *S. pneumoniae* and the diseases associated with it, belong to the most frequent causes of death worldwide. [4, 7, 23-24] The more than 1.5 million deaths *per annum* occur mainly in the elderly and the very young, where the frequency of each serotype as well as the increasing antibiotic resistance varies from country to country. [4, 7, 23-24] This is exemplified in Figure 4, representing the most-recent situation in Austria in 2009. Interestingly, some serotypes are more prevalent in bacteremia whereas others are more associated with meningitis. [25]

A - INTRODUCTION

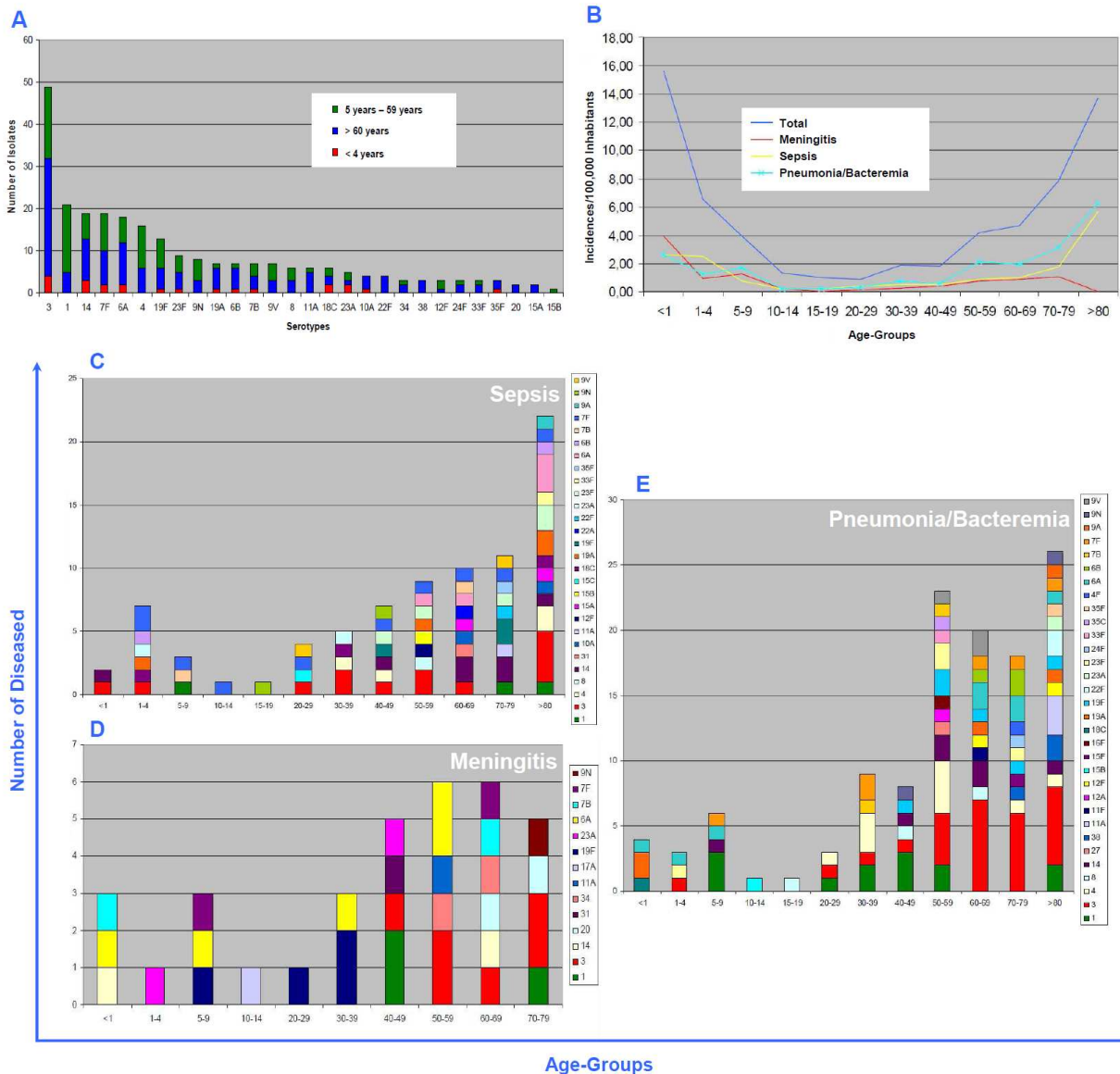


Figure 4: Statistical Surveillance of *S. pneumoniae*. 303 invasive pneumococcal diseases were reported in Austria (2009) with a strong similarity of the age-related serotype distribution to the international picture (A). Highest numbers of invasive-diseases referred to at-risk populations, namely children and elderly (B). The numbers of disease-associated serotypes were depicted as: 28 serotypes in 80 isolates from sepsis-cases (C), 14 serotypes in 32 isolates from meningitis-cases (D) and 32 serotypes in 110 isolates from cases of pneumonia/bacteremia (E). (modified from [25])

S. pneumoniae invasion of the nasopharyngeal lumen is followed by the influx of neutrophils one to three days later as host immune-response to the infection. [7, 26-27]

The innate immune response is based on the recognition of highly conserved pathogen-associated molecular patterns (PAMPs) of *S. pneumoniae* by Pathogen Recognition Receptors (PRRs) such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs). [22, 28] This interaction induces cellular (*i.e.* dendritic cells, macrophages, neutrophils and monocytes) and humoral immune responses (complement cascades, chemokines, cytokines, C-reactive protein (CRP),

etc.). [22, 28] Pneumococci are *inter alia* recognized by TLR2 (*e.g.* lipoteichoic acid (LTA) and peptidoglycan [29-36]), TLR4 (*e.g.* PLY [37-56]), TLR9 (endosomal, unmethylated CpG [57-59]) and by the activation of predominantly the classical (CRP bound to phosphorylcholine activates C1q) but also the alternative complement cascade [7, 60-62].

Whilst the innate immune response gives rise to an unspecific repertoire of host mechanisms recognizing and fighting certain classes of pathogens, the adaptive immunity represents a very specified and effective defensive-system in eukaryotes. [22, 28] The time-delayed adaptive immune response is typically activated by innate immune mediators such as chemokines and cytokines, four to seven days post infection and induces immunologic memory. [22, 28] This level of host-defense is mainly based on the functionality of specific antibodies, recognizing bacterial surface antigens, thus activating the classical (IgM and IgG) or the alternative (IgA) pathway of complement activation. [22, 28] Phagocytosis – after opsonization with serotype-specific antibodies and complement component C3b – leads to a host-mediated killing of pneumococci. In addition to humoral immunity, CD4⁺ T-cells secreting interleukin-17 (IL-17) are reported of being necessary for efficient pathogen clearance. [7, 27, 63-66] The IgA1 protease activity of *S. pneumoniae* diminishes the clearance by antibody until sufficient amounts of other (sub-)classes of specific antibodies have been generated or high levels of IgG are present. [67-70]

S. pneumoniae is resistant to innate immune responses stimulated by itself which leads to clearance of other microbial species in the same niche. [71] For example the pneumococcal capsule reduces the complement deposition of iC3b on the bacterial surface and thus inhibits clearance of the pathogen by the host's defense. [7, 72-74]

2 PREVENTION AND TREATMENT OF *S. PNEUMONIAE* INFECTIONS

2.1 *State of the Art Vaccines Against Pneumococcal Infections*

Worldwide, several pneumococcal vaccines are in different stages of development, and three capsular polysaccharide (CPS) vaccines have already been in use in the last years: (i) 23-valent purified CPS vaccine (Pneumovax®, Merck [75]), (ii) 13-valent CPS vaccine conjugated to diphtheria CRM₁₉₇ protein (Prenvar®, Wyeth/Pfizer [76]) and (iii) 10-valent CPS vaccine conjugated to protein D from *H. influenzae* (Synflorix™, GSK [77]).

CPS-based vaccines have a lower immunogenicity and require repeated immunizations, since polysaccharides do not induce T-cell mediated immunological memory, making them

unsuitable for use in young children and toddlers. The protein conjugated-polysaccharide vaccines induce enhanced immune response and memory, however just provide protection against a limited number of pneumococcal serotypes contained in the respective preparation. Therefore, these vaccines carry an inherent risk of replacement with evading serotypes, which are not included in the vaccines, or resistance due to serotype switching. [13, 78-82]

There is a new generation of pneumococcal vaccines, based on non-polysaccharide antigens, hence common proteins such as pneumococcal surface adhesin A (PsaA, SP1650), pneumolysin (PLY, SP1923), pneumococcal surface protein A and C (PspA, SP0117 and PspC, SP2190) under development, which have an improved efficacy regarding the aforementioned drawbacks of existing vaccines. [13, 81-86] Currently, a recombinant-protein-based vaccine, containing PsaA, secreted 45 kD protein (PcsB or Usp45, SP2216) and serine/threonine protein kinase (StkP, SP1732), which are very well conserved in all pneumococcal serotypes, are immunogenic during pneumococcal infections and induce opsonophagocytic antibodies, is under development in Intercell's IC47-program. [87-88]

2.2 *The History of the Prevention of Pneumococcal Infections*

Long before vaccination against the pneumococcus was established, the concomitant diseases associated with pneumococcal infections were attempted to be cured by bleeding, hydro- or oxygen-therapy or mechanical ventilation as well as administration of digitalis, morphine, quinine, strychnine or even alcohol. [1, 89-90]

It was the German brothers Felix and Georg Klemperer who in 1891 achieved the first breakthrough by immunizing rabbits intravenously (*i.v.*) with heat killed pneumococcal isolates. Passive transfer of these hyperimmune sera was shown to be protective against infection, which made their work instrumental for the definition of the humoral immune response, as it is known today. [1, 91]

About 20 years after this discovery, vaccination with heat killed pneumococci was tested in clinical trials in African miners, where the finding of the existence of the different disease-causing serotypes indicated a serotype specific protection. This subsequently led to the administration of a trivalent vaccine during World War I and serum therapy as treatment against pneumonia (Figure 5). [1, 92-94]

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vi

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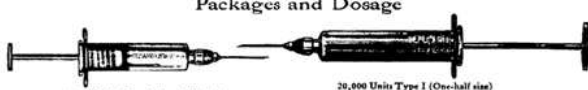
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Figure 5: Advertisement for a Pneumococcal Serum Therapy in 1933. This antiserum was manufactured by the Lederle Laboratories, Inc. against *S. pneumoniae* induced type I pneumonia and was comparably expensive at that time. (modified from [94])

As these therapeutics were mostly based on hyperimmune sera from horse or rabbit, severe side-effects such as hypersensitivity or serum sickness arose. This and the advent of chemotherapeutics (e.g. sulphonamides and penicillin), which were considered as the ultimate therapy at this time, lead to the abolishment of serum-based therapies, until an emergence of penicillin- and multidrug-resistant pneumococci was realized in the 1970s. [1]

Today, a worldwide trend of increasing penicillin-resistance (e.g. 14.3 % in North America^[95], 16.7 % in Greece^[96], more than 25 % in Burkina Faso^[97] and 47 % in Northern African countries^[98]) and multidrug-resistance (e.g. 30.1 % in North African countries^[98] or approximately 33 % in Malawi^[99]) is observed in *S. pneumoniae* isolates. In Austria (most recent data from 2009) this trend in antibacterial resistance was shown for 268 isolates to be as following: erythromycin/clarithromycin (15.30 %), penicillin (7.84 %), chloramphenicol (2.24 %) and tetracycline (1.12 %). [25]

Therefore, next to putting huge efforts in the discovery of novel antibiotics, the development of vaccines, based on e.g. pneumococcal-polysaccharides or conserved antigens

was revived. [88, 100-101] Also, the recent focus on new therapeutic approaches based on fully human (monoclonal) antibodies promise alternative treatment options of pneumococcal infections in the future. [102-107]

3 POTENTIAL PNEUMOCOCCAL ANTIGEN TARGETS FOR A *mAb*-BASED THERAPY

3.1 Protein Virulence Factors and Cell-Surface Proteins of *S. pneumoniae*

Many different pneumococcal cell surface components have been described and some of the most promising vaccine candidates among these are illustrated in Figure 6. Virulence factors, such as PLY, and cell-surface proteins, like choline-binding proteins, PspA, PspC, and LytA, but also the cell wall constituents wall teichoic acid (WTA) and LTA, are common to all serotypes of *S. pneumoniae*. [7, 24]

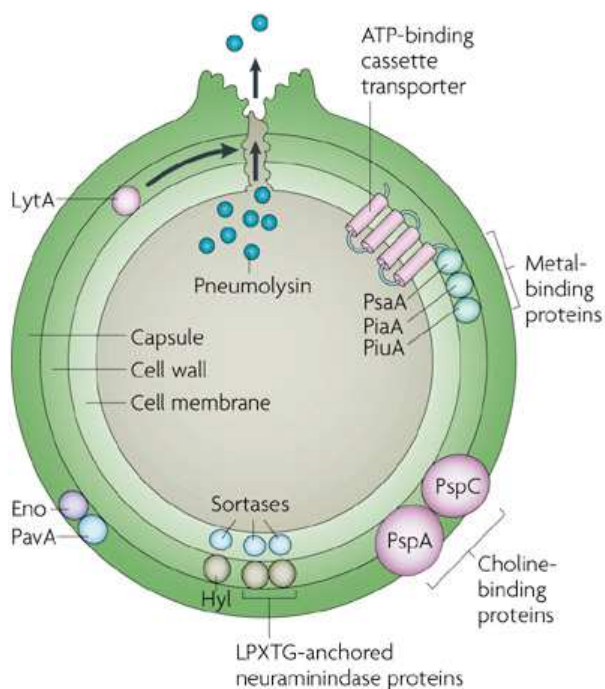


Figure 6: Important Pneumococcal Virulence Factors. The virulence of *S. pneumoniae* is diversified due to virulence factors such as the capsule, the cell wall, choline-binding proteins, metal-binding proteins, surface proteins, LPXTG-anchored proteins and PLY.[7]

It was shown that immunization with PiaA and PiuA (both divalent pneumococcal metal-ion-binding lipoproteins) protected against invasive disease in mice [7]. LPXTG-anchored proteins such as neuraminidases are also present in *S. pneumoniae*. Naturally developed antibodies to PsaA are protective against colonization in mice, whereas the antibodies against PspA can reduce bacteremia and pneumonia. [7, 24]

3.2 *Lipoteichoic acid (LTA)*

3.2.1 *Structural Features of LTA*

The core of the pneumococcal cell wall consists of the murein (peptidoglycan) – a network of peptide-cross-linked glycan strands.^[108] In many Gram-positive bacteria, teichoic acids - WTA and LTA in *S. pneumoniae* - are linked to and extend through these peptidoglycan layers, which *inter alia* allows the protein trafficking in pneumococci. ^[63, 108-109]

The first description of teichoic acid (C-polysaccharide; re-named to WTA after the identification of the presence of ribitol phosphate and sugars ^[110-113]) and LTA (F-antigen) lead to the differentiation based on their lipophilic character, hence their Forssmann antigenicity. ^[63, 108, 114-118]

Both, WTA and LTA, are common antigens in different pneumococcal serotypes (compared to capsular polysaccharides) and are expressed in similar amounts as murein in the cell wall, where approximately 90 % thereof are covalently linked to murein as WTA and approximately 10 % are anchored *via* lipid anchors as LTA to the glycolipids of the cytoplasmic membrane (Figure 7). ^[63, 108, 119-120]

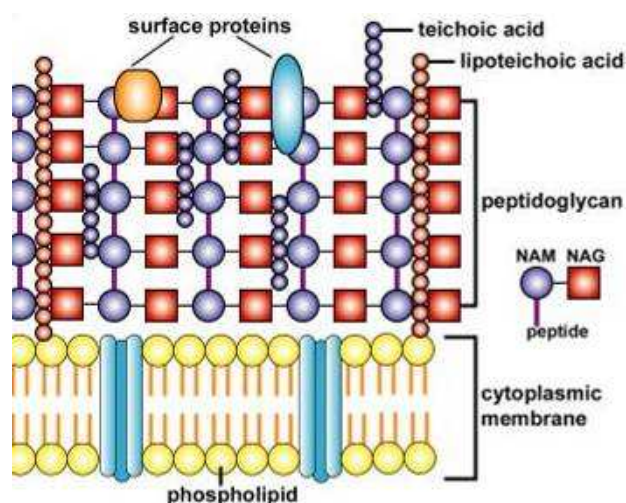


Figure 7: Schematic Drawing of the Gram-Positive Cell Wall Depicting WTA and LTA ^[120]

The identical and highly complex chemical composition of the repeating units of WTA and LTA (Figure 8) as well as their decoration with choline, makes the pneumococcal teichoic acids unique compared to other species. Both were already chemically synthesized as smaller subunits as well as full-length structures, which will allow even more diversified possibilities for investigation *e.g.* in the course of vaccine development. ^[63, 112, 121-132]

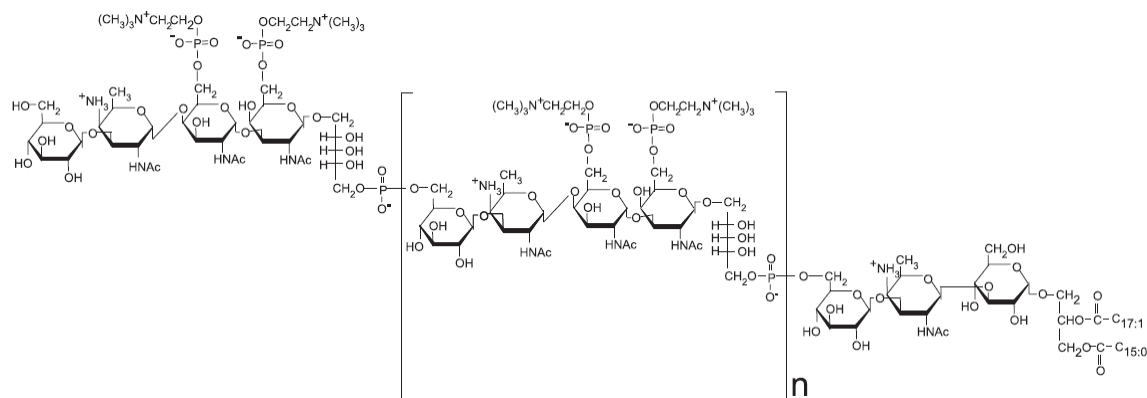


Figure 8: The Structural Model for Pneumococcal LTA. The currently accepted model of pneumococcal LTA is depicted. At present more models, revising and confirming ascertained structural features, are suggested. [127]

The choline-decoration (phosphorylcholine, *PCho*) of WTA and LTA acts as an anchor for choline-binding proteins such as murein hydrolases (LytA, LytB and LytC), phosphorylcholine esterase (*Pce*), *PspA* and *PspC*, serves as a host-binding factor for *e.g.* CRP, myeloma proteins, the receptor of the platelet-activating factor (PAF) and is reported to act as a receptor for bacteriophages. [63, 128, 133-140]

Compared to other pneumococcal and bacterial adhesins, that specifically bind to host cells, LTA is reported of allowing the adhesion and invasion of *S. pneumoniae* and other Gram-positive bacteria with lower cell-type specificity. [63, 140-147]

3.2.2 The Diverse Roles of LTA

LTA is essential in Gram-positive bacteria, by shaping the physicochemical surface properties, for controlling the autolysin activity and biofilm formation and for positioning the cell division machinery. Further, LTA is a receptor for phages, induces the susceptibility and/or resistance to antimicrobial peptides and killing in *e.g.* neutrophil extracellular traps, plays a role in the maintenance of cation homeostasis (*e.g.* storage of magnesium) and serves as an attachment site for bacterial surface proteins. [119, 148-150]

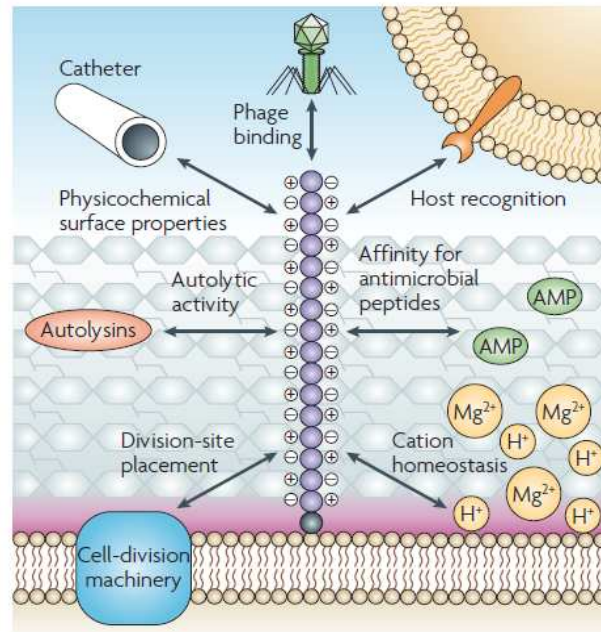


Figure 9: The Several Roles of WTA and LTA in Gram-Positive Bacteria [150]

The *PCho* moiety on LTA of *S. pneumoniae* is also present on several other respiratory pathogens (e.g. *Neisseria spp.*, *Haemophilus influenzae*, *Pseudomonas spp.*, *Streptococcus agalactiae*, *Streptococcus pyogenes*) and shares structural similarity to PAF, targeted in acute phases of inflammation by CRP and also activates the PAF-receptor, inducing inflammation and the release of tumor necrosis factor-alpha (TNF- α). [36, 63, 134, 138, 151-157]

Pneumococcal cell wall components, and LTA activate the alternative pathway of complement activation upon binding to erythrocytes, hence recruit leukocytes and are also involved in the adherence of pneumococci during the host-pathogen interaction of the *PCho* and the PAF receptor on endothelial cells. LTA is further involved in the binding of classical C-type lectins such as mannose binding lectin and L-ficolin, inducing the lectin pathway of complement activation. [63, 138, 150, 158-163]

Pneumococcal LTA was shown to act as a synergistic PAMP in the course of host innate immune responses. Thus it is recognized *via* TLR2 and CD14 – which is amplified by endogenous TLR4 ligands – leading to: (i) loss of transepithelial resistance, (ii) activation of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- κ B), (iii) activation of p38 mitogen-activated protein kinase (p38 MAPK), and (iv) signaling of transforming growth factor beta (TGF- β). [29-31, 33-36, 63] Recently pneumococcal LTA was reported to lead to a TLR2-dependent but TLR4-independent aggregation of platelets *via* activation of the

A - INTRODUCTION

phosphoinositide 3-kinase/Ras-proximate-1 (PI3K/RAP1) pathway and induction of glycoprotein IIb/IIIa (GPIIb/IIIa), leading to thrombocytopenia in sepsis-patients. [164]

Also LTA from other Gram-positive bacteria induces opsonization through specific antibodies and plays a role in the major histocompatibility complex (MHC) class II presentation of zwitter-ionic glycopolymers and the activation of CD4⁺ T-cells, leading to IL-10 expression. [29,

31, 63, 150, 165-170]

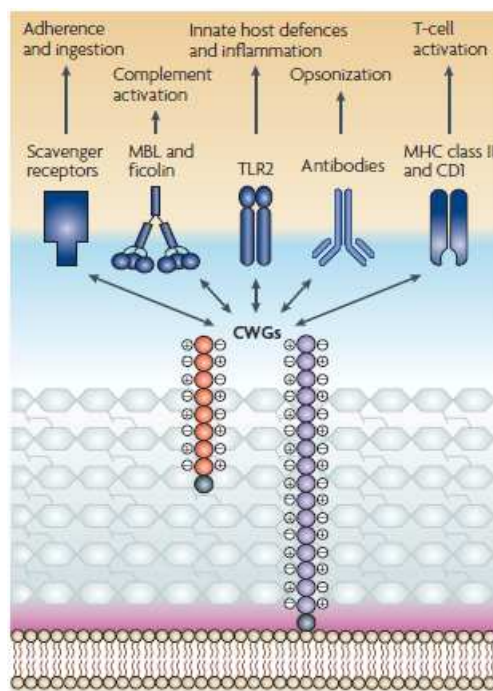


Figure 10: The Interactions of WTA and LTA with the Eukaryotic Host [150]

3.2.3 The Potential of Anti-LTA-Antibodies

In *S. pneumoniae*, LTA is a species common antigen, present in all strains, independent of the serotype, and shows multiple biological functions as elucidated in the previous sections.

Therefore, antibodies against LTA would not only have opsonizing activity but also induce neutralizing effects by *e.g.* interfering with the TLR2 induced inflammation. LTA-specific antibodies were shown to protect against *Staphylococcus aureus* and *Enterococcus faecalis* but were detrimental in *Staphylococcus epidermidis* infections. [63, 171-172] Commercial intravenous immunoglobulin containing anti-carbohydrate IgG antibodies (*Sandoglobulin*[®], *Privigen*[®], CSL Behring and *Gamunex*[®], Talecris), was also reported to recognize pneumococcal LTA. [173]

3.3 *Pneumococcal surface protein A (PspA, SP0117)*

3.3.1 *Basic Structural Features of PspA*

Pneumococcal surface protein (PspA, SP0117) is an approximately 67-99 kDa highly immunogenic cell-surface protein of *S. pneumoniae*. This mosaic-gene encoded protein is highly variable but present on all pneumococcal strains characterized today, which makes it highly valuable as a potential vaccine antigen with broad effectiveness. [174-178]

The protein was originally defined by its recognition of different epitopes of PspA by protective mouse mAbs. [179-180] PspA is non-covalently anchored to choline moieties of teichoic acids in the bacterial cell wall, *via* a highly conserved, C-terminal choline binding region (CBR) of ten 20-amino-acid repeats, which is similar in *e.g.* pneumococcal LytA and PspC. [174-175, 177, 180-186] The CBR is followed by a slightly hydrophobic sequence of 17 amino-acids, ten 20-amino-acid repeats, a flexible proline-rich (PR) domain which may span the cell wall, and the N-terminal half, consisting of a signal peptide, and a highly charged, α -helical, anti-parallel, coiled-coil domain, which forms a heptad motif, and protrudes outside the capsule. [136-137, 176, 179, 183-184, 187-192]

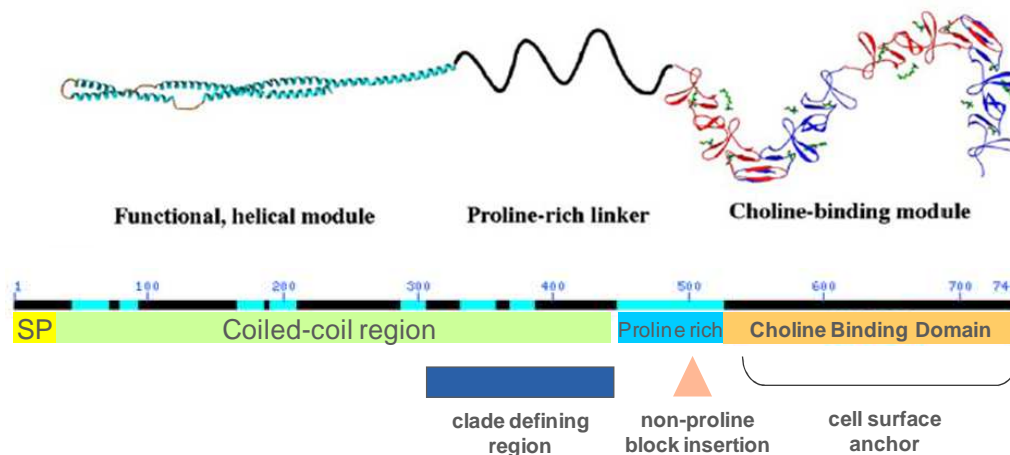


Figure 11: Protein Structure of PspA. The signal peptide (SP) and the N-terminal coiled-coil region are separated from the C-terminal choline-binding domain by a Pro-rich region. (modified from [185, 193])

Due to an accumulation of mutations, the N-terminal half is variable in different strains, but is eliciting protection by its epitopes. For example, the full-length protein as well as the N-terminal half, the C-terminal 104 and the N-terminal 115 amino-acid repeats of the α -helical region were shown to induce protective immunity. [136, 176, 183-184, 190, 194-199]

The PR-region, consisting of irregular repeats of Pro-residues every two to three amino acids, gives rise to sequence motifs such as PAPAP (interrupted by PKP) and PEKP, and is characteristic for PspA as well as for PspC (being structurally similar to PspA). This region is

interrupted (in 56 % of all PspAs) by a highly conserved non-proline block (NPB) of 33 non-proline-amino acids in about 90 % of pneumococci, where the proline-repeats impact the cross-reactivity of anti-PspA antibodies.^[136, 176, 193, 200-203] It was shown that the PR-region of the pneumococcal strain TIGR4 (serotype 4, Clade 3) is recognized by human antibodies which was confirmed by antibody-mediated protection against infection in mice.^[24, 202, 204] Therefore, the unmasked PR and NPB epitopes are on the pneumococcal surface, accessible for mAbs, which were shown to be protective in mouse-challenge models ^[186, 202]

3.3.2 The Families and Clades of PspA

The clade-defining region (or B region), which is known to have cross-protective epitopes, is approximately located between amino acid 192 and 270 (based on the TIGR4 sequence), upstream of the PR-region. It was mapped by epitopes of protective mAbs and led to the identification of six clades (monophyletic groups differing in >20 % of their amino-acid sequences), which are further subdivided according to DNA- and protein sequence-similarity into three families: Family 1 consists of Clades 1 and 2, Family 2 of Clades 3 to 5 and Family 3 is equivalent to Clade 6 (Figure 12) ^[176, 183, 205]. It is noteworthy that more than 98 % of PspA-types found so far belong to Family 1 and 2. ^[176, 183] Epidemiologic studies - showing the variability of the PspA-family coverage in isolates - emphasize the importance of monitoring population-based data during the development of vaccines and therapeutic approaches. ^[206-212]

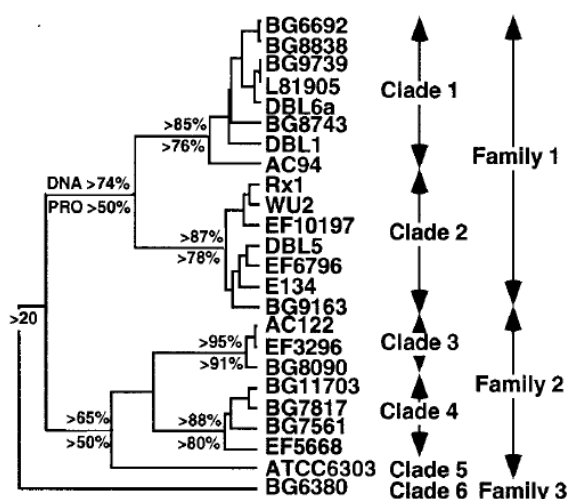


Figure 12: The Families and Clades of PspA. This cladogram was generated by mapping epitopes recognized by protective mAbs, thus identifying six clades which were further subdivided into three families. ^[176]

3.3.3 *The Functional Role of PspA*

Pneumococci require iron for growth and proliferation which they are capable of acquiring *via* PspA through binding to human lactoferrin (hLF) – an iron-sequestering glycoprotein in mucosal secretions, phagocytic cells, and serum during inflammation. Strains expressing PspA specifically bind to hLF *via* the C-terminal half of the α -helical domain. This leads to interference with host immune functions and to an increased acquisition of iron, whereas mutants lacking PspA were shown to be unable to bind to hLF. [176, 185, 213-215]

Lactoferrin and its iron depleted form, apolactoferrin (ALF), are both bactericidal against pneumococci but are limited in their function, hence being blocked at their active sites, by PspA. In the case of hLF, PspA directly and specifically binds through its lactoferrin-binding domain to the N-lobe of hLF, inhibiting the penetration of the bacterial membrane by the bactericidal peptide. [216-217]

Another role of PspA was found to be next to the inhibition of C3b deposition onto pneumococci, also the inhibitory effect to the formation of functional alternative pathway C3 convertase. Thus a reduction of the pathogen-clearance by the complement-dependent host defense system and phagocytosis is mediated. This family independent, anti-complementary function of PspA just requires the surface accessibility as prerequisite for proper function. [176, 185, 203, 218-219]

3.3.4 *The Role of PspA During Host-Infection*

PspA was shown to be expressed *in vivo* in mice and to elicit cross-reactive and cross-protective antibodies against strains expressing different PspA-clades. [183, 220] The protein is known to be necessary for full virulence of pneumococci and for slowing down clearance. Immunization with PspA protects mice against fatal sepsis, bacteremia, lung infection and carriage. [174, 176, 178, 180, 183, 203, 221-223]

Healthy and diseased humans elicit clade cross-reactive antibodies to PspA, where strong immune responses develop during convalescence and highly effective T-cell mediated responses are elicited. [224-227]

In vitro studies revealed a stimulatory effect of PspA together with other pneumococcal surface proteins on A549 cells, human neutrophils and hPBMCs by stimulating the extracellular signal-regulated kinase (ERK), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha ($I\kappa B$ - α), c-Jun N-terminal kinase (JNK), nuclear factor-kappa B (NF- κ B) and

A - INTRODUCTION

p38 MAPK signaling cascades, hence the early cytokine (IL-6, IL-8) and chemokine (CCL2, CCL4 and CCL5) secretion. [228-230]

3.3.5 Reported *in vivo* Efficacy of Anti-PspA-Antibodies

Since PspA was discovered as a very potent antigen target, several studies, based on immunizations with DNA, proteins, peptides, *etc.* in active immunization and passive transfer models were conducted. A huge number of different application routes and adjuvants were already tested for their protective ability in several mouse strains and challenge-models. (Table 1) [176, 183, 191, 197-198, 203, 231-248]

The potential use of PspA as a vaccines component against *S. pneumoniae* is *inter alia* given due to its T-cell-dependent nature, hence an induction of immunogenicity in infants, presence in all strains, and relatively low production costs compared to existing polysaccharide vaccine. [183]

Table 1: Examples of the Most Promising Vaccination Approaches Based on PspA

Antigen	Comments	Sources
<i>PspA</i> -DNA (<i>plasmid, truncated gene, etc.</i>)	<ul style="list-style-type: none"> » Pneumococcal clearance 24h post immunization » IgG1, IgG2a, IFN-γ, TNF-α, Th1 response » Cross-reactivity (Family 1 and Family 2), » Increased complement deposition 	[191, 231-234]
<i>PspA</i> -recombinant protein	<ul style="list-style-type: none"> » Higher antibody-titers compared to DNA, » Strong antibody-binding to pneumococci, » IL-4 secretion (Th2), » Immunization of healthy humans showed cross-reactive antibodies <i>in vitro</i> » Combination with PLY-mutant, PspC or ClpP or PspA-DNA: enhanced protection with complementing effects; » Increase of IgG2a even by parental immunization 	[183, 203, 233, 235-241]
<i>PspA</i> -recombinant protein + different adjuvants	<ul style="list-style-type: none"> » Nontoxic A subunit mutant of cholera toxin (S61F) induces high levels of PspA-specific IgG and IgA in serum and CD4+ Th2-type cells » <i>Lactobacillus casei</i>: cross-reactive antibodies, IL-17 and IFN-γ secretion in lung and spleen, TNF-α in the respiratory tract » CTB-PspA': protection against sepsis » IL-12: lung IgG2a mRNA levels, systemic and respiratory IgG1, IgG2a and IgA levels, OPK, increased » Low level LPS, whole cell <i>Bordetella pertussis</i> vaccine: protection against lethal respiratory challenge, high levels of systemic and mucosal IgG2a and IgA » Low level LPS, diphtheria-tetanus pertussis vaccine: induction of IgG2a, significant protection and survival » Loss of TLR4-function: protection against infection, clade cross-reactive and cross-protective antibodies induced 	[242-247]
Passive Transfer of Sera	<ul style="list-style-type: none"> » Mice protected when passive immunization with mouse, rabbit, monkey sera and human (phase I) sera 	[176, 197-198, 248]

3.4 Pneumolysin (PLY, SP1923)

3.4.1 Basic Structural Features of PLY

Pneumolysin (PLY, SP1923) is a cholesterol-dependent, cytoplasmic, thiol-activated cytolysin, which binds to cholesterol-containing membranes of eukaryotic cells. It is released by pneumococci and reported of being an essential virulence factor of *S. pneumoniae*. Sequence comparison of PLY indicates limited homology to human CRP and high sequence similarity to perfringolysin O, mitilysin and pseudopneumolysin. [13, 38, 63, 249-263]

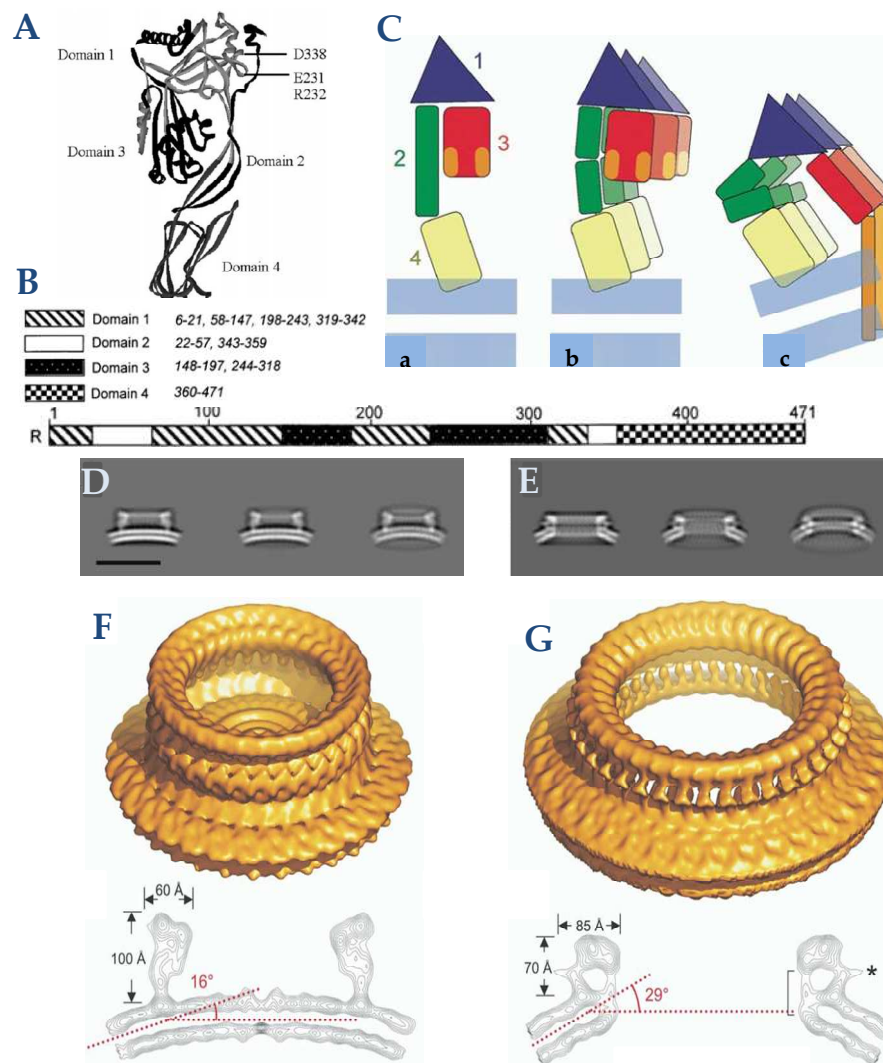


Figure 13: The Structure of PLY. The crystal structure of PLY (A) is illustrated by the amino-acids giving rise to the four domains of this antigen (B). A schematic drawing represents the binding of the cell-binding domain 4 to the cholesterol-containing membrane (C-a), refolding of several domains (C-b) and the pore-formation (C-c). Rejections obtained from cryo-electron microscopy show the 38-mer prepore (D) and the subsequently formed pore (E). Computational 3D-reconstructions illustrate the prepore (F) and pore (G) with the corresponding cross-sections below each. (modified from [254, 281, 284])

After its first description as a hemolysin in 1905, PLY – a 52.8 kDa (470 amino acid) large protein – was extensively investigated towards its toxic nature and the loss of toxicity due to binding of sterols and cholesterol. PLY shares a high inter-strain gene-conservation in different pneumococcal serotypes. The exact secretory pathway that leads to release of PLY is unknown to date but was shown to be independent of LytA, LytB and LytC activity. [63, 251, 264-276]

The amino-acid sequence of PLY gives rise to four domains, where domain 4 and here especially Trp433 is known to be crucial for binding of PLY to cholesterol, the insertion into the eukaryotic cell membrane *via* β -hairpins and hence the formation of ring-shaped, oligomeric pores consisting of up to 44 monomers, leading to the formation of a transmembrane channel, lined by 176 β -strands (Figure 13). [63, 254, 277-283]

3.4.2 *The Functional Roles of PLY*

Next to its cytolytic, pore-forming ability which is described in the previous section, PLY exerts diverse functions in the context of bacterial pathogenesis and host response.

It was shown that a *ply* knock out strain can be complemented by PLY-release from co-infection with wild-type cells and that it has a critical role in acute sepsis in the early phases of pneumococcal infection. [63-64, 271, 285-286] *ply* mutant strains show slower growth during lung infection and give rise to chronic bacteremia (compared to wild-type cells which induce acute sepsis). [63-64, 286-289] The expression of PLY accelerates the pneumococcal transmission into the blood, although it is not required for successful pneumococcal colonization of the nasopharynx. [63-64, 289-291]

Although complement-activation through PLY is important during lung infection, the cytotoxicity of PLY becomes more crucial when the disease progresses towards a systemic infection. [50, 63-64, 292-293]

Severe effects on host cells, in the early stages of a pneumococcal infection and during the subsequent progression towards pneumococcal bacteremia or meningitis, are often associated with the cytolytic activity of PLY. Sublytic concentrations block the proliferation of stimulated lymphocytes (*i.e.* reduced lymphokine and antibody expression), inhibition of the respiratory burst and a reduction of the migration capacity of cells of the human immune system such as PMNLs. [50, 63-64, 294-302] PLY induces the inhibition of cilia-beating (*i.e.* on the respiratory tract epithelium as well as ependymal lining of brain slices), damage of mitochondria (*e.g.* leading to neuronal cell death), irreversible damage of cochlear hair cells (*i.e.* sensorineural hearing loss),

permeabilization of the alveolar endothelium and/or the pulmonary endothelium as well as damage of lung tissue (e.g. due to the activation of PAF and downstream signaling pathways) and the blood brain barrier. [297, 303-317]

PLY aids the evasion from human dendritic cells and leads to an activation of lung mast cells mediating pneumococcal killing. It is further reported, that sublytic concentrations of PLY induce a Src-kinase dependent, rapid microtubule bundling and stabilization. In addition PLY was reported to activate the two GTPases – Ras homology gene family member A (RhoA) and Ras-related C3 botulinum toxin substrate 1 (Rac1) – leading to actin remodeling (stress-fibers, filopodia and lamellopodia) which is reversible by using specific inhibitors and pre-incubation of PLY with cholesterol. [318-321]

PLY and also its non-hemolytic mutant forms are reported of activating the classical pathway of complement activation as well as activating TLR4 as PAMPs, including the downstream-signaling *via* myeloid differentiation primary response gene 88 (MyD88) and ERK and subsequently the release of TNF- α , IL-1 β , IL-6 and IL-17 leading to an inflammatory response. In addition to the activation of TLR4, PLY acts *via* NF- κ B, the NLR family, pyrin domain containing 3 (NLRP3) inflammasome and MAPK signaling, inducing IL-1 β , CCL (2, 4, 5, and 8) and CXCL (8 and 10). PLY activates the nuclear factor of activated T-cells (NFAT) signaling pathway, induces caspase-1-dependent cytokines and leads to degradation of C3 *in vitro* and *in vivo*. Its role during infection and inflammation is further potentiated by (i) its induction of cellular phospholipase A₂-activity which subsequently leads to pro-inflammatory and cytotoxic metabolites, (ii) the interferon- γ (IFN- γ) dependent induction of NO, (iii) the activation of intracellular oxygen radicals (*i.e.* reactive oxygen species, ROS) and (iv) the mobilization of matrix metalloproteinases (8 and 9) from neutrophils. [37-56, 300]

It is further reported that the binding-ability of PLY to the Fc portion of IgG prevents the effective opsonophagocytosis and hence the killing of *S. pneumoniae in vivo*. [38, 63, 322-323]

The immunization of mice with PLY, pneumolysoids (PLY-based toxoids with no or reduced hemolytic activity) and both being conjugated to capsular polysaccharides, or administered in combination with other antigens, repeatedly showed increased survival after challenge with different *S. pneumoniae* strains. Studies with *ply* mutant strains showed the essentiality of PLY as a pneumococcal virulence factor. [37, 63, 250, 272, 324-331]

3.4.3 Reported *in vivo* Efficacy of Anti-PLY-Antibodies

As aforementioned, many studies were conducted using PLY in active immunization model in naïve *Mus musculus*, showing prolonged survival rates after challenge with *S. pneumoniae*. Affinity-purified pneumolysoid-specific human antibodies were shown to protect mice against invasive disease due to the toxin-neutralizing ability of the antibodies and not due to direct opsonization of pneumococcal cells. [323]

Humans are reported of exhibiting a PLY-specific antibody-response due to natural exposure to pneumococci, with increasing antibody-titers during infection. Patients are reportedly predisposed to pneumococcal pneumonia when lacking high levels of antibody. [332-335]

Purified human polyclonal antibodies, rat polyclonal IgG as well as hyperimmune serum targeting PLY showed protection against lethal challenge and other diseases such as pneumococcal keratitis in passive immunization models. [323, 332, 336-337]

Several mAbs, that either inhibit pore-formation (*e.g.* PLY-4) or binding of the toxin to cells (*e.g.* PLY-5 and PLY-7), were generated, characterized and showed promising effects such as (i) the protection of ependymal cilia which are severely affected by PLY during meningitis, (ii) the protection of lung tissue which is profoundly affected during pneumococcal pneumonia, and (iii) a significant protection against a lethal *intranasal (i.n.)* challenge with pneumococci (with a synergistic effect when used in combination), which substantiates the usefulness of PLY specific mAbs for *e.g.* the use in adjunctive therapies with antibiotics. [284, 301, 313, 323, 338-340]

4 MONOCLONAL ANTIBODIES – THE GOLDEN BULLET FOR FIGHTING INFECTIOUS DISEASES?

4.1 Technologies for the Generation of mAbs

Since the development of the first mAbs by Koehler and Milstein, where spleen cells of immunized hosts are fused with a cancer cell-line (*e.g.* myeloma), yielding a continuous cell-culture secreting mAbs, several new and more effective technologies were established for the generation of highly specific mAbs. [28, 341-342]

The major drawback of the original hybridoma technology is mainly the immunogenicity of rodent mAbs in humans. [94, 343] This problem was abolished when phage-display technologies were developed and the design of chimeric mouse-human antibodies, humanized mAbs or fully human mAbs, was implemented. [94, 344-348]

In the recent years, several new approaches such as the pre-selection of antigen-specific B-cells based on their Ig-receptor ^[349], or the generation of heteromyeloma cell lines based peripheral blood lymphocytes of vaccinated humans ^[350] were developed for the generation of fully human mAbs. Also the use of transgenic animals, expressing a complete repertoire of human immunoglobulins ^[351-352], or conditionally immortalized cells expressing fully human mAbs controlled by a MHC-promoter ^[353], as well as the molecular cloning of functional immunoglobulin genes from single plasma cells for target-selective homologous recombination ^[354], were established.

Today, display technologies based on phage-, ribosome-, microbial- and protein-DNA-display not just allow the engineering and expression of fully human (monoclonal) antibodies from several libraries, but also the generation of antibody-fragments such as single-chain variable fragments (scFv). The cloning of these fragments allows the generation of constructs being more stable than Fv-fragments, where the antibody-specificity is still maintained.^[355-368]

Sindbis Virus Based Mammalian Cell Surface Display

As the generation of fully human mAbs against the target antigens of this project is aimed to be based on a novel technology developed by Cytos Biotechnology AG ^[369], emphasis is put on this in the following.

The technology is based on screening for antigen-specific memory B-cells from isolated hPBMCs, the generation of scFv-libraries, and selection by mammalian cell surface display *via* a Sindbis virus expression system (Figure 14).^[369]

Briefly, hPBMCs are isolated from healthy human blood donors, and subsequently sorted for antigen-specific human memory B-cells by means of fluorescence activated cell sorting (FACS) using fluorescently labeled antigens as bait (virus like particles fused to the antigen of interest; VLP-Ag). Following RNA isolation from memory B-cells, a scFv-library is generated, which is used to generate a high-titer Sindbis virus based expression-library. This virus-library is subsequently used for infection of baby hamster kidney (BHK) cells at a low multiplicity of infection (MOI), leading to a pool of infected cells expressing one specific antibody on each cell surface. After a single-cell sorting for cells expressing a functional antibody by FACS, a BHK-cell monolayer is generated, expression of gene-specific antibodies is verified, the variable regions (VRs) are cloned and the antibody is expressed in any desired format (*e.g.* scFv-Fc or IgG). ^[369]

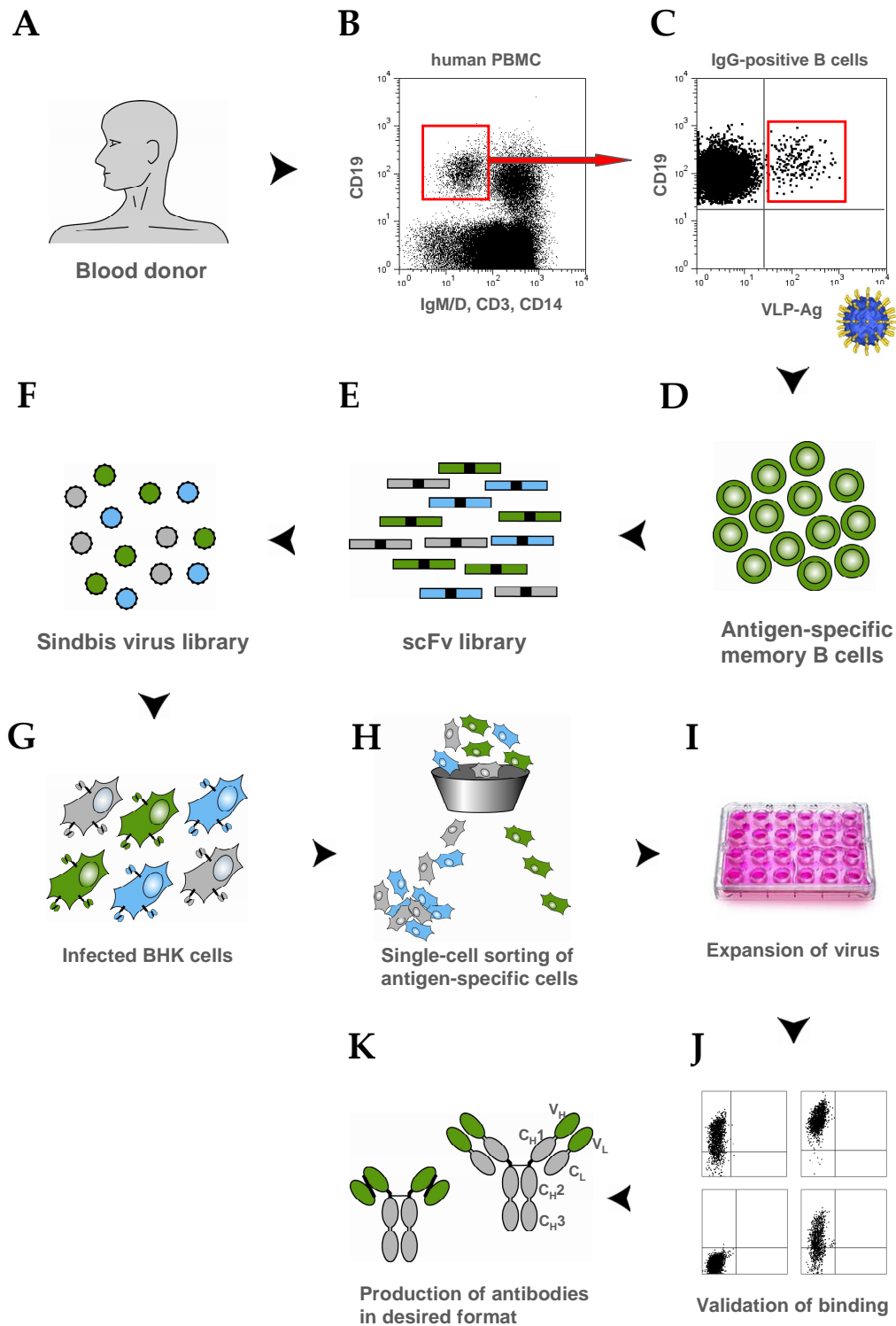


Figure 14: Schematic of the Sindbis Virus Based Mammalian Cell Surface Display Technology. Fully human mAbs are generated using serum from healthy human blood donors (A), isolating hPBMCs (B) and sorting with antigen-coupled virus-like particles (C) for antigen-specific memory B-cells (D). Following the construction of a scFv-library (E) and a Sindbis virus-library (F), BHK-cells are infected (G) which is followed by single-cell sorting for antigen-specific BHK-cells (H). After successful virus-expansion (I), the binding of gene-specific antibodies is validated (J) and antibodies are finally produced in the desired format (K). (modified from ^[369])

4.2 *The Potential of Antibody-Based Therapies*

The increasing emergence of penicillin- and multidrug-resistant *S. pneumoniae* strains and other bacterial species revives the idea of antibody-based therapies against infectious diseases.

[348, 370-374]

Hypersensitivity against antisera, which supported the abolishment of serum therapy when the antibiotic era arose in the 1930's, is just one of the disadvantages associated with non-human antibodies in therapy. In addition hyperimmune sera were reported to comprise hazards in transmission of infectious diseases, to show great lot-to-lot variations, and to imply low concentrations of specific antibodies. [370, 372, 375-376]

In contrast to polyclonal antibodies, mAbs represent an exhaustless pool of homogenous antibodies with markedly higher specificity and therefore greater therapeutic efficacy. [370, 372, 375-377] MAbs exhibit a greater uniformity, allow the combined use in therapy, and eliminate the risk of transmission of blood-borne infectious diseases due to the feasibility of the generation *in vitro*. [370, 372, 375-377]

The main advantages for the use of mAbs in therapeutic settings are high pathogen specificity, enhancement of immune functions and favorable pharmacokinetics of this type of drug. [102, 370-372, 378-379] Native human IgG was shown to have a great pharmacokinetic profile (tissue penetration, half-life and host tolerance), which is even greater than pharmacokinetics observed for chimeric and humanized antibodies. [102, 370]

Antibodies are part of the natural defense system and allow – due to somatic mutations and rearrangements – the specific targeting of antigens of theoretically all pathogens.[379] MAbs therefore impair a versatile repertoire of functions such as inhibition of microbial attachment, agglutination, complement activation, opsonization, enhancing effects on host effector-cells, depletion of immune effectors, and toxin neutralization. Thus they have a great potential by directly targeting bacterial pathogens or neutralizing the toxic products of infection even at physiological extremes *inter alia* at low pH. [102, 370-372, 377]

There are also drawbacks associated with mAbs such as high manufacturing costs, the immanent need of early and precise diagnostics, the requirement of systemic administration and the limited usefulness against mixed infections, which exacerbate the development of antibody-based therapies to date. [370, 372, 377] The given risk of microorganisms to adapt to antibodies over time, may require to target the mutated epitopes with other mAbs, an administration of mAb-cocktails, or even the aiming towards other conserved antigens. [102, 370, 377]

A - INTRODUCTION

Nevertheless, it was shown that a mAb-based therapy is even effective against brain infections such as meningococcal meningitis, as inflammation makes the blood-brain barrier permeable to pathogens. ^[370, 372] At present there are several antibody-based passive therapies, using murine, chimeric, humanized or fully human mAbs, approved by the FDA. This huge field of application from cancer-therapy, inhibition of transplant rejection, treatment of rheumatoid and autoimmune diseases, as well as the therapy of infectious diseases, emphasizes the great potential of mAbs in antibody-based therapies. ^[102, 368, 380-387]

B - AIMS OF THE STUDY

Due to increasing antibiotic resistance and poor vaccine responses in at-risk populations new treatment strategies such as passive immunotherapy with human mAbs are under investigation. With respect to this, three conserved antigens of *S. pneumoniae* – LTA, PspA (SP0117) and PLY (SP1923) – were selected for in depth analyses. Consequently the specific aims of this project were to:

- a) express and purify PspA and PLY as well as domains thereof in recombinant form,
- b) generate hyperimmune sera against all target antigen-constructs in *M. musculus*
- c) determine the *in vivo* virulence of several pneumococcal strains in different routes of infection
- d) optimize purification, detection and quantification methods of LTA and to subsequently isolate and purify native LTA from different pneumococcal strains,
- e) perform complementary *in vitro* validations of all three target antigens, hence to assess the antigen binding in ELISA and Western blot analyses and to determine their accessibility to antibodies on the surface of distinct pneumococcal strains by flow cytometry,
- f) set-up and optimize cell-based functional assays to scrutinize the *in vitro* activity of the generated antibodies
- g) investigate the *in vivo* efficacy of the antigen-specific antibodies in various mouse models of infection and to correlate these findings with their *in vitro* activity
- h) optimize the staining conditions for the sorting of PLY-specific human B-cells
- i) identify healthy human donors for the isolation of memory B-cells by fluorescence activated cell sorting and for cloning and expression of human mAbs against all target antigens *in vitro*.

In summary, the overall aim of the present study was to generate antibodies against the pneumococcal antigens – LTA, PspA and PLY – and to investigate the characteristics and potentials of both – the antigens and the antibodies – *in vitro* and *in vivo* and thus to obtain a substantial knowledge for their application spectrum as targets for human mAb development.

C - MATERIALS AND METHODS

1 BACTERIA

Table 2 represents an overview of *S. pneumoniae* wild-type strains used in the course of this project. Wild-type pneumococcal strains were grown in Todd Hewitt Broth supplemented with 0.5 % yeast extract (THY) or on blood-agar (Columbia agar supplemented with 5 % sheep blood, bioMérieux) at 37 °C and 5 % CO₂.

Table 2: *S. pneumoniae* Strains used in this Study. PspA family and clade type were determined by sequence analysis and grouping according to Hollingshead et al. [176]

Strain	Serotype	PspA-Family	PspA-Clade	Origin
D39	2	1	2	Peter Andrew; University of Leicester, UK
A66.1	3	1	2	David Briles, University of Alabama, USA
WU2	3	1	2	David Briles, University of Alabama, USA
TIGR4	4	2	3	Birgitta Henriques-Normark, SMI, Sweden
4D2341-94	4	2	3	Eddie Ades, CDC, USA
PJ1324	6B	2	3	Birgitta Henriques-Normark, SMI, Sweden
EF3030	19F	1	1	David Briles, University of Alabama, USA

Gene deletion strains were generated in a previous study [24] and were cultured in media supplemented with 250 µg kanamycin per ml.

Escherichia coli was cultured in LB-medium (PAA) or on LB-agar plates, both supplemented with the required antibiotics and incubated at 37 °C (150 rpm shaking in the case of liquid cultures).

2 EUKARYOTIC CELL LINES

HL-60 cells are myeloid and promyelocytic cells derived from a female patient with acute myeloid leukaemia and established as a cell line in 1977. [388] In culture, these cells are ovoid or round but occasionally express pseudopodia and are heterogenous in size (9 to 25 µm in diameter). HL-60 cells double every 24 h in an actively growing culture, although doubling time is about 72 h immediately after recovery from the frozen stock. The cells largely resemble promyelocytes, however they spontaneously and slowly differentiate into other cell types of granulocytic phenotype. [388]

HL-60 cells were cultured at 37 °C and 5 % CO₂ in RPMI-1640 Medium (Sigma) supplemented with 20 % FCS, 1 % penicillin/streptomycin solution and 1 % L-glutamin. When the cells were approximately 80 % confluent they were diluted 1:5 in fresh medium.

HEK-Blue™ hTLR4 (InvivoGen) cells are HEK293 cells, stably transfected with the human TLR4 gene and the co-receptors MD-2 and CD14. By stimulation of the receptor(s), an NF-κB/AP-1-induced transcription of secreted embryonic alkaline phosphatase (SEAP) is induced, which is followed by an expression-level dependent color change of **QUANTI-Blue™** (InvivoGen) from pink to purple/blue.

The cells were cultured at 37 °C and 5 % CO₂ in DMEM-medium (GIBCO) supplemented with additives such as growth factors, antibiotics, antimycotics, *etc.*, according to manufacturer's instructions (InvivoGen; HEK-Blue-hTLR4- Technical Datasheet; *page 1-3*)

3 ISOLATION AND PURIFICATION OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS AND RED BLOOD CELLS

Human peripheral blood mononuclear cells (hPBMCs) and red blood cells (hRBCs) were isolated from freshly drawn blood under aseptic conditions. Blood was drawn using a Vacutainer® blood collection and Luer-Lock® set (Becton Dickinson, N° 367326 and N° 364902) and sodium-heparin blood-collection tubes (17 IU/ml; Becton Dickinson, N° 368480) according to general blood-collection standards. Blood from two tubes was diluted approximately 1:1 with DPBS and carefully layered on top of Lymphocyte-Separation Medium (LSM, LSM 1077, PAA) in 2:1 ratio without mixing the two phases. After centrifugation at 680 x g for 20 min and RT without brakes, the upper-most layer – containing the plasma and most of the platelets – was carefully removed and discarded. The hPBMCs were collected from the interphase without penetrating the LSM layer from above. The remaining LSM was removed and the hRBCs at the bottom of the tube were collected. The hPBMCs and the hRBCs were washed twice with DPBS – centrifugation at 420 x g for 10 min at RT without brakes – and then counted using a Neubauer-chamber and Trypanblue life/dead-staining.

The purified hRBCs were stored at 4 °C until further use, whereas the hPBMCs were re-suspended in FCS with 10 % DMSO at 10⁷ cells per ml and frozen at – 80 °C using a freezing container (Nalgene Cryo 1 °C Freezing Container).

4 GENERATION OF RECOMBINANT ANTIGENS

All pneumococcal antigens were cloned into a pET28b+ vector, subsequently expressed in recombinant form with an N- or C-terminal penta-His-tag and purified *via* an immobilized metal ion affinity chromatography (IMAC).

4.1 General DNA- and Protein- Methods

Purification of gDNA from S. pneumoniae

Genomic DNA (gDNA) was purified from pneumococcal strains grown in THY overnight using the “Wizard® Genomic DNA Purification Kit” (Promega), following the manufacturer’s protocol for Gram-positive bacteria (Technical Manual; page 16 ff).

Agarose Gel Electrophoresis

DNA was separated on agarose gels containing 1-2 % agarose (Invitrogen) in TAE-buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.3) and 0.5 µg/ml Ethidium bromide. All DNA-samples were mixed 1:6 with loading dye (Fermentas) and for size estimation a 1kb DNA ladder (*GeneRuler™*, Fermentas) was used. Gels were run for 35 min at 130 V and scanned (*AlphaDigiDoc™*, RT).

SDS-PAGE

For the separation of proteins, polyacrylamide-gels (4-20 %, Tris glycine, PAGEr® Gold Precast Gels, 1 mm, Lonza) were loaded with samples, mixed 1:5 with sample buffer (*Non-Reducing Lane Marker Sample Buffer*, ThermoScientific, supplemented with 1 mM DTT if required) and run in a *Mini-PROTEAN II* (BioRad) system for 45 min at 200 V in electrophoresis buffer (196 mM glycine, 0.1 % SDS in 50 mM Tris-HCl pH 8.3). Then the gels were either stained with Coomassie or used for Western blot analysis. The protein size was estimated using a prestained protein ladder (*PageRuler™ Plus*; Fermentas)

For Coomassie Staining, gels were rinsed with dH₂O post PAGE and subsequently stained using the *SimplyBlue™ SafeStain* (Invitrogen), which is based on the Coomassie® G-250 stain, according to the manufacturer’s instructions (Instructions Manual - *SimplyBlue™ SafeStain*, Invitrogen)

4.2 Generation of Bacterial Expression Hosts for Protein Expression

Cloning of Constructs into the pET28b+ Vector

Prior to cloning, the genes of interest were amplified by genomic PCR – using the *Expand High Fidelity PCR System* (Roche) according to manufacturer's instructions, adding 2 mM MgCl₂, 200 μM dNTP-mix, 1 μM sense- and anti-sense primers (Supplementary Table 1) and 1.75 U of *Expand High Fidelity Enzyme Mix* in DNase/RNase-free reagents. Following the DNA-amplification using the standard PCR-cycling program – 95 °C for 5 min (1 cycle), 95 °C for 30 sec, 52 °C for 30 sec, 72 °C for 60 sec (35 cycles), and 72 °C for 5 min; depending on the desired product, annealing temperature and extension times were adjusted – the amplicons and the vector were digested with appropriate restriction enzymes (according to manufacturer's instructions, NEB), separated by agarose-gel electrophoresis and purified using the *QIAquick Gel Extraction Kit* (QIAGEN). The purified, linearized vector (pET28b+) and insert DNA were ligated overnight at 16 °C with T4 DNA-ligase (Invitrogen) according to manufacturer's instructions. The ligated DNA was precipitated with 1 μl glycogen, 2.5 volumes of 100 % EtOH and 0.1 volume of 3 M NaAc for 10 min at -80 °C, pelleted by centrifugation at 15,000xg for 10 min, washed three times with 70 % EtOH and finally dissolved in 5 μl dH₂O before transformation into electro-competent DH5-α cells (ElectroMAX™ DH5-α-E™, Invitrogen).

Growing clones were subjected to colony-PCR using Taq-polymerase (Promega), applying reagents and buffers according to manufacturer's instruction and following the standard PCR-program described before. The size of the amplicons was subsequently checked by agarose-gel electrophoresis.

Transformants that were positive by colony-PCR were subjected to plasmid purification. The purified plasmids was subsequently digested using the appropriate restriction enzymes (according to manufacturer's instructions, NEB) in order to verify the expected fragment sizes by agarose-gel electrophoresis. The insert from positive clones was confirmed by sequencing (MWG Biotech).

Plasmids containing the desired inserts were transformed into chemically competent *E. coli* BL21-CodonPlus® Competent Cells (Stratagene) according to the manufacturer's protocol.

4.3 *Small-Scale Expression Analysis*

Single colonies of the *BL21* transformants were used to inoculate 5 ml LB-medium supplemented with the appropriate antibiotics – dependent on *E. coli* host strain and the vector. The next day this culture was diluted 1:10 with fresh growth medium and incubated at 37 °C and 150 rpm for 90 min until the logarithmic growth phase was reached. Protein-expression was induced by addition of 0.1 mM IPTG and the culture was grown for another 3 h.

2 ml of this culture were then harvested by centrifugation for 10 min at 15,000 x g. The pellet was lysed in 200 µl *BugBuster™ Protein Extraction Reagent* (Novagen) for 20 min rotating. A 50 µl aliquot of the crude lysate (CL) was kept for further analyses and the remaining solution was centrifuged for 10 min at 15,000 x g. The supernatant (soluble fraction, SOL) was kept for analysis and the pellet was dissolved in 150 µl 8 M urea and 500 mM NaCl in 50 mM TrisHCl pH 8.0 (insoluble fraction, INSOL).

4 µl of all three fractions (CL, SOL and INSOL) were analyzed for protein-expression by SDS-PAGE followed by Coomassie-staining and Western blot analysis (using anti-Penta-His as primary antibody).

4.4 *Large-Scale Expression and Purification of Recombinant Proteins*

Cultivation of Expression Cell Lines

A 100 ml o/n-culture of the *E. coli* protein expression strain was prepared in 12.5 ml “pimped medium” and 87.5 ml LB medium supplemented with the appropriate antibiotics. Therefore, 1 liter of “pimped medium” was aseptically prepared by adding 10 g yeast extract, 13.73 g *D*-glucose*H₂O, 1.16 g KH₂PO₄, 6.27 g K₂HPO₄*3H₂O, 2 % glycerol, 0.25 g MgSO₄*7H₂O, 0.8 mg FeCl₃*6H₂O, 0.1 g CoCl₂, 0.05 g CuCl₂, 0.1 mg ZnCl₂, 0.1 mg Na₂MoO₄*2H₂O, 0.025 mg H₃BO₄ and 5 µl HCl(cc) to 1000 ml LB medium (PAA).

The o/n-culture was used to inoculate 2 liters of pre-warmed fermentation medium in a baffled flask which was incubated for 2 h at 37 °C and 150 rpm. The protein production was induced by the addition of 0.1 mM IPTG and the culture was grown for another 4 h.

The biomass was harvested by centrifugation (Beckman Avanti J-25) at 4 °C for 15 min at 9000 x g. The pellet was washed by re-suspension in approximately 30 ml DPBS, centrifuged for 15 min at 4000 rpm and stored at -20 °C until further use.

High-Pressure Homogenization (HPH)

After thawing the biomass, it was diluted 1:5 to 1:30 in HPH-lysis buffer containing 50 mM Tris-HCl pH 8.0, 500 mM NaCl and 0.1 % Triton X-100. Subsequently 1 mM DTT (if required) and protease inhibitors were added from a 100 x stock containing 1 mg/ml leupeptin, 0.1 mg/ml pepstatin A, 0.5 mg/ml aprotinin, 4 mM bestatin, 1 mM E-64 and 100 mM EBSF in dH₂O.

The bacterial suspension was filled into the high-pressure homogenizer (HPH; *Panda 2K*; Niro Soavi) and lyzed in five cycles according to standard protocol (Intercell-Department for Protein Expression and Purification) which was followed by centrifugation at 4 °C for 15 min at 9000 x g. 100 µl of sample were drawn after each step and analyzed for protein content by SDS-PAGE and subsequent Coomassie-staining. The fraction containing the recombinant protein were kept and used for protein purification.

Immobilized Metal Ion Affinity Chromatography (IMAC)

Following the estimation of the amount of recombinant protein (post HPH), the soluble fraction was rotated over-night at 4 °C with the appropriate volume of washed and equilibrated Ni-sepharose (Amersham). This resin was then subjected to IMAC according to standard protocol (Intercell-Department for Protein Expression and Purification) and applying the following buffer-gradient (Table 3). All fractions were finally analyzed by means of SDS-PAGE and subsequent Coomassie-staining. The EA-fraction of the IMAC was dialyzed three-times against DPBS or buffer containing 50 mM Tris-HCl pH 8.0 and 150 mM NaCl at 4 °C using *Spectra/Por® Dialysis Membranes* with the appropriate molecular-weight cut-off (Spectrum Laboratories).

Table 3: Gradient for IMAC-Purification of Recombinant Proteins.

Purification Step	Buffer	Column Volumes
FT	o/n rotated Ni-sepharose resin	
W0	50mM Tris/HCl pH 8.0 + 500mM NaCl + 0,1% TX100	15
W0TX	50mM Tris/HCl pH 8.0 + 500mM NaCl	10
W20	20mM Imidazol in 50mM Tris/HCl pH 8.0 + 500mM NaCl	5
W40	40mM Imidazol in 50mM Tris/HCl pH 8.0 + 500mM NaCl	5
EA	250mM Imidazol in 50mM Tris/HCl pH 8.0 + 150mM NaCl	3
EE	250mM Imidazol in 50mM Tris/HCl pH 8.0 + denaturing agent (6M GuaHCl or 8M urea) + 500mM NaCl	3

N.B. in each step, except of EA, 1 mM DTT may be added if required

Removal of Endotoxin via Membrane-Filtration

In order to remove bacterial lipopolysaccharide (LPS) from the recombinant proteins, the protein-solutions were filtered through *Acrodisc® Units with Mustang™ E Membranes* (PALL) according to manufacturer's instructions. Following the LPS-depletion, the content of endotoxin in the sample was determined using the *Limulus Amebocyte Lysate (LAL) QCL-1000® Test* (Lonza), according to manufacturer's instructions.

Qualification and Quantification of Purified Recombinant Proteins

The purified recombinant protein was qualified by SDS-PAGE and subsequent Coomassie-staining and Western blot analysis using mouse anti-penta-His antibody (Quiagen) as primary antibody. For the quantification of the prepared protein a *BCA Protein Assay* (Pierce) was used according to manufacturer's instructions.

4.5 *Coupling of Antigens to FluoSpheres®*

Fluorescent microspheres (Carboxylate-Modified, Amine-Modified or Sulfate and Aldehyde-Sulfate FluoSpheres®, Molecular Probes, Invitrogen) were coupled with 1 mg of the required protein according to manufacturer's instructions ("*Working with FluoSpheres® Fluorescent Microspheres*", page 1-5) and the coupling-success was checked by means of SDS-PAGE and subsequent Coomassie-staining.

5 *ISOLATION, PURIFICATION AND QUANTIFICATION OF LTA*

5.1 *Small-Scale Optimization using LTA from S. pyogenes*

Hydrophobic Interaction Chromatography (HIC)

In order to optimize the conditions for the HIC-based purification of pneumococcal LTA [126], 500 µl of *Octyl Sepharose™* (GE Healthcare) were rotated over-night at 4 °C with 100 µg LTA from *S. pyogenes* (L 3140, SIGMA) in 0.1 M NH₄Ac-buffer, pH 4.7 (HIC-buffer) with no, 5 % and 15 % n-propanol (SIGMA) added. The incubated resins were transferred into *BioRad Micro Bio-Spin® Chromatography Columns* (BioRad) and the *S. pyogenes* LTA was eluted using an n-propanol gradient from 0 % to 100 % in HIC-buffer. Thus the column-resin was briefly incubated with 500 µl of eluent and centrifuged at 4 x g for 1 min into a fresh collection tube. All fractions were stored at -20 °C until further use.

ELISA-Based Quantification using GAS-LTA Specific Antibodies

100 µl of LTA from *S. pyogenes* (1 mg/ml) or 100 µl of each HIC-fraction (see previous section) were spiked with 0.1 M NaHCO₃-buffer, pH 9.3 and used in 5-fold dilutions for overnight coating of *MaxiSorp*TM plates (Nunc) at 4 °C. The next day, the coating solution was discarded and 100 µl of blocking buffer containing 2 % BSA in 1xDPBS were added per well and incubated for 1 h at room-temperature. The plates were washed three-times with 300 µl of 0.1 % Tween[®]20 in DPBS (PBS-T) per well and incubated for 1 h at room-temperature with 50 µl of a 1:200 dilution of primary antibody ("*Lipoteichoic Acid Antibody, Rabbit Polyclonal, anti-S. pyogenes, 18-783-77138*", GenWay) in 1 % BSA in DPBS (dilution buffer). After incubation, the plates were washed three-times with 300 µl of PBS-T per well and incubated for 1 h at room-temperature with 50 µl of a 1:1000 dilution of secondary antibody ("*Anti-rabbit IgG, peroxidase-linked species specific whole antibody (from donkey)*", AmershamBiosciences) in dilution buffer. The plates were finally washed three-times in the "overflow"-mode with 300 µl of PBS-T per well. Following the light-protected incubation for 30 min at room-temperature, with 50 µl ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); SIGMA) per well, the OD₄₀₅ was determined.

5.2 Purification of LTA from *S. pneumoniae*

Pneumococcal LTA was purified using an n-butanol extraction and subsequent hydrophobic interaction chromatography (HIC) as described [126].

Bacterial Culture

The *S. pneumoniae* strains TIGR4 and PJ1324 (6B) were inoculated and grown in 100 ml of THY pre-culture until the end of the log-phase at 37 °C and 5 % CO₂. 50 ml of this pre-culture were then used to inoculate 5 liters of fresh THY and bacteria were grown for 16-18 h at 37 °C and 5 % CO₂ before harvesting by centrifugation at 4 °C for approximately 30 min at 4400 rpm.

Cell Disruption and LTA-Isolation

The pelleted bacterial biomass was re-suspended in 50 ml of 1 mM Tris-HCl, pH 8.0 and incubated for 1 h at 37 °C with 0.25 mg/ml lysozyme (SIGMA) to break up the cell wall. 5 mM MgCl₂ was then added to the solution, which was further incubated for 1 h at 37 °C with 50 µg/ml RNase A (Promega) and 10 µg/ml DNase I (Roche), followed by the addition of 0.5 % SDS (SIGMA) and over-night incubation at 55 °C with 50 µg/ml ProteinaseK (Invitrogen).

C – MATERIALS AND METHODS

After the denaturation of ProteinaseK for 10 min at 95 °C, 25 ml of 50 mM citrate-buffer, pH 4.7 were added and the solution was sonicated twice for 5 min on ice. The bacterial lysate was mixed with an equal volume of n-butanol (Merck) under stirring for 20 min at room-temperature. After centrifugation at 4 °C for 45 min at 17,200 x g the lower, aqueous phase was collected before an equal volume of fresh citrate-buffer was added to the solvent-phase for a second extraction as described.

These steps were repeated and the three aqueous phases obtained were pooled, frozen at - 80°C and subsequently lyophilized.

Hydrophobic Interaction Chromatography (HIC)

The lyophilized LTA-extracts were re-suspended in 35 ml of chromatography start buffer containing 15 % n-propanol in 0.1 M NH₄Ac-buffer, pH 4.7. After centrifugation at 4 °C for 60 min at 26,900 x g, the supernatant was filtered through a 0.2 µm membrane filter. The filtrate was subjected to HIC with 54 ml *Octyl Sepharose*TM (GE Healthcare) using an ÄKTAexplorerTM (GE Healthcare) chromatography system. LTA was eluted using a linear gradient of 15-90 % n-propanol in 0.1 M NH₄Ac-buffer, pH 4.7 and 5 ml fractions were automatically collected.

Quantification based on the Phosphomolybdenum-Blue Reaction

Post HIC, the fractions were screened for their phosphate content. High phosphate concentrations were expected in the flow-through fractions, representing DNA and proteins not bound to the chromatography column and in a second peak from later fractions that should contain LTA. ^[126]

The phosphate determination is based on the formation of phosphomolybdenum blue from phosphate. 50 µl of a 0.65 mM phosphate standard (SIGMA) and 100 µl of each fraction were mixed with 200 µl ashing solution containing 2 M H₂SO₄ and 0.44 M HClO₄. Following incubation in open glass vials (Hirschmann) for 2.5 h at 146 °C, 1 ml of reducing solution containing 3 mM ammonium molybdate, 0.25 M sodium acetate and 1 % ascorbic acid was added and the fractions were incubated for 2 h at 45 °C. The OD₇₀₀ of 250 µl of each sample was subsequently measured.

PAGE-Based Quantification

For the PAGE-based quantification of isolated and purified LTA from *S. pyogenes* and *S. pneumoniae*, 30-40 % Tris-Borate-EDTA gels (JULE Inc.) were pre-run at 4 °C for 20 min at 100 V in running buffer containing 90 mM Tris, 80 mM boric acid and 2.6 mM EDTA, pH 8.3. LTA-fractions in loading dye – 2 mg/ml Orange G in 40 % sucrose or 4 mg/ml Xylene cyanol and 4 mg/ml Bromophenol blue in 40 % sucrose – were loaded and run over-night at 4 °C and 100 V.

Post PAGE, the gels were stained by a combined AlcianBlue (SIGMA) – Silver staining (BioRad) following the literature protocols ^[389-390] and the manufacturer's instructions (*BioRad Silver Stain*) and were subsequently scanned (GS-800, Calibrated Densitometer, BioRad).

Additionally, were some gels immediately fixed with 50 % MeOH and 10 % acetic acid after electrophoresis and subsequently just stained using the *Bio-Rad Silver Stain* kit (BioRad).

As the precast gels from JULE Inc. were extremely fragile (*e.g.* ripping-off the slots when removing the comb or disrupting of the whole gel when placing the gel-cassette into the electrophoresis chamber, *etc.* happened very easily), 4-20 % Tris-Borate-EDTA gradient-gels (Lonza) were purchased and used as described.

ELISA-Based Quantification using GAS-LTA Specific Antibodies

The HIC fractions were screened for their LTA-content *via* an LTA-ELISA that was based on the initial setup but using (i) rabbit polyclonal antibody *vs.* LTA from *S. pyogenes* (1:200, GenWay, 18-783-77138) or (ii) mouse mAb *vs.* Gram Positive Bacteria (1:1000, GenWay, 20-511-241416) and 1:1000 dilutions of the appropriate secondary antibodies conjugated to horse-raddish peroxidase (HRP, rabbit anti-mouse-HRP (P0260), DAKO; donkey anti-rabbit-HRP (NA934), AmershamBiosciences) for a detection following the ABTS-staining protocol at OD₄₀₅.

LTA-Deacylation-Procedure

For a more effective PAGE-based quantification and qualification of the LTA-purification, 20 µg GAS-LTA (as an initial optimization step) and subsequently, 20 µl of isolated pneumococcal LTA were primarily chemically decayed (cdLTA) by a mild NH₄OH-treatment (according to the original procedure ^[391]). This decay was followed by a limited HCl-hydrolysis where the cdLTA (1/2 of the remaining material; the other half was used for PAGE to check for

the success of the NH₄OH treatment) and 20 µl of 2 M HCl were incubated at 80 °C for 0, 1, 5, and 10 min, followed by an immediate cooling step on ice, the removal of HCl by flushing with dry-air on ice and subsequent dissolving of the chemically decomposed LTA in 10 µl dH₂O. Samples from all steps were subsequently subjected to LTA-PAGE and a combined Alcian Blue and Silver-staining.

6 ANIMAL STUDIES

6.1 Animal Guidelines – Tierversuchsantrag

All animal-studies in *M. musculus* carried out during this project were approved according to Austrian guidelines (*Tierversuchsgesetz BGBl. Nr. 501/1989*) in the “*Tierversuchsantrag N° 18 – Kapitel II*”, “*Tierversuchsantrag N° 27 – Kapitel I*” and “*Tierversuchsantrag N° 27- Kapitel III*”.

For all studies, female C3H/HeN mice were purchased from Harlan Winkelmann (Borchen, Germany) or Elevage Janvier (Le Genest Saint Isle, France) and used at the age of six to eight weeks with a body-weight of approximately 23 g.

6.2 Generation of Hyperimmune Sera in *M. musculus*

For the generation of hyperimmune sera (experiment-N°: CGI/LST3742 and LST3885; according to “*Tierversuchsantrag-N° 18, Kapitel II*”), female C3H/HeN mice (5 mice/group) were immunized subcutaneously (*s.c.*; flank) three times at two-week intervals with 50 µg of purified recombinant protein per mouse and immunization, formulated with ALUM (Aluminium Hydroxide Gel Adjuvant, Alhydrogel; Brenntag) or emulsified in CFA/IFA (Adjuvant Complete Freund (1st immunization) and Adjuvant Incomplete Freund (2nd and 3rd immunization), Difco Laboratories) after taking pre-immune sera one day before the first immunization. On day 35, mice were anesthetized with isofluran and terminally bled – retro-orbital plexus – for the collection of hyperimmune sera.

6.3 Dose Finding Studies for Lethal Infection of *M. musculus* with Pneumococcal Strains

For the determination of the optimal challenge dose of different *S. pneumoniae* strains, which were to be used *via* different application routes in passive-transfer and challenge models, naïve, female C3H/HeN mice (5 mice/group) were challenged with different bacterial cfu in DPBS – *intraperitoneally* (*i.p.*, 100 µl/mouse), *i.n.* (40 µl/mouse), or *i.v.* (100 µl/mouse). The

challenge dose was confirmed by plating on blood agar and the survival of the mice was monitored for 15 days post challenge, being compared to appropriate control groups. Surviving mice were sacrificed on day 15 post challenge *via* cervical dislocation. (experiment-N°: GNA3876 and LST3898; according to “*Tierversuchsantrag-N° 27, Kapitel III*”)

6.4 *In vivo Efficacy Testing of Polyclonal Antibodies*

The *in vivo* efficacy of polyclonal antibodies, generated against several recombinant pneumococcal antigens, was tested in models of *i.p.* passive transfer of hyperimmune-sera and *i.p.*, *i.n.*, or *i.v.* lethal challenge with different pneumococcal strains. Therefore 10 µl of hyperimmune serum diluted in 490 µl DPBS were injected *i.p.* into groups of 5 naïve, female C3H/HeN mice followed by a lethal challenge 24 h post immunization. The challenge dose was confirmed by plating on blood agar and the survival of the mice was monitored for 15 days post challenge, being compared to appropriate control groups. Surviving mice were sacrificed on day 15 post challenge *via* cervical dislocation.

7 IN VITRO ASSAYS

7.1 *Western Blot Analysis*

Following the SDS-PAGE, the separated proteins were transferred onto a nitrocellulose membrane (iBlot® Gel Transfer Stacks, Invitrogen) using a dry transfer system (iBlot™ Gel Transfer Device, Invitrogen), according to manufacturer’s instructions. After Ponceau-staining (Ponceau S solution, SIGMA) and quick-documentation, the membranes were destained and blocked – 1 h at room-temperature or over-night at 4 °C – with 5 % milk powder in DPBS. After a brief rinsing-step with 0.1 % Tween®20 in DPBS (PBS-T), the membranes were incubated for 1 h at room-temperature with a 1:5000 dilution of primary antibody in 5 % milk, before the membranes were washed three-times with PBS-T for 10-15 min at room-temperature. Following incubation for 1 h at room-temperature with a 1:5000 dilution of secondary antibody (*Peroxidase-conjugated AffiniPure F(ab')₂ fragment Goat anti-mouse IgG(H+L)*, Jackson Immuno Research) in DPBS, the membranes were washed four-times with PBS-T for 10-15 min at room-temperature and once with DPBS for 5 min at room-temperature before the blots were developed (ChemiGlow™, Alpha Innotech), according to manufacturer’s instructions, and analyzed (FluorChem™ SP, Alpha Innotech).

7.2 Enzyme Linked Immunosorbant Assay (ELISA)

Determination of Serum-Antibody-Titers

For the determination of serum-antibody-titers, each well of a *MaxiSorp*TM plate (NuncTM) was coated with 50 µl of a 1 µg/ml antigen-dilution in 0.1 M NaHCO₃-buffer, pH 9.3 and incubated over-night at 4 °C. The next day, the coating solution was discarded and 100 µl of blocking buffer containing 2 % BSA in DPBS was added per well and incubated for 1 h at room-temperature. After incubation, the plates were washed three-times with 300 µl of 0.1 % Tween[®]20 in DPBS (PBS-T) per well and incubated for 1 h at room-temperature with 50 µl of pre-diluted serum – 5-fold dilutions in 1 % BSA in DPBS (dilution buffer) – per well. After incubation, the plates were washed three-times with 300 µl of PBS-T and incubated for 1 h at room-temperature with 50 µl of pre-diluted secondary antibody (“*Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP*”, DAKO, 1:1,000 in dilution buffer, or “*Goat Anti-Human IgG-HRP*, SouthernBiotech, 1:4,000 in dilution buffer). The plates were finally washed three-times in the “overflow”-mode with 300 µl of PBS-T per well. Following the light-protected incubation for 30 min at room-temperature, with 50 µl ABTS (SIGMA) per well, the OD₄₀₅ was determined and the data were analyzed (Gen5TM, BioTek).

Peptide ELISA

For the determination of the peptide-recognition-profile of hyperimmune-sera generated against several PspA-constructs, pre-coated streptavidin-plates (NuncTM) were washed three-times using 300 µl of 0.1 % Tween[®]20 in DPBS (PBS-T) per well and subsequently coated with 100 µl of the appropriate peptides (1 µg/ml) in PBS-T and incubated over-night at 4 °C.

The next day, the coating solution was discarded and 100 µl of blocking buffer containing 2 % BSA in DPBS were added per well and incubated for 1 h at room-temperature. After incubation, the plates were washed three-times with 300 µl of PBS-T per well and incubated for 1 h at room-temperature with 50 µl of pre-diluted serum – 5-fold dilutions in 1 % BSA in DPBS (dilution buffer) – per well. After incubation, the plates were washed three-times with 300 µl of PBS-T per well and incubated for 1 h at room-temperature with 50 µl of a 1:1000 dilution of secondary antibody (“*Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP*”, DAKO) in dilution buffer. The plates were finally washed three-times in the “overflow”-mode with 300 µl of PBS-T per well. Following the light-protected incubation for 30 min at room-temperature, with 50 µl

ABTS (SIGMA) per well, the OD₄₀₅ was determined and the data were analyzed (Gen5™, BioTek).

IL-6 ELISA

The IL-6 levels of pre- and hyperimmune-sera from *M. musculus* were determined using the “Mouse IL-6 ELISA Ready-SET-Go!®” kit (eBioscience) according to the manufacturer’s protocol (Technical Data Sheet “Mouse IL-6 ELISA Ready-Set-Go!®” ; page 1-6) and the data were analyzed (Gen5™, BioTek).

7.3 Surface Staining of Live Pneumococci

For the surface staining and subsequent flow cytometric analysis, 5 ml THY over-night cultures of several *S. pneumoniae* strains were grown from glycerol stock at 37 °C and 5 % CO₂. The next day, 10 ml of pre-warmed THY were inoculated with 0.5 ml of the over-night culture and the bacteria were grown to OD₆₂₀ = 0.3. 2 ml of this culture were centrifuged at 4 °C for 5 min at 1000 x g, the pellet was washed twice with 2 ml of 2 % BSA in HBSS and re-suspended. The bacterial cells were diluted to 5x10⁵ cells in 100 µl of 2 % BSA in HBSS, added to a 96-well plate (U-bottom, Nunc™) and subsequently incubated at 4 °C for 45 min with primary antibody or serum. Following washing with 150 µl of 2 % BSA in HBSS per well and centrifugation at 4 °C for 5 min at 1000 x g, the secondary antibody was added and incubated at 4 °C for 45 min in the dark. This was followed by another washing and centrifugation step, and an incubation at 4 °C for 10 min in the dark with 0.05 µl of SYTO®60 (Invitrogen) in 100 µl HBSS. After a final washing and centrifugation step, the cells were fixed over-night with 200 µl of 2 % para-formaldehyde per well at 4 °C. The stained cells were measured (FC-500 Analyzer, BeckmanCoulter) and the data were evaluated (FCSExpress; DeNovoSoftware).

7.4 Surface Staining of Eukaryotic Cells

Several eukaryotic cells (HL-60, HEK 293T, hybridoma and hPBMCs) were used for surface-staining for flow-cytometric analyses according to the following protocol, which was slightly modified in some cases if required (see Results and Discussion where done).

Briefly, the eukaryotic cells were washed in FACS-buffer containing 2 % FCS and 0.01 % NaN₃ in DPBS, centrifuged for 10 min at 1000 x g and diluted in FACS-buffer to 1x10⁶ cells/ml. 1x10⁵ cells/well were subsequently incubated with the required dilutions of

primary antibody or serum for 45 min, on ice, in the dark. Following centrifugation for 10 min at 1000 x g, the cells were washed with 150 µl of FACS-buffer and 100 µl of secondary antibody, diluted in FACS-buffer, were added and incubated for 45 min on ice, in the dark. After a final washing step with 150 µl of DPBS and centrifugation for 10 min at 1000 x g, the cells were re-suspended in 200 µl of DPBS and subjected to flow-cytometric analysis (FC-500 Analyzer, BeckmanCoulter) and the data were evaluated (FCSEXPRESS; DeNovoSoftware).

7.5 Hemolysis-Inhibition Assay

Purification of IgG from M. musculus Sera

After thawing, heat-inactivation at 56 °C for 45 min and centrifugation at 4 °C for 5 min at full-speed, the mouse-sera were filtered through a 0.2 µm syringe filter (PALL). Following a dilution of 1:3 in Binding Buffer pH 5.0 (Pierce) the sera were loaded onto *BioRad Micro Bio-Spin® Chromatography Columns* (BioRad), packed with washed – 10 column volumes of Binding Buffer pH 5.0 – “*Ultralink Immobilized Protein G*” (Pierce; gel capacity approx. 20 mg IgG/ml). After collecting the flow-through, the column was washed with 20 column volumes of Binding Buffer pH 5.0 before the bound IgG was eluted with 5 column volumes of Elution Buffer pH 2.5 (Pierce) and immediately neutralized by adjusting the pH with 100 µl of 1 M Tris buffer pH 8.0 per 1000 µl eluate. After two cycles of dialysis against DPBS, in a *Slide-A-Lyzer® Dialysis Cassette* (10,000 MWCO, Pierce), SDS-PAGE with subsequent Coomassie-staining was performed.

Determination of the Hemolytic Unit [HU] of PLY

The setup of the hemolysis (inhibition) assay is based on literature protocols ^[301, 392] being modified regarding the following procedure.

200 µl of freshly isolated hRBCs in DPBS (5×10^8 cells/ml) were incubated with 50 µl of a serial dilution series of PLY in triplicates for 1 h at 37 °C and constantly slow shaking. After a centrifugation step at 4 °C for 5 min at 4400 rpm, the supernatant was transferred into a 96-flat bottom plate (Nunc) and the OD₅₄₁ was determined. Using dH₂O as positive control (100 % hemolysis) and DPBS as blank (0 % hemolysis) as standards in triplicates, the hemolytic unit of PLY was calculated, where 1 HU was defined as the concentration of PLY required to induce 50 % hemolysis of hRBCs.

The Optimized Hemolysis-Inhibition Assay

After the HU of the recombinant PLY was determined (which should be determined for all isolations of hRBCs prior to the inhibition-assay), the hemolysis-inhibition assay was setup according to the following scheme (Figure 15). Briefly, several fold-dilutions of antibody (*e.g.* mAb or IgG from serum) were incubated with 2 HU of PLY for 1 h at 37 °C and constantly slow shaking before 200 μ l of freshly isolated hRBCs (5×10^8 cells/ml in DPBS) were added and incubated for 1 h at 37 °C and constantly slow shaking. Following a centrifugation step at 4 °C for 5 min at 4400 rpm, the supernatant was transferred into a 96-flat bottom plate (Nunc), the OD₅₄₁ was determined and the capacity of hemolysis-inhibition of the antibody was determined.

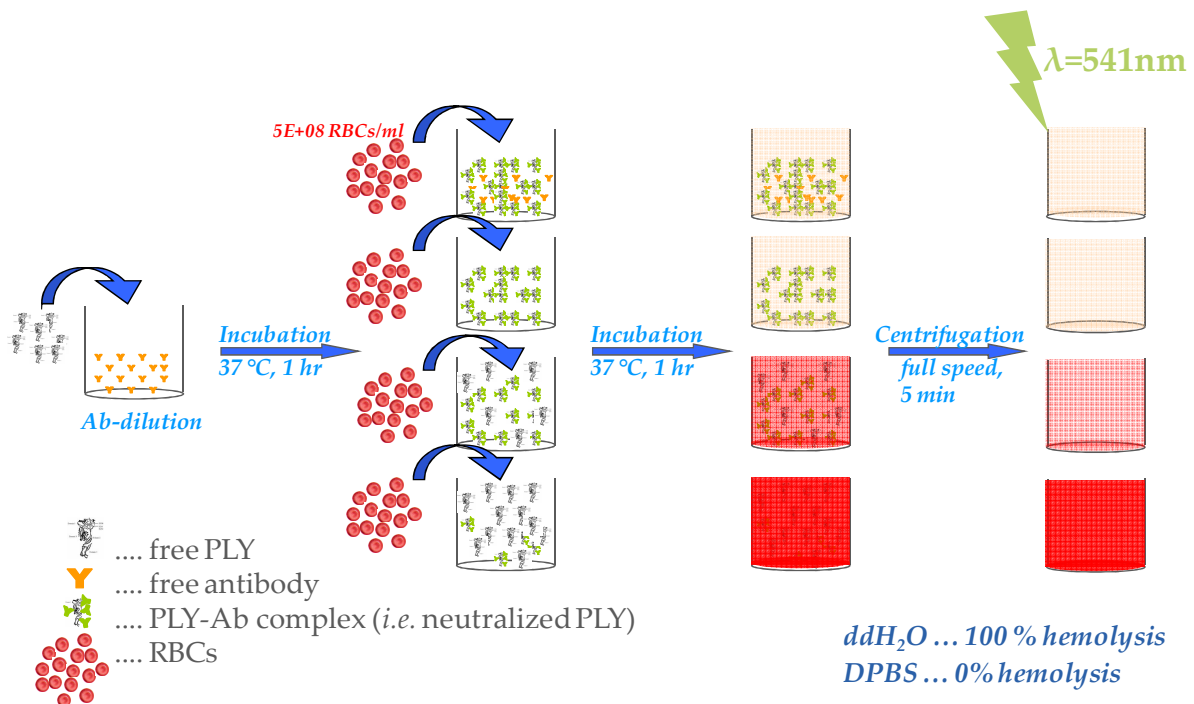


Figure 15: Schematic Representation of the Hemolysis-Inhibition Assay. (© Lukas Stulik)

7.6 TLR4-Reporter Assay

The TLR4-Reporter Assay was setup according to manufacturer's instructions (InvivoGen) using 1×10^6 HEK-Blue™ hTLR4 cells/ml which were extremely carefully treated (*e.g.* no scraping, no trypsinization of the culture) in order to avoid stress-response and unspecific reporter-activation. Furthermore, just endotoxin (LPS) free reagents and qualified FCS (Gibco) were used in all assay-steps.

For the quantification and assay-control, an *E. coli* LPS (K12, InvivoGen) standard-calibration as well as unrelated antibodies and proteins, in the same buffer and the same LPS-content as the recombinant PLY, were used in each assay.

D - RESULTS

1 GENERATION OF RECOMBINANT PROTEINS

In order to obtain the target antigens for this project, PspA (SP0117), PLY (SP1923) and subconstructs thereof were expressed in recombinant form with a C-terminal His-tag by *XhoI/NcoI* cloning into the pET28b+ expression vector. The whole protein and domains, that were shown to be the most protective and reactive regions in *in vivo* and *in vitro* settings in previous experiments, were used.

1.1 Sequence Information and Designing of the Recombinant Constructs

The expression and purification of recombinant PspA and several subconstructs thereof, was based on the PspA-sequences of the pneumococcal wild-type strains WU2 (PspA Family 1, Clade 2) and TIGR4 (PspA Family 2, Clade 3), as these two strains were shown to be representatives for two different PspA-clades within two different families [163].

A

M³¹EESPQVVEKSSLEKKYEEAKAKADTAKKDYETAKKKAEDAQKKYEDDQKRTEEKARKEAEASQKLNNDVALVVQNAY
KEYREVQNQRSKYKSDAEYQKKLTEVDSKIEKARKEQQDLQNKFNVEVRAVVVPEPNALAETKKKAAEEAKAEEKVAKRK
YDYATLKVVALAKKEVEAKELEIEKLYEISTLEQEVATAQHQVDNLKLLLAGADPDDGTEVIEAKLKKGEAELNAKQA
ELAKKQTELEKLLDSDLPEGKTQDELDKAEFAEELDKKADELQNKVADLEKEISNLEILLGGADPEDDTAALQNKLA
KKAELAKKQTELEKLLDSDLPEGKTQDELDKAEFAEELDKKADELQNKVADLEKEISNLEILLGGADSEDDTAALQNK
LATKKAELKTEKELDAALNELGPDGDEEETPAPAPQPEQPAPAPKPEQPAPAPKPEQPAPAPKPEQPAPAPKPEQPA
PAPKPEQPAKPEKPAEPTQPEKPATPKTGWKQENGMWYFY⁵³⁸LEHHHHH*

B

M³¹EESPVASQSKAEKDYDAAVKKSEAAKKAYEEAKKALEEAKVAQKKYEDDQKKTEEKAELEKEASEAIAKATEEVQQ
AYLAYQRASNKAEAAKMIIEEAQRRENEARAKFTTIRTTMVVPEPEQLAETKKKAAEEAKAKEPKLAKKAAEAKAKLEEA
EKKATEAKPQVDAEEVAPQAKIAELENQVHRLEQELKEIDESESEDYAKEGFRAPLQSKLDAKKAKLSKLEELSDKID
ELDAEIAKLEDQLKAVEENNNVEDYSTGLEKTI AAKKTELEKTEADLKKAVNEPEKSAEEPSQPEKPAEEAPAPEQP
TEPTQPEKPAEETPAPKPEKPAEQPKAEKTDDQQAEDYARRSEEEYNRLTQQQPPKAEKPAPAPQPEQPAPAPKTGW
KQENGMWYFYNTDGSMATGWLQNNGSWYYLNSNGAMATGWLQYNGSWYYLNANGAMATGWAKVNGSWYYLNANGSMAT
GWVKDGTWYYLEASGAMKASQWFKVSDKWYVNSNGAMATGWLQYNGSWYYLNANGAMATGWAKVNGSWYYLNANGS
MATGWVKDGTWYYLEASGAMKASQWFKVSDKWYVNSGSLAVNTTVDGYTVNENGEWV⁶³⁵LEHHHHHH*

Figure 16: Amino Acid Sequences of Recombinant PspA_FL_T4 (A) and PspA_FL_WU2 (B). The recombinant proteins PspA_FL_T4 and PspA_FL_WU2 cover the amino acid sequences (normal letters) 32 to 538 (T4) and 32 to 635 (WU2) of the corresponding native proteins. All amino acids highlighted in gray originate from the pET28b+ vector backbone.

The alignment of all published PspA-sequences [390] of different pneumococcal backgrounds indicated that both strains express PspA with a sequence variation within the clade-defining region (“B”) (Supplementary Figure 1) as described in the literature [163, 170, 193-199]. Sequence alignments of the proline-rich regions (Supplementary Figure 2 and Supplementary Figure 3) further confirmed that just PspA from WU2 and not from TIGR4 contains the non-

proline block within the proline-rich region (Supplementary Figure 3), which is described in literature as another fundamental difference of the two types of PspA. [124, 163, 180, 187-190]

Recombinant PspA (SP0117) from *S. pneumoniae* strain TIGR4 (serotype 4; PspA Family 2, Clade 3) and WU2 (serotype 3; PspA Family 1, Clade 2) were designed with expected molecular weights of 56.5 kDa (corresponding to amino acids 32 to 538 (Figure 16-A) of the native protein) and 67.6 kDa (corresponding to amino acids 32 to 635 (Figure 16-B) of the native protein) respectively avoiding the hydrophobic C-terminal cell wall binding domain.

For the determination of the *in vivo* and *in vitro* protective and functional characteristics, several subconstructs of PspA were also cloned and expressed in recombinant form from the *S. pneumoniae* TIGR4 and WU2 backgrounds (Figure 17). For each genetic background one construct comprising the proline-rich region, one covering the clade defining region (“B”) and one consisting of the immunodominant epitope of the clade defining region and the proline-rich region (see Figure 17 for details), was generated.

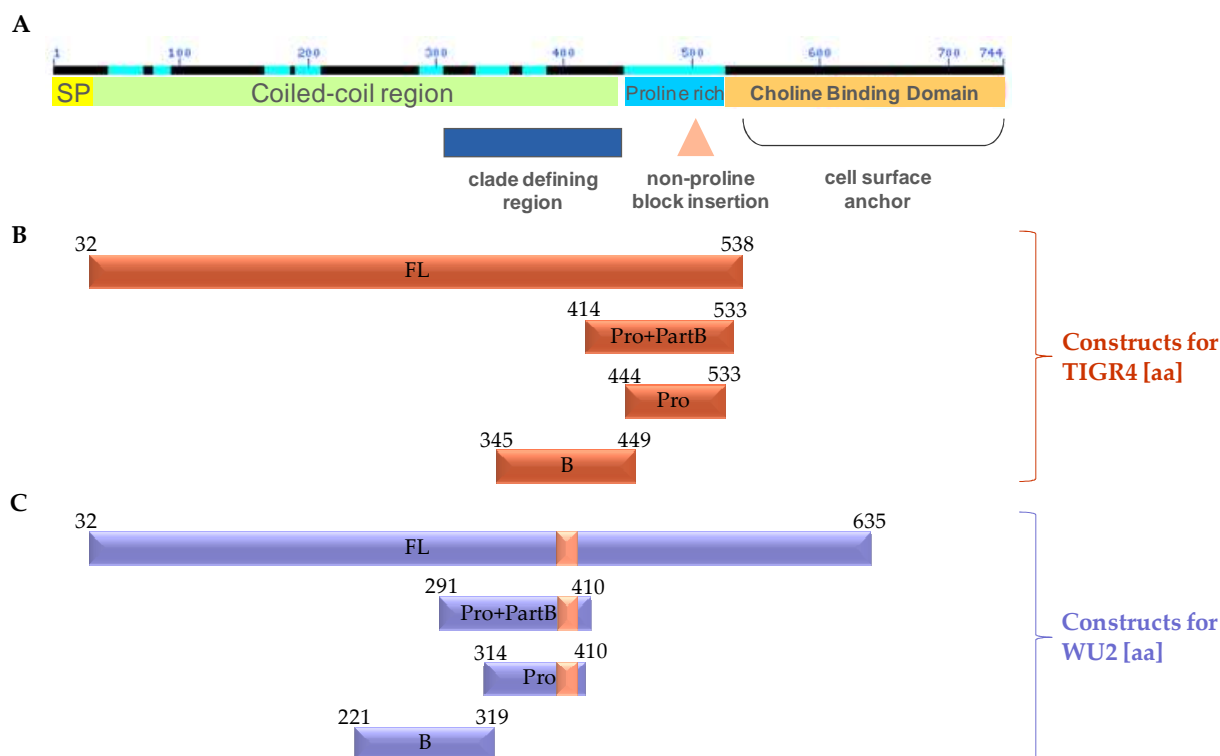


Figure 17: Recombinant Constructs of PspA (SP0117). Aligned to the native protein structure of PspA from *S. pneumoniae* TIGR4 (A), the generated recombinant protein constructs for TIGR4 (B) and WU2 (C) are indicated. Based on the low sequence similarity of WU2 to TIGR4, this figure just indicates the sizes of the WU2 constructs, including the non-proline block insertion (pink box) and not the real positions thereof in the native protein structure. (modified from [185, 193])

D – RESULTS

Likewise, PLY and variants thereof were expressed and purified in recombinant form only from the *S. pneumoniae* TIGR4 background as this protein is completely conserved across all pneumococcal strains (Figure 18).

M¹MANKAVNDFILAMNYDKKKLLTHQGESIENRFIKEGNQLPDEFVVIERKKRSLSTNTSDISVTATNDSRLYPGALLVVDETLLENNPTLLAVDRAPMTYSIDLPGGLASSDSFLQVEDPSNSSVRGAVNDLLAKWHQDYGVNNVPA¹⁴⁶RMQYEKITAHSMEQLKVKFGSDFEKTGNSLDIDFNSVHSGEKQIQIVNFKQIYYTVSVDVAVKNPGDVFQDTVTVEDLKQRGISAERPLVYISSVAYGRQVYLKLETTSKSDEVEAAFEALIKGVKQTEWKQILDNTEVKAVILGGDPSSGARVVTGKVD
MVEDLIQEGSRFTADHPGLPISYTTSFRLDNNVATFQNSTDYVETKVTAYR³⁵⁹NGDLLLDHSGAYVAQYYITWNELSY
DHQGKEVLTTPKAWDRNGQDLTAHF'TTSIPLKGNVRNLSVKIRECTGLAWEWWR'TVYEKTDLPLVRKRTISIWGTTLYP
QVEDKVEND⁴⁷¹LEHHHHH*

Figure 18: Amino Acid Sequences of Recombinant PLY and Constructs Thereof. The recombinant protein PLY covers the full amino acid sequence 1 to 471 of the corresponding native protein. For PLD, the site of deletion of A146 is highlighted in red and the sequence for domain 4, which was deleted in PLY Δ D4, is underlined. All amino acids highlighted in gray originate from the pET28b+ vector backbone.

Thus the wild-type toxin (PLY, 52.9 kDa, corresponding to amino acids 1 to 471 of the native protein), as well as the mutant toxoids PLD (52.8 kDa, corresponding to amino acids 1 to 471 of the native protein with a deletion of A146^[393]) and PLY Δ D4 (43.5 kDa, corresponding to amino acids 1 to 359 of the native protein, with the cell-binding domain 4 being deleted) were generated.

1.2 Cloning and Protein Expression

In the following the main steps for cloning and protein purification are outlined and exemplified on the procedure for the recombinant proteins PspA_B_T4 and PspA_FL_T4. The obtained quantitative and qualitative information for each recombinant protein generated are summarized at the end of this chapter (Table 4).

After cloning of the insert-sequences into the expression vector pET28b+, the recombinant plasmid DNA was amplified in DH5 α cells and subjected to colony PCR using T7 promoter and terminator specific primers in order to screen for positive clones (Figure 19). The colony PCR proves the expected fragment sizes and positive insertion of insert DNA into the pET28b+ vector for several clones (lanes 1,4,7,8,10,12,14 and 15) and reveals negative clones (lanes 2, 3, 5, 6, 9, 11 and 13) which were not further processed.

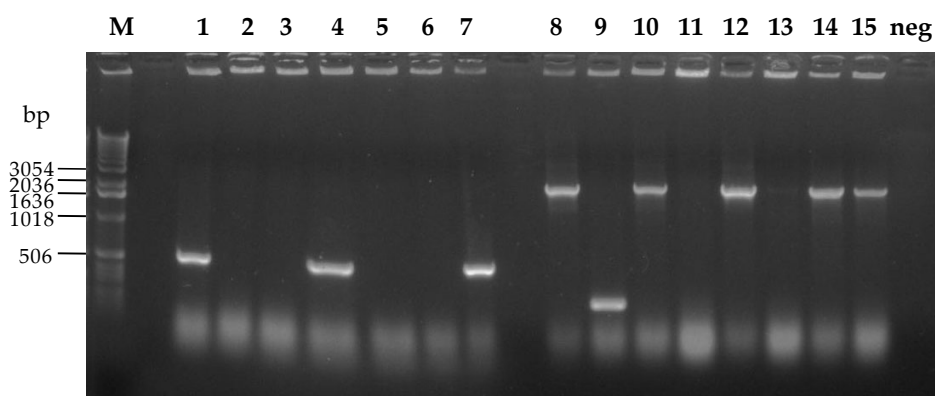


Figure 19: Colony PCR. Clones for *PspA_B_T4* (lanes 1-7; expected insert size: 314 bp) and *PspA_FL_T4* (lanes 8-15; expected insert size: 2139 bp) were randomly picked and used as template in colony PCR with T7 promoter and terminator specific primers. Water was included as negative control (neg).

The plasmid DNA from positive DH5 α -clones was subsequently amplified, purified (Figure 20-A) and subjected to a double-restriction digest with *XhoI* and *NcoI* to reanalyze the successful DNA-insertion into the pET28b+ backbone by comparing the computationally expected fragment sizes and the fragment sizes obtained following the digest and agarose gel electrophoresis (Figure 20-B).

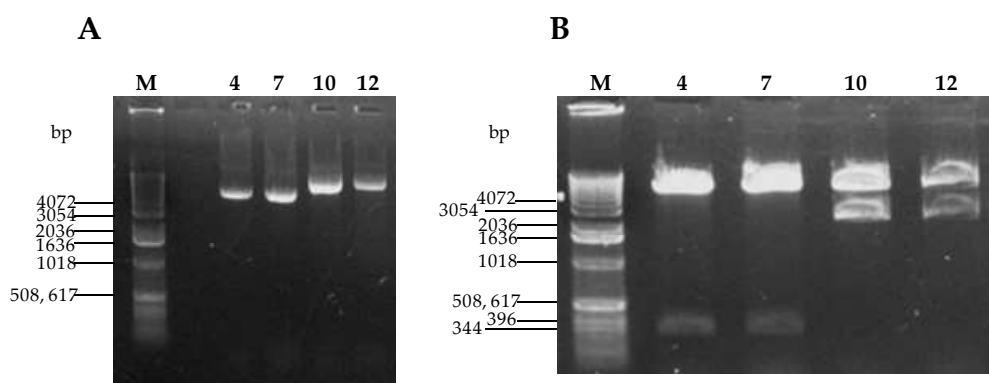


Figure 20: Plasmid Purification and Double-Restriction Digest. A. Successful plasmid purification was confirmed by agarose gel-electrophoresis for the recombinant plasmid DNA of *PspA_B_T4* (lanes/clones 4, 7) and *PspA_FL_T4* (lanes/clones 10, 12) B. Restriction digest of the plasmids the fragment size were analyzed on an agarose gel. The expected fragment sizes for the inserts were 314 bp for *PspA_B_T4* and 2139 bp for *PspA_FL_T4* and 5368 bp for the pET28b+ backbone after the double digest.

To indemnify the sequence-accuracy, positive plasmid DNA was finally sent for sequencing to MWG Biotech.

The sequenced, insert-positive, plasmid DNA was further used to transform BL21(DE3) *E. coli* expression host cells. Following successful transformation, these cells were used for small-scale expression experiments *via* IPTG induction, hence the checking for the expression of the protein of interest in recombinant form. For this purpose the total bacterial lysate (t),

D – RESULTS

soluble (s) and insoluble (i) fractions were separated by SDS-PAGE and proteins were visualized by Coomassie-staining (Figure 21-A) and Western blot analysis (Figure 21-B).

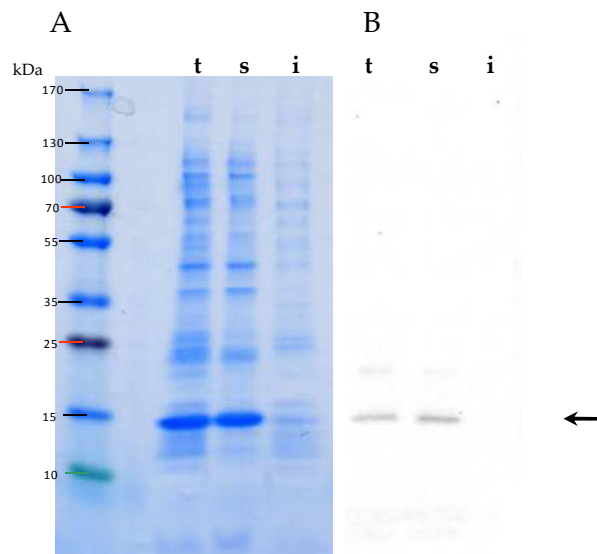


Figure 21: Example for a Small Scale Expression - PspA_B_T4. The successful small scale expression of recombinant PspA_B_T4 was confirmed by Coomassie-staining (A) and Western blot analysis (B). The expected protein size of approximately 12 kDa is indicated by an arrow.

Following a successful small-scale expression, the recombinant proteins were expressed on large scale and purified *via* IMAC using an imidazole gradient. Fractions were collected after each step and 5 μ l of each were subjected to SDS-PAGE analysis in order to monitor the purification (Figure 22).

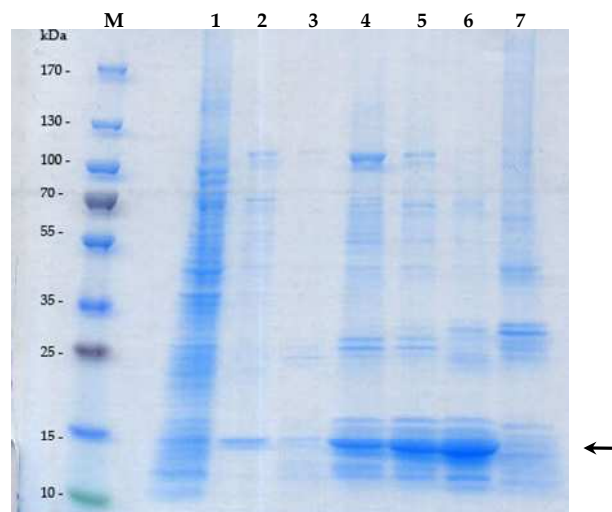


Figure 22: Monitoring of the IMAC-Purification. The purification success of PspA_B_T4 is exemplified, where the fractions of the IMAC-purification using an imidazole-gradient were analyzed via SDS-PAGE and subsequent Coomassie-staining (lanes 1-7).

The fractions containing the target protein, were subsequently pooled and dialyzed, centrifuged, filtered and subjected to SDS-PAGE and Coomassie-staining to analyze the protein quality (for an example see Figure 23).

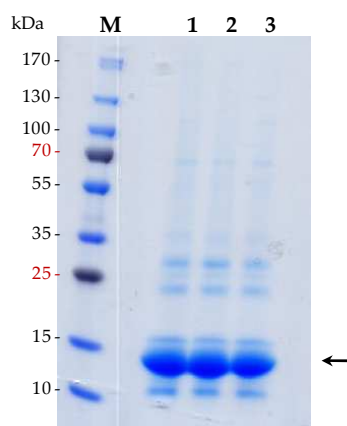


Figure 23: Quality Control Post Dialysis. Purified PspA_B_T4 is shown as representative: First the protein was dialyzed (lane 1), then precipitated protein was removed by centrifugation and the remaining soluble fraction was loaded (lane 2) and filtered through a MustangE membrane to reduce the endotoxin content (lane 3).

The concentration and endotoxin content were determined as summarized in Table 4 for all proteins that were expressed in the course of this study.

Table 4: Overview of All Proteins Purified in Recombinant Form.

Protein	MW [kDa]	pI	Fraction	Concentration [mg/ml]	Endoxin (LPS) [EU/mg]
PLY_WT	52.898	5.19	soluble	3.10	182
PLY_MUT (= PLD; deletion of A146)	52.827	5.19	soluble	1.65	733
PLYΔD4	43.513	5.68	soluble	1.14	167
PspA_B_T4	11.391	4.22	soluble	0.52	97
PspA_B_WU2	11.225	4.63	soluble	18.79	6
PspA_Pro_T4	9.443	4.64	soluble	1.20	107
PspA_Pro_WU2	11.192	4.38	soluble	3.00	3748
PspA_Pro+partB_T4	12.662	4.81	soluble	1.91	106
PspA_Pro+partB_WU2	13.619	4.58	soluble	6.00	3960
PspA_FL_T4	56.468	4.75	soluble	2.81	502

Solely the aimed expression of PspA_FL_WU2 was not successful, although the cloning into the expression vector was found to work as expected (data not shown). Thus, a new reconstruction and amplification of the sequence from the gDNA of WU2 as well as the cloning

D – RESULTS

into different protein-expression hosts such as Rosetta™ 2(DE3)pLysS cells might be useful in the future.

2 GENERATION AND SCREENING OF MOUSE HYPERIMMUNE SERA

For the generation of hyperimmune sera five mice per group were immunized with recombinant variants of PspA and PLY *s.c.*. Following terminal bleeding, the antibody titers (EC50) of individual mice were determined and compared to the corresponding pre-immune sera in ELISA-based screenings (exemplified in Figure 24).

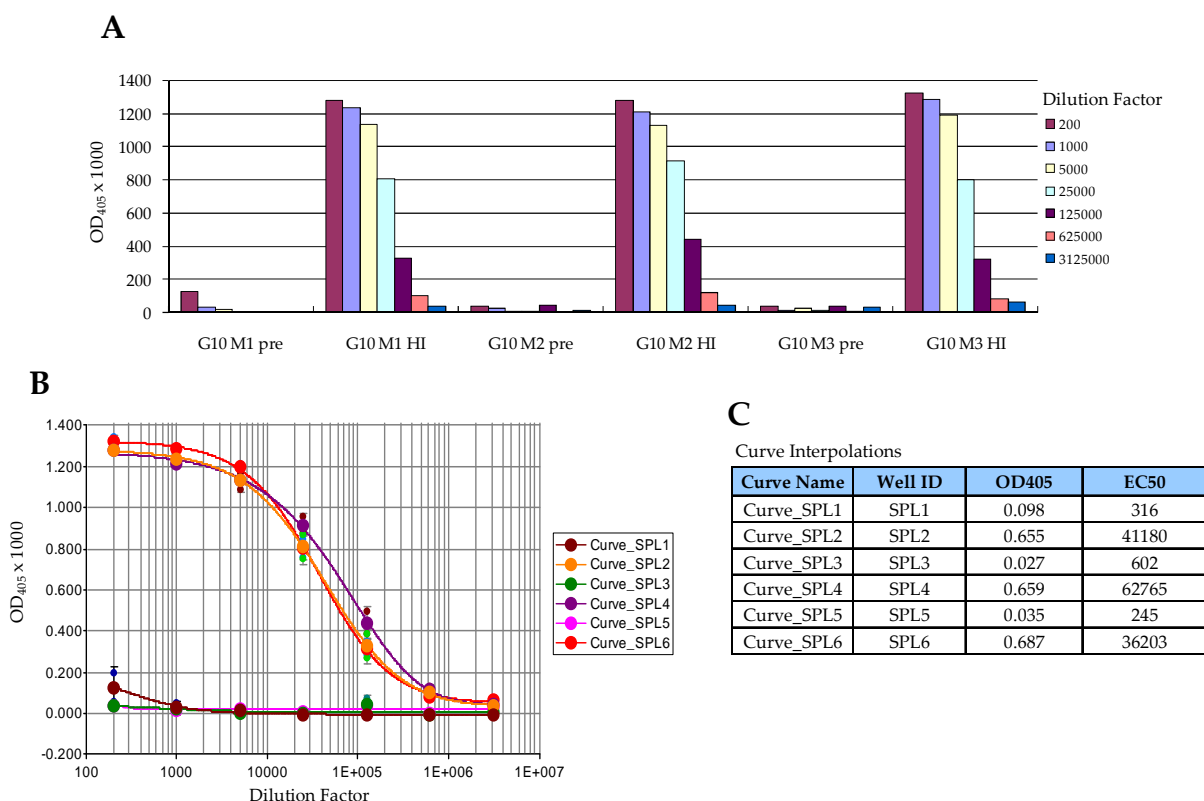


Figure 24: Results of the Titer-Screening of Hyperimmune Sera. The serum screening of three mice immunized with PspA_B_T4 (CGI/LST3742 Group-N° 10) is exemplified by showing the obtained serum-titration graphs for pre- and hyperimmune-sera (A), the calculated log-curves thereof (B) and the results-table, indicating the EC50 values (C).

It was noted that during the immunization-procedure, mice of different groups died or had to be sacrificed after the second immunization on day 14 as some mice showed severe reactions (*e.g.* ventral position, pulmonary ventilation rates of 28 bpm, being catatonic, *etc.*). These observations, which were not consistent in-between both experiments, cannot be explained or traced back on any operational-errors being made.

None of the mice had pre-existing antibodies specific for the immunogens used and all surviving mice developed very high hyperimmune serum titers, hence were successfully immunized with the constructs applied (summarized in Supplementary Figure 4 to Supplementary Figure 6).

3 THE *IN VIVO* VIRULENCE OF PNEUMOCOCCI IN MOUSE VIA DIFFERENT APPLICATION ROUTES

The *in vivo* virulence of *S. pneumoniae* is not only dependent on the strain *i.e.* the genetic background but also on the routes of infection, thus for all strains that were subsequently used for passive immunization studies with polyclonal mouse antibodies, the LD₉₀ (lethal dose 90) was determined in dose titration experiments – all dose-titrations carried out in the course of this project are summarized in Supplementary Table 10.

It was previously determined that the strain PJ1324 (6B) was virulent when used *i.p.* but avirulent in an *i.n.* challenge (data not shown), and the results obtained for the *i.v.* dose-titration experiments indicate that the strain PJ1324 (6B) was not virulent in this setting (Figure 25-A).

The highly virulent character of the *S. pneumoniae* D39 indicated by the high lethality in the *i.v.* and *i.p.* model (Figure 25-C and E) was also observed when using this strain in an *i.p.* lethal sepsis model with just 10 cfu – as discussed for the *in vivo* efficacy of anti-PspA and anti-PLY antibodies in the following chapters. As a lower challenge dose was not feasible this strain could not be used as challenge strain.

The pneumococcal strains TIGR4 and WU2 were virulent in the chosen setting without being too lethal at higher challenge doses (Figure 25-B and D respectively). Hence, these strains were used in *i.v.* models of bacterial challenge (5x10³ cfu/mouse for TIGR4 and 5x10⁵ cfu/mouse for WU2) as representatives for Family 1 and 2 PspA strains in order to determine the *in vivo* efficacy of anti-PspA and anti-PLY polyclonal antibodies as discussed in the following chapters.

D – RESULTS

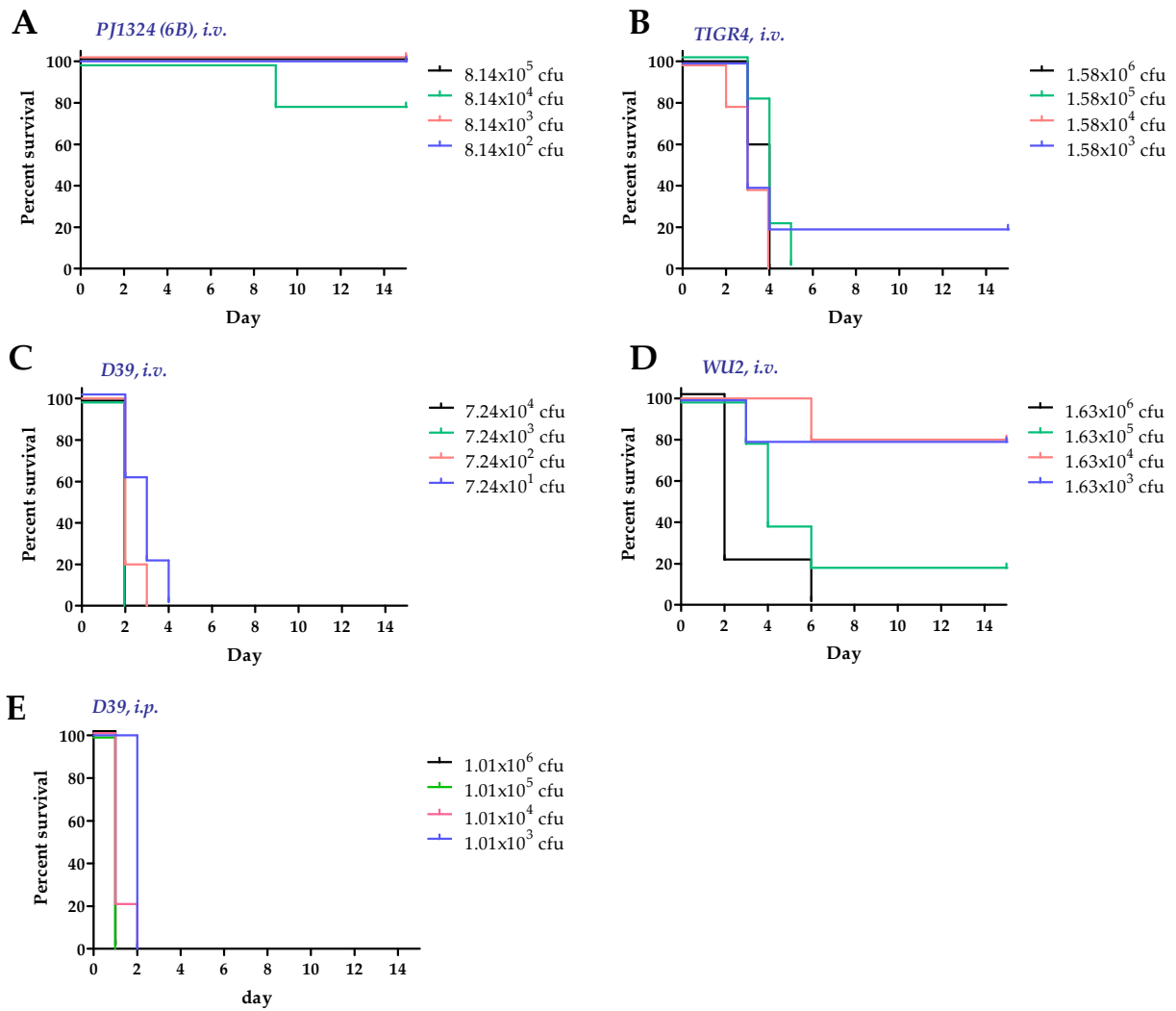


Figure 25: Dose Titration for the i.v. and i.p. Challenge with *S. pneumoniae*. The *in vivo* virulence was tested by infecting 5 mice per group with the strains PJ1324 (A), TIGR4 (B), D39 (C) and WU2 (D) *i.v.* and with the strain D39 (E) *i.p.*. The LD₉₀ dose for each strain was determined by injecting four different challenge doses (cfu).

4 ANTIGEN SPECIFIC ASSAYS AND EXPERIMENTS

4.1 Lipoteichoic acid (LTA)

4.1.1 Optimization of the Purification and Quantification of LTA

Optimization of the HIC-Purification Procedure

In order to determine the optimal elution conditions for the hydrophobic interaction chromatography (HIC) with *Octyl Sepharose™* for the purification of LTA from *S. pneumoniae* [126], commercially available, purified and quantified LTA from *S. pyogenes* (group A streptococcus; GAS) was used in a small scale experiment.

For this purpose triplicates of GAS-LTA (100 µg each) were diluted in three different HIC starting buffers (0 %, 5 % and 15 % n-propanol added) and subjected to HIC-purification with an n-propanol gradient (0-100 %), before all fractions were subsequently screened for their LTA-content *via* a GAS-LTA-specific ELISA.

The ELISA read-outs (absorbance at OD₄₀₅) for all three runs (Figure 26) indicate that all three starting-buffer-compositions are suitable for the purification of GAS-LTA by means of HIC. Furthermore the most quantitative elution was achieved using an n-propanol content of 30-60 % (indicated by the red box in Figure 26).

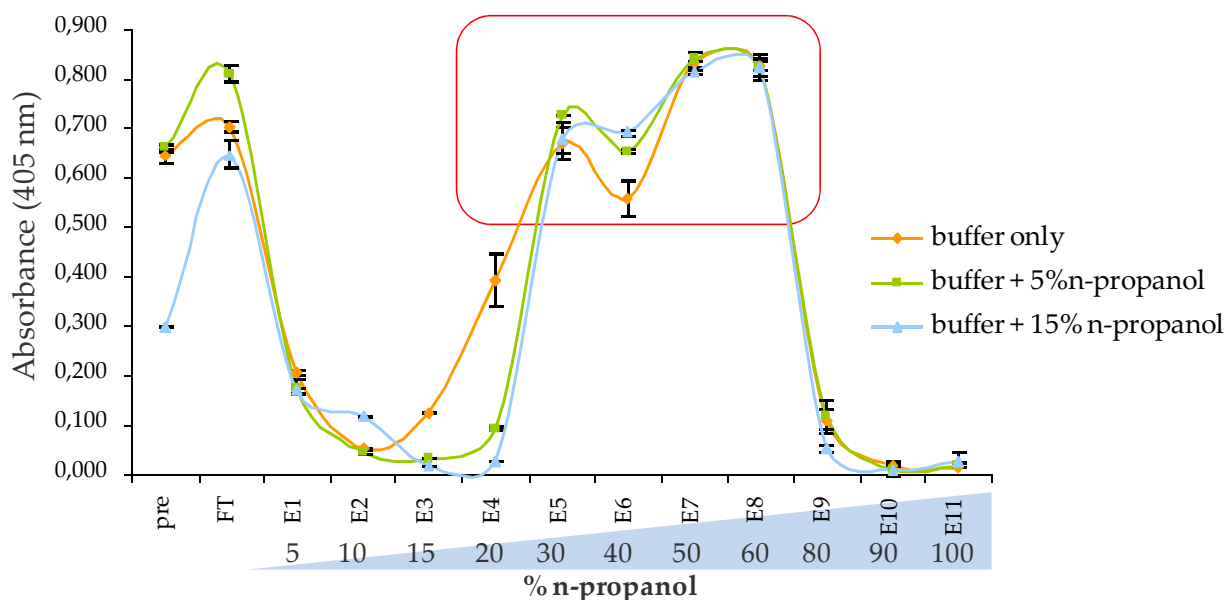


Figure 26: ELISA-Read-Outs of the HIC-Optimization with GAS-LTA. The quantitative elution of GAS-LTA from the *Octyl Sepharose™* resin was optimized using different compositions of starting buffer and an increasing n-propanol gradient. Eluted fractions were analyzed for their LTA-content by an LTA-specific ELISA.

Based on these results, a HIC starting-buffer without n-propanol was selected for the re-hydration of the lyophilized extracts of pneumococcal LTA, and a gently inclining n-propanol-

D – RESULTS

gradient between 30 % and 70 % with higher fraction-numbers and lower fraction-volumes, was used in order to maximize the yield and quality for all HIC-runs.

Optimization of the Quantification Procedure

By applying the established HIC-purification protocol, LTA from the *S. pneumoniae* strains PJ1324 (6B) and TIGR4 was aimed to be purified in the course of eight different purification-approaches.

All fractions obtained from four different runs – exemplified by the chromatogram of run-number 1 in Figure 27 – were subsequently screened for their phosphate content by means of the *phosphomolybdenum-blue* reaction described in the literature. [126]

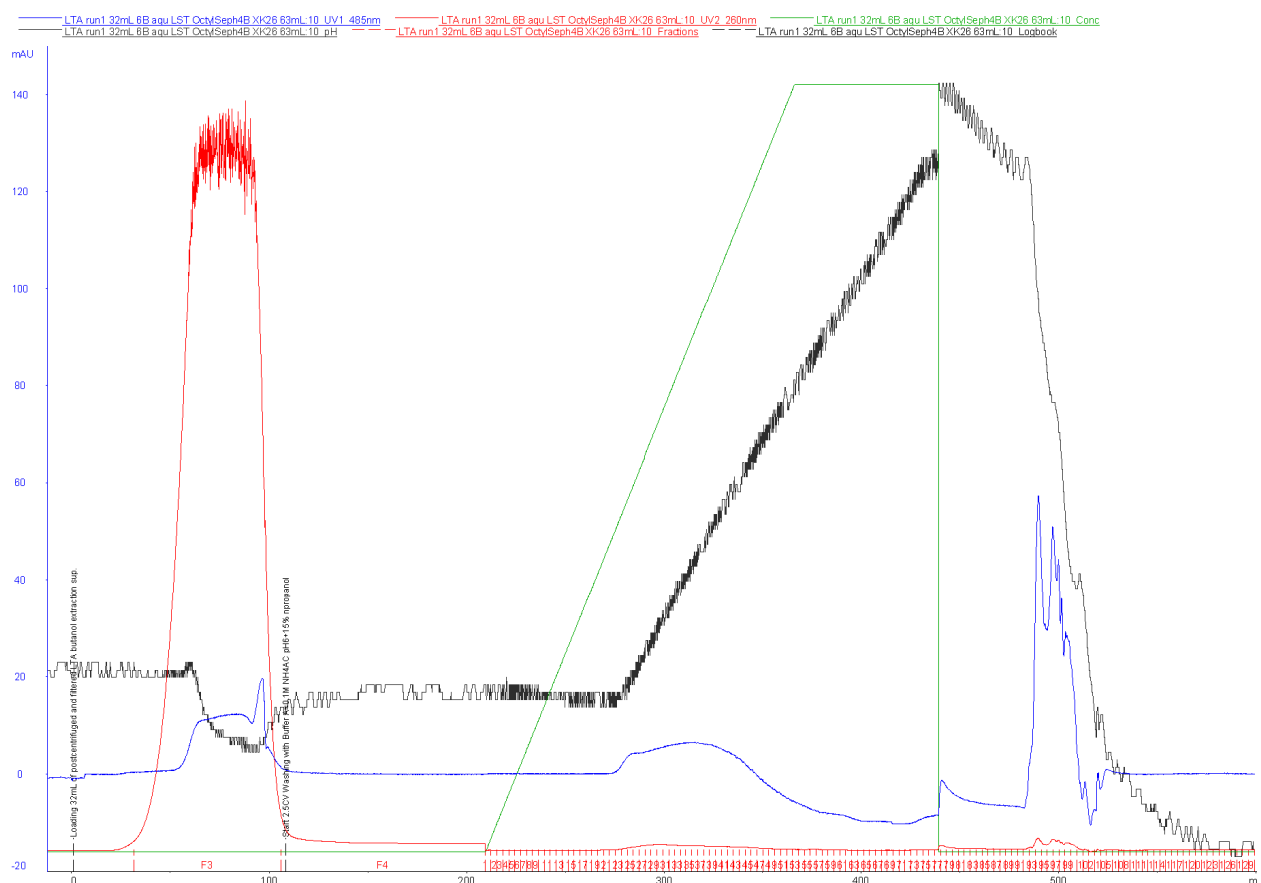


Figure 27: Chromatogram of a HIC-Run. The HIC-based purification of LTA was monitored by recording the absorbance at 260 nm (dark red) and 485 nm (blue), the conductivity (brown), the solvent-gradient-concentration (green) and the fraction number (red). This is exemplified by the chromatogram recorded for the purification of LTA from 500 ml of an over-night culture of the *S. pneumoniae* strain PJ1324 (6B).

In order to quantify the purified pneumococcal LTA, a standard calibration using GAS-LTA (ranging from 1×10^0 mg/ml to 5.12×10^{-7} mg/ml LTA; exemplified in Figure 28) was established and included in each phosphomolybdenum-blue screening of the HIC fractions.

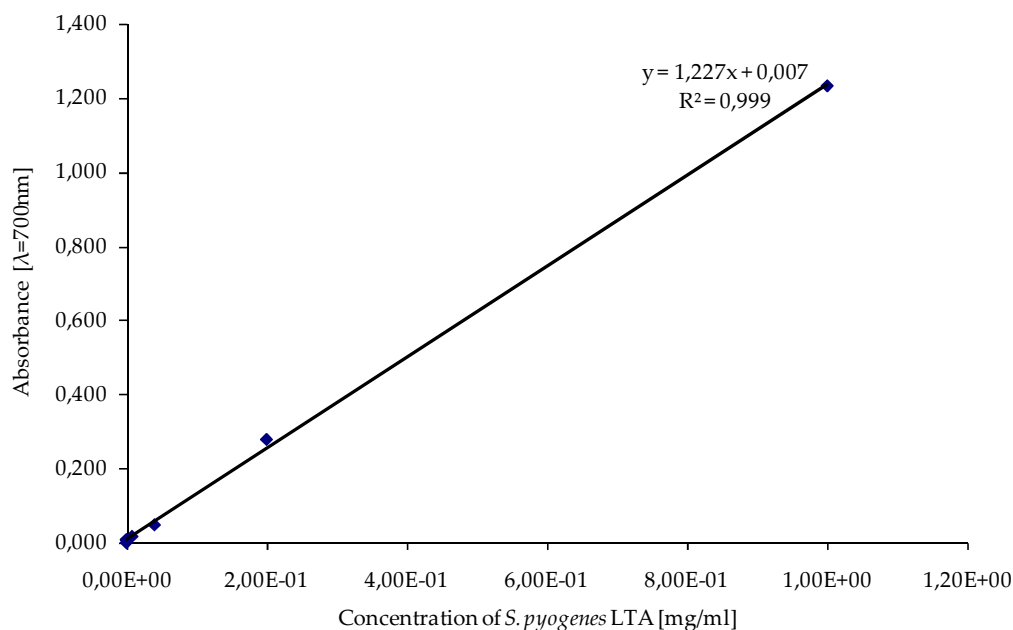


Figure 28: Standard Calibration for the Phosphomolybdenum-Blue Reaction. The standard calibration which was included in all detection approaches of pneumococcal LTA post HIC was set-up and used in a broad concentration-range between 1×10^0 mg/ml to 5.12×10^{-7} mg/ml of GAS-LTA.

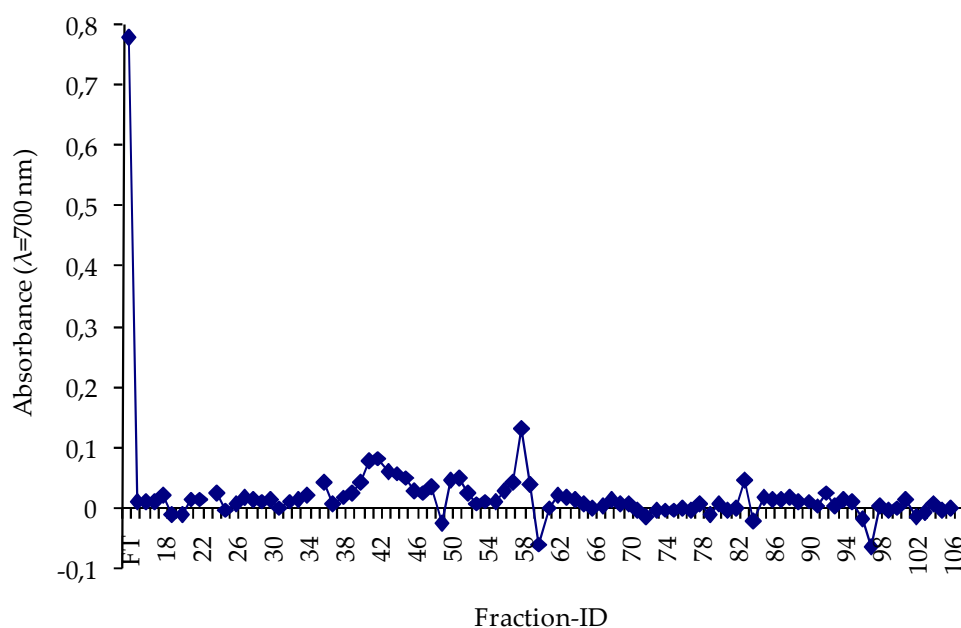


Figure 29: Result for the Phosphomolybdenum-Blue Reaction of Pneumococcal LTA. The phosphate-screening is exemplified for all HIC-fractions obtained from 500 ml of an o/n culture of the *S. pneumoniae* strain PJ1324 (6B).

The fractions of all HIC-runs that were subjected to the phosphomolybdenum-blue reaction, showed the same picture as exemplified for run-number 1 in Figure 29. The high background and the low detection-efficiency were found to be independent of the culture volume – 500 ml to 10 L of o/n cultures were tested – and the *S. pneumoniae* strain used.

D – RESULTS

Although the phosphomolybdenum-blue based quantification method was described as relatively sensitive standard screening procedure during LTA isolation in the literature [126], the detection limit was above the concentration of pneumococcal LTA in the HIC fractions. Based on the calibration with GAS-LTA, a concentration below 100 µg/ml could not be detected with sufficient reliability thus it was concluded that this method was not suitable for the detection of pneumococcal LTA.

In order to establish an alternative suitable LTA-detection method, an *ELISA-based quantification* approach using GAS-LTA-specific primary antibodies was set-up. For this purpose several LTA-specific antibodies were cross-titrated with different concentrations of GAS-LTA to determine their optimal working concentration (data not shown). The antibody with the highest sensitivity (Gram Positive Bacteria Antibody, Mouse Monoclonal IgG1 Antibody, GenWay; working dilution of 1:200) was henceforth used for all ELISA-based LTA-screenings. The limit of detection for GAS-LTA was found to be 0.1 ng/ml at a 1:200 dilution of the primary antibody (data not shown).

This ELISA-based detection – optimized and functional for GAS-LTA – was also shown to allow the detection of pneumococcal LTA in several purification approaches. An example for the purification of TIGR4-LTA is given in Figure 30.

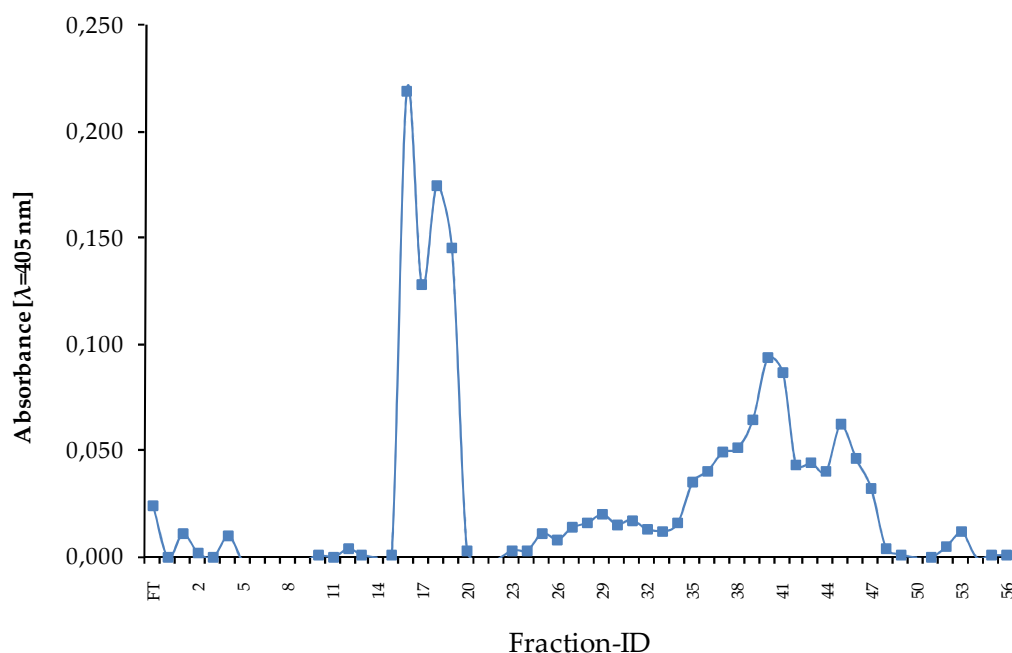


Figure 30: ELISA-Based Screening of HIC-Fractions of Pneumococcal LTA. The ELISA-screening is exemplified for all HIC-fractions obtained from 10 L of an o/n culture of the *S. pneumoniae* strain TIGR4.

Finally, in order to optimize the qualification and quantification of isolated and purified pneumococcal LTA a *PAGE-based separation* with a subsequent, *combined Alcian-Blue and silver-staining* was investigated.

For the initial set-up of this method, Tris-Borate-EDTA gels (30-40 %) were loaded and run with different concentrations of LTA from *S. pyogenes* before staining. As these precast gels were extremely fragile, the quality of the gels and therefore the results obtained therewith were not satisfactory (data not shown).

Thus subsequently LTA from *S. pyogenes* was separated on Tris-Borate-EDTA gradient-gels (4-20 %) loaded with two different loading dyes (native loading dye and OrangeG+sucrose), (Figure 31-A) and silver-stained without previous Alcian-Blue staining (Figure 31-B).

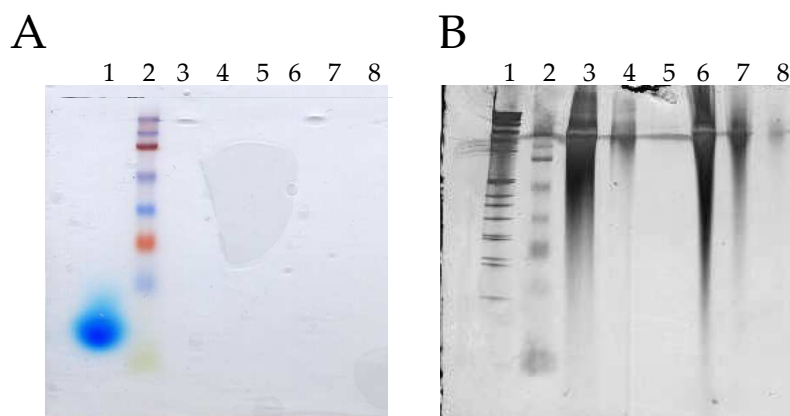


Figure 31: PAGE-Separation (A) and Silver Staining Approach (B) for GAS-LTA. The PAGE-based purification of LTA from *S. pyogenes* was carried out using a 4-20 % TBE-Gel without Alcian-Blue staining prior to silver-staining. Next to the GeneRuler 1kb DNA-Ladder (lane 1) and PageRuler Plus Prestained Protein Ladder (lane 2), GAS-LTA was loaded in three concentrations (10 µg, 1 µg and 0.1 µg) in native loading dye (lanes 3-5) and OrangeG+sucrose (lanes 6-8).

The stained gel (Figure 31-B) showed that both loading dyes were suitable for loading of the gel but that the use of the native loading dye (lanes 3-5) led to a more band-like separation as described in the literature ^[389, 394-395] - compared to the OrangeG+sucrose which led to cone-like lanes (6-8). Further can be observed that the detection-limit for GAS-LTA was reached when loading and separating 1 µg of this antigen, which is approximately 10,000-fold lower than achieved with the GAS-LTA specific ELISA described before.

As a smear of the native GAS-LTA was visible after silver staining of the polyacrylamide gel (Figure 31-B), GAS-LTA (100 µg) was subjected to a literature-based deacylation procedure according to the published protocol ^[391]. This procedure should result in a banding pattern after

D – RESULTS

separating and visualizing different fractions of the chemically treated LTA *via* PAGE and silver-staining. When evaluating the PAGE-gel post staining (data not shown), it was noted that no traces of any chemically decayed LTA were evident, although the markers and the native GAS-LTA-control showed the same profiles as in the initial setup (Figure 31). Therefore it was concluded that the apparent loss of LTA during the chemical treatments, possibly yields amounts of decayed LTA being lower than the detection limit of the PAGE and silver-staining. Alternatively the chemical treatment might have employed too harsh conditions that led to a total decomposition of the native LTA.

Taken together the results obtained with the PAGE-based visualization of quite high amounts of GAS-LTA, were not promising with regards to further application of this procedure for the qualification and quantification of pneumococcal LTA. Thus, this approach was not followed up further in the course of this project.

4.2 *Pneumococcal surface protein A (PspA, SP0117)*

4.2.1 *In vitro Assays using Polyclonal Antibodies Generated vs. PspA Constructs*

Peptide ELISA

In order to determine if hyperimmune-sera generated against different recombinant PspA-constructs based on the *S. pneumoniae* strains WU2 and TIGR4 recognize linear epitopes of PspA, an ELISA using 25 aa long peptides covering the expressed PspA_FL variant from TIGR4 with a 5 aa overlap was carried out. As controls, the recombinant proteins PspA_FL_T4 and PspA_Pro+PartB_WU2, purified polyclonal mouse IgG generated against PspA_FL_T4 and a mouse mAb – 3G3E8E3 recognizing the PspA peptide IEKLQYEISTLEQEVATAQHQVDNLKLLA (peptide N° 8) – were included.

All hyperimmune-sera recognized the two recombinant protein-controls used (data not shown). The mAb- and IgG-controls bound to the peptides as expected, showing specific recognition of peptide N° 8 by mAb 3G3E8E3 and a broad recognition of multiple peptides, with strongest binding in the proline-rich region by the polyclonal IgGs.

The data (Figure 32) indicate that hyperimmune sera against PspA_FL_T4, PspA_B_T4 and PspA_Pro+PartB_T4 contain antibodies recognizing the majority of the peptides distributed over the whole sequence. These sera also recognized linear epitopes in the coiled-coil region. In general, hyperimmune sera against PspA_B_WU2 and PspA_Pro_WU2 showed only low reactivity with the peptides tested, likely based on the fact that the peptides were not generated

against the PspA-sequence of WU2 (PspA Family 1, Clade 2) but for TIGR4 (PspA Family 2, Clade 3). All other sera tested, showed a medium to high reactivity within different regions of PspA independent of the constructs sequence.

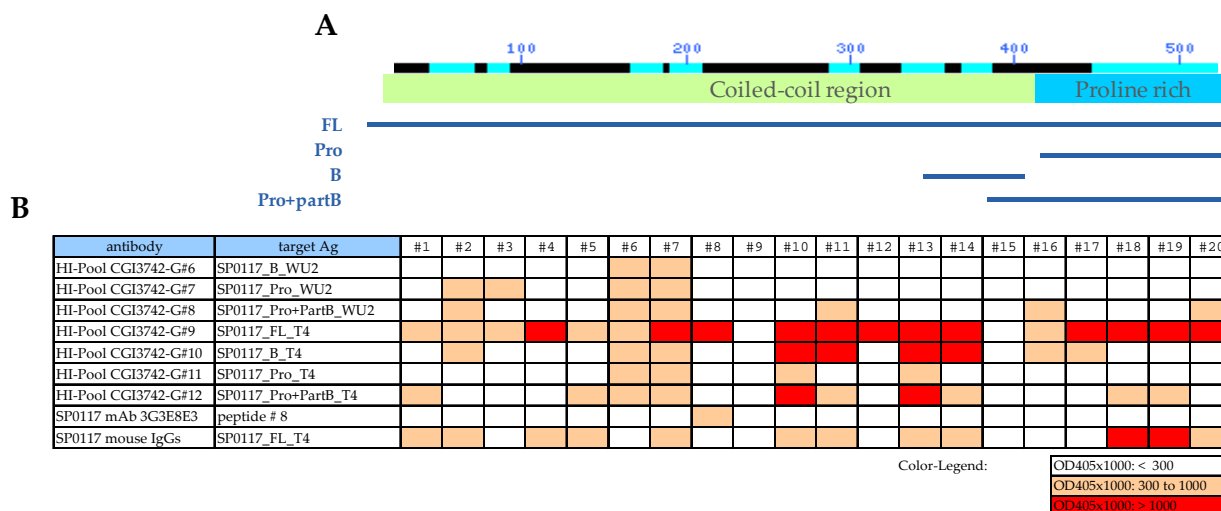


Figure 32: Results for the Peptide ELISA. The overlapping 25 aa long peptides were generated based on the amino-acid sequence of PspA from *S. pneumoniae* TIGR4. (A) Structural features of PspA and the antigen-constructs used for the generation of the hyperimmune-sera are represented. (B) The peptide-recognition by the HI-sera and the control antibodies used is depicted with the most strongly recognized regions highlighted in red.

Surface-Staining of Live Pneumococci

Hyperimmune-sera generated in experiment CGI/LST3742 were subsequently checked for their capability of recognizing PspA on the surface of different live *S. pneumoniae* strains by means of flow cytometry.

The results (Table 5) suggest a clade-specific recognition of PspA on the pneumococcal surface, reflected by the strong surface staining signal of the TIGR4wt strain with hyperimmune-sera generated against the TIGR4 (PspA Family 2, Clade 3) constructs and D39wt with those against the WU2 (PspA Family 1, Clade 2) constructs respectively.

The specific recognition of PspA on TIGR4wt was confirmed by the absence of staining on a TIGR4ΔPspA strain (lacking PspA expression) using the hyperimmune-sera generated against the recombinant TIGR4-constructs. In addition to that staining by anti-WU2-PspA antibodies was reduced on a TIGR4ΔPspC strain – lacking PspC but still presenting PspA on its surface.

D – RESULTS

Table 5: Results for the Surface Staining of Live *S. pneumoniae* using HI-Sera (CGI/LST3742). The recognition of different pneumococcal wild-type and mutant strains by hyperimmune-sera was compared to the corresponding pre-immune sera. Results are represented using a color-coding for positive recognition of not less than 49.5 % (+++), not less than 29.5 % (++), not less than 14.5 % (+) and less than 14.5 % (-).

		TIGR4 WT		TIGR4 ΔPspA		TIGR4 ΔPspC	
		Serotype	PspA-clade	Serotype	PspA-clade	Serotype	PspA-clade
		4	3	4	-	4	3
HI-Serum ID	Target Antigen	Surface Recognition		Surface Recognition		Surface Recognition	
CGI/LST3742-N° 6	PspA_B_WU2	-		-		+	
CGI/LST3742-N° 7	PspA_Pro_WU2	+		+		-	
CGI/LST3742-N° 8	PspA_Pro+PartB_WU2	++		++		+	
CGI/LST3742-N° 9	PspA_FL_T4	+++		-		+++	
CGI/LST3742-N° 10	PspA_B_T4	+++		-		+++	
CGI/LST3742-N° 11	PspA_Pro_T4	++		-		++	
CGI/LST3742-N° 12	PspA_Pro+PartB_T4	+++		-		+++	

		EF3030 WT		EF3030 ΔPspA		D39 WT	
		Serotype	PspA-clade	Serotype	PspA-clade	Serotype	PspA-clade
		19F	1	19F	-	2	2
Serum ID	Target Antigen	Surface Recognition		Surface Recognition		Surface Recognition	
CGI/LST3742-N° 6	PspA_B_WU2	-		+		+++	
CGI/LST3742-N° 7	PspA_Pro_WU2	-		+		++	
CGI/LST3742-N° 8	PspA_Pro+PartB_WU2	+		+		+++	
CGI/LST3742-N° 9	PspA_FL_T4	+		+		+	
CGI/LST3742-N° 10	PspA_B_T4	-		+		-	
CGI/LST3742-N° 11	PspA_Pro_T4	-		+		-	
CGI/LST3742-N° 12	PspA_Pro+PartB_T4	-		+		-	

4.2.2 *In vivo* Efficacy Testing of Polyclonal Antibodies Generated vs. PspA Constructs

In order to assess the protective efficacy of hyperimmune-sera, generated against different PspA-constructs in the experiments CGI/LST3742 and LST3885, these were examined in passive immunizations studies in mice. 5 mice per group received polyclonal serum 24 h prior to a lethal bacterial challenge. Different *S. pneumoniae* strains – representing different serotypes and PspA clades – and challenge-routes were tested in the course of this study.

The survival of the animals was monitored for 15 days post infection and data thus obtained were statistically evaluated using a Log-rank (Mantel-Cox) Test (data represented in Supplementary Table 12). Mice mock immunized with mouse pre-immune serum and PBS served as negative and PspA_FL polyclonal rabbit serum as positive control.

In two experiments with *i.p.* challenge with strain PJ1324 (serotype 6B; PspA Family 2, Clade 3) (Figure 33) antisera generated against recombinant PspA_FL_T4, PspA_B_T4, PspA_B+Pro_T4 derived from the *S. pneumoniae* strain TIGR4 (serotype 4, PspA Family 2, Clade 3) showed a clade specific protection. Antibodies against the proline-rich region alone

(PspA_Pro_T4) were not fully protective and no cross-clade protection was obtained when using hyperimmune sera generated against the WU2-constructs (serotype 3; PspA Family 1, Clade 2). Although anti-PspA_Pro_T4 antibodies were not shown to be fully protective, statistical comparison with the mock-immunized PBS-control group indicated that all TIGR4-constructs significantly prolonged the survival of the mice ($p < 0.01$).

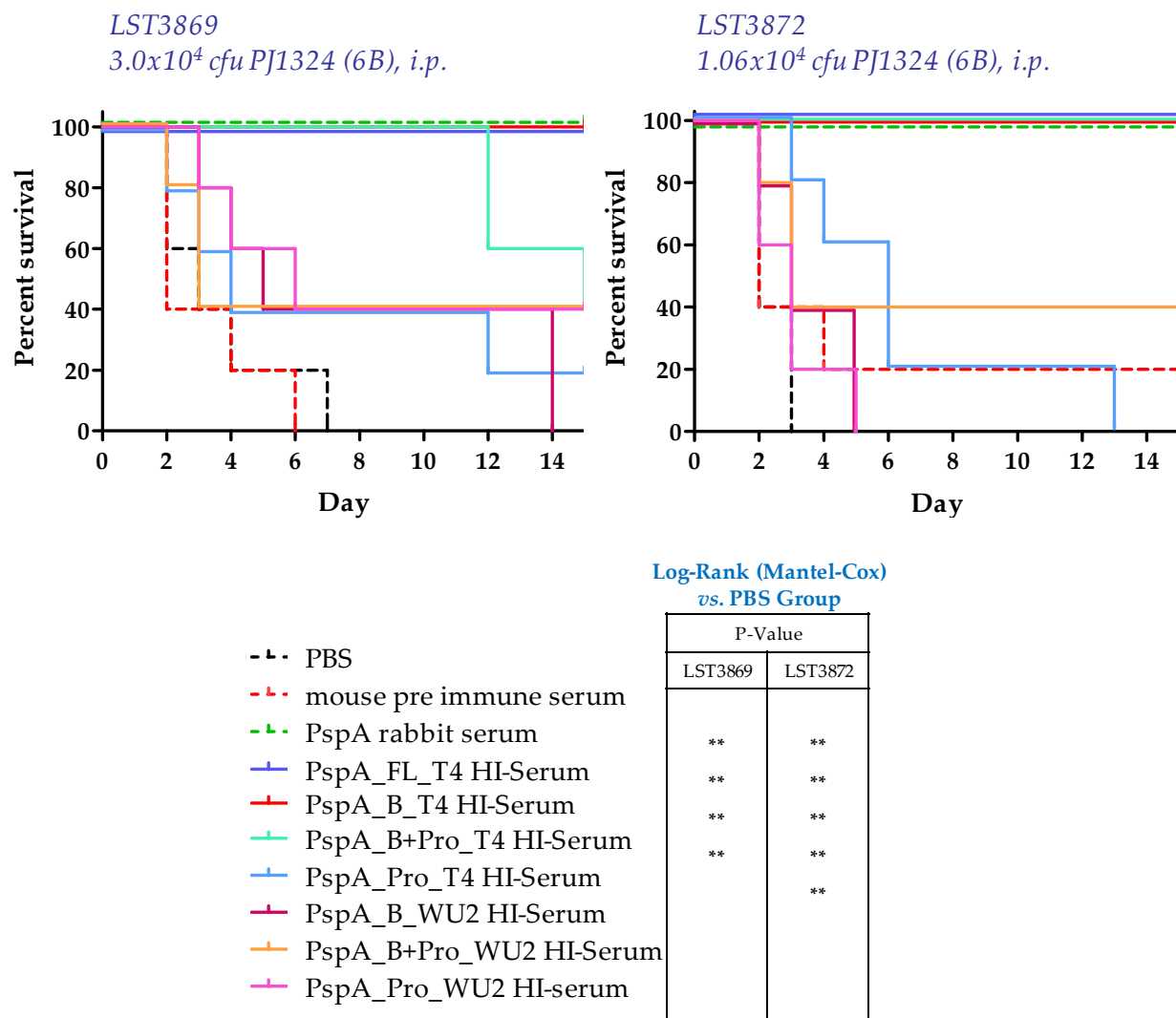


Figure 33: In vivo Efficacy of Polyclonal Anti-PspA Antibodies Against i.p. Challenge with the *S. pneumoniae* Strain PJ1324 (6B). The survival of individual mice was monitored for 15 days post challenge. All survival-curves were statistically compared to the mock-immunized PBS-control group, using the Log-rank (Mantel-Cox) test where $p < 0.01$ is indicated with **.

The *in vivo* efficacy of the hyperimmune sera was re-examined in an *i.p.* challenge model using the *S. pneumoniae* strain D39 (serotype 2, PspA Family 1, Clade 2) and anti-PspA_FL_T4 serum – shown to be fully protective in all previous experiments – as the positive control. A clade-specific protection of 40 % with anti-PspA_B+Pro_WU2 serum was observed (Figure 34).

D – RESULTS

Further, some extent of cross-clade protection (20 %) was achieved with the hyperimmune-sera generated against PspA_FL_T4, PspA_B+Pro_T4 and PspA_Pro_T4. The positive control, anti-PspA_FL_T4 serum, was not shown to be fully protective in the given setting and the statistical evaluation of the data-sets indicates no significant difference in the survival curve of any of the groups compared to the mock-immunized PBS-group.

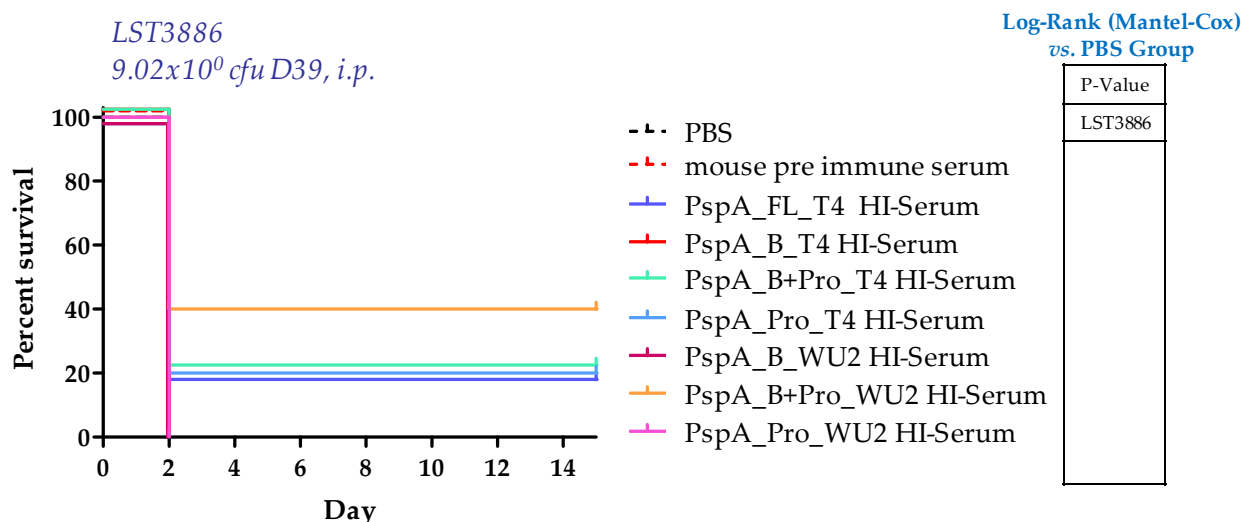


Figure 34: *In vivo* Efficacy of Polyclonal Anti-PspA Antibodies Against i.p. Challenge with the *S. pneumoniae* Strain D39. The survival of individual mice was monitored for 15 days post challenge. All survival-curves were statistically compared to the mock-immunized PBS-control group, using the Log-rank (Mantel-Cox) test.

The hyperimmune sera were subsequently tested in a more natural setting, hence their protective potential was examined in a lethal model of pneumonia using an *i.n.* infection with the pneumococcal strain A66.1 (serotype 3; PspA Family 2, Clade 3) which was shown to specifically induce pneumonia and no other related disease [396].

In the experiment LST3897 (Figure 35, left graph) a cross-clade protection with anti-PspA_Pro_T4 serum (20 %), but no protection against lethal challenge with any of the other hyperimmune sera used was observed. Both, the positive control (PspA rabbit serum) as well as the anti-PspA_Pro_T4 serum significantly prolonged the challenge-survival ($p < 0.01$), compared to the mock-immunized group.

As the amounts of remaining hyperimmune sera from the experiment CGI/LST3742 were not sufficient, the experiment LST3897 was repeated in the study LST3909 with another batch of hyperimmune sera generated with exactly the same antigen-preparations (LST3885).

In this experiment LST3909 (Figure 35, right graph) a clade-specific protection using anti-PspA_B_WU2 serum (60 %) and some extent of cross-clade protection with hyperimmune sera

raised against PspA_B+Pro_T4 (40 %), PspA_B_T4 (20 %) and PspA_Pro_T4 (20 %) was observed. Statistical analysis further indicated significant prolonged survival of the positive control PspA_FL polyclonal rabbit serum ($p < 0.01$), anti-PspA_B_WU2 serum ($p < 0.01$) and anti-PspA_Pro_WU2 serum ($p < 0.05$), but no significant protection with any antibodies against the TIGR4-constructs.

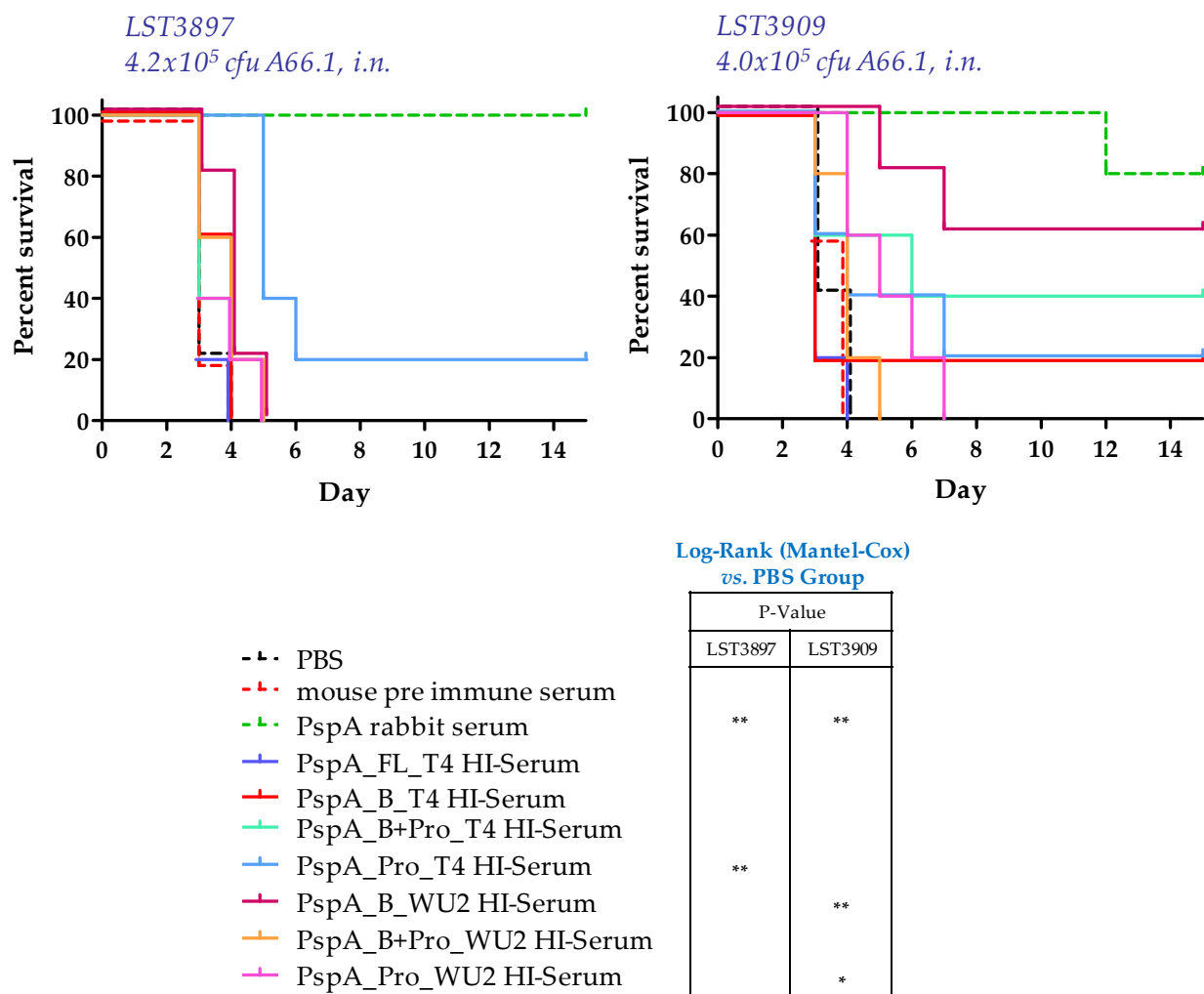


Figure 35: In vivo Efficacy of Polyclonal Anti-PspA Antibodies Against i.n. Challenge with the *S. pneumoniae* Strain A66.1. The survival of individual mice was monitored for 15 days post challenge. All survival-curves were statistically compared to the mock-immunized PBS-control group, using the Log-rank (Mantel-Cox) test where $p < 0.01$ (**) and $p < 0.05$ (*) are indicated.

Finally, in a different experimental setting, mice were infected *i.v.* in a lethal sepsis model with the strains TIGR4 (serotype 4, PspA Family 2, Clade 3) and WU2 (serotype 3, PspA Family 1, Clade 2) using the optimized (LST3898) challenge-doses of 5×10^3 cfu/mouse and 5×10^5 cfu/mouse respectively.

D – RESULTS

The hyperimmune sera generated in LST3885 were not protective and did not induce a statistically significant prolonged survival against *i.v.* challenge with the strain TIGR4 (Figure 36, left graph). Nonetheless after *i.v.* challenge with the strain WU2 (Figure 36, right graph) anti-PspA_Pro_WU2 and anti-PspA_B+Pro_WU2 hyperimmune sera showed full protection with a p-value below 0.05 but antibodies against the B-region alone showed no effect.

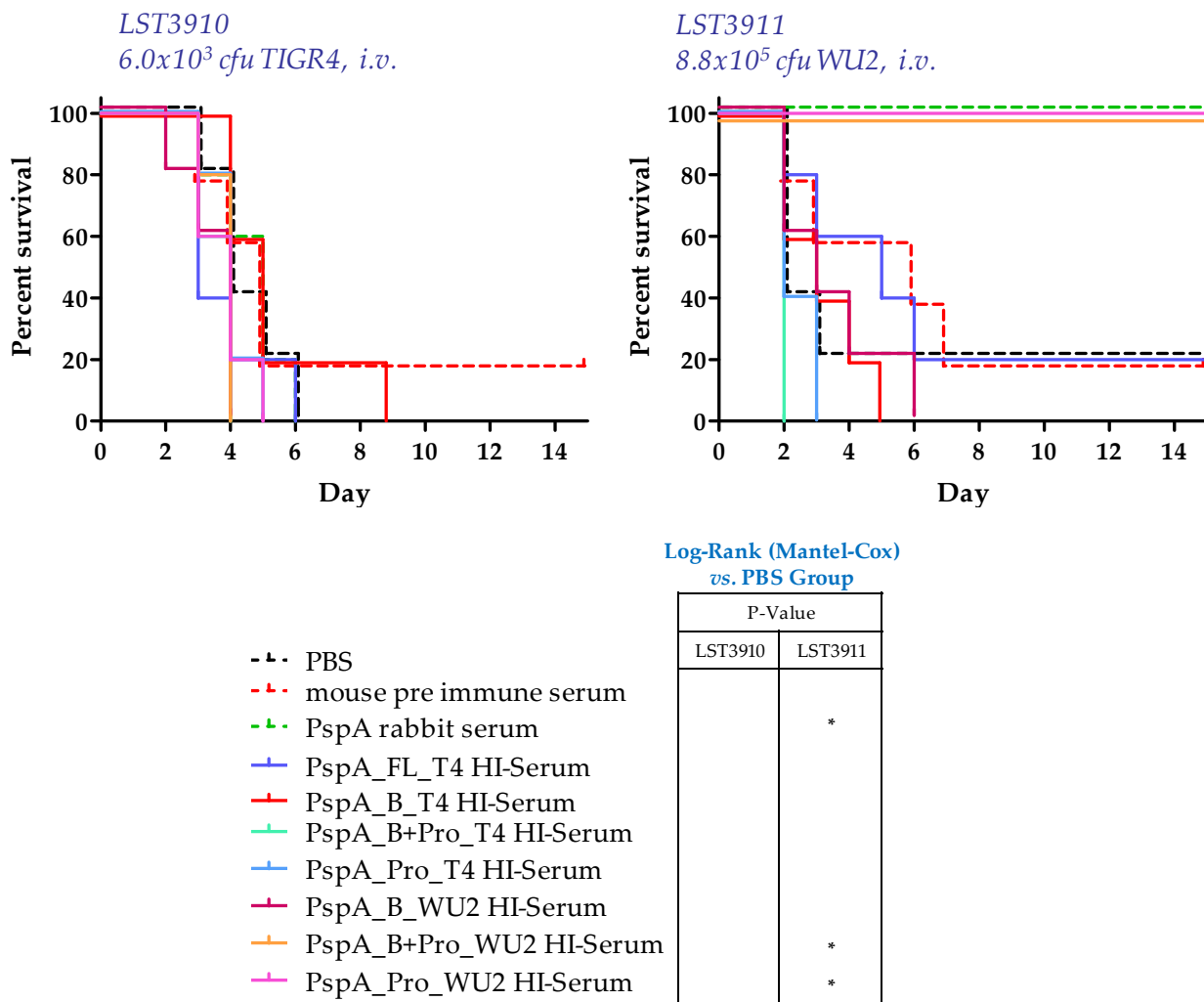


Figure 36: In vivo Efficacy of Polyclonal Anti-PspA Antibodies Against *i.v.* Challenge with the *S. pneumoniae* Strains TIGR4 and WU2. The survival of individual mice was monitored for 15 days post challenge. All survival-curves were statistically compared to the mock-immunized PBS-control group, using the Log-rank (Mantel-Cox) test where $p < 0.05$ is indicated with *.

4.3 Pneumolysin (PLY, SP1923)

4.3.1 In vivo Efficacy Testing of Polyclonal Antibodies Generated vs. PLY Constructs

In order to determine the protective efficacy of hyperimmune-sera generated against PLY, PLD and PLY Δ D4, these were tested in passive immunizations of C3H/HeN mice in the experiments CGI/LST3742 and LST3885. 5 mice per group received 10 μ l of the PLY specific

serum each 24 h prior to lethal bacterial challenge with different *S. pneumoniae* strains and challenge-routes.

In repeated *i.p.* challenge experiments anti-PLY and anti-PLD sera generated in CGI/LST3742 showed some protection against the pneumococcal strain PJ1324 (serotype 6B) (Figure 37). The protective potential of the polyclonal antibodies generated against PLY was elicited by challenge survivals of 0 % (CGI/LST3742-N°2), 20 % (CGI/LST3742-N°3 and CGI/LST3742-N°4) and 40 % (CGI/LST3742-N°1). As the observed protection differed between the groups, the IL-6 response of mice, immunized with these hyperimmune-sera, was determined 24 h post challenge (LST3872; data presented and discussed in 4.3.2).

A statistically significant prolonged survival ($p < 0.05$) was observed for two of the anti-PLY groups, namely CGI/LST3742-N°2 and CGI/LST3742-N°3. 40 % of the mice that received polyclonal antibodies against PLD survived, resulting in a significantly prolonged survival ($p < 0.05$) compared to the mock-immunized PBS-control group.

The commercially available mAb 1F11 also protected 40 % of the mice, giving rise to a statistically significant different survival curve ($p < 0.05$) compared to the mock-immunized control.

PspA_FL polyclonal rabbit serum, which was used as positive control in both experiments, was repeatedly shown to be fully protective and to significantly extend the challenge-survival ($p < 0.01$) compared to the PBS-control group.

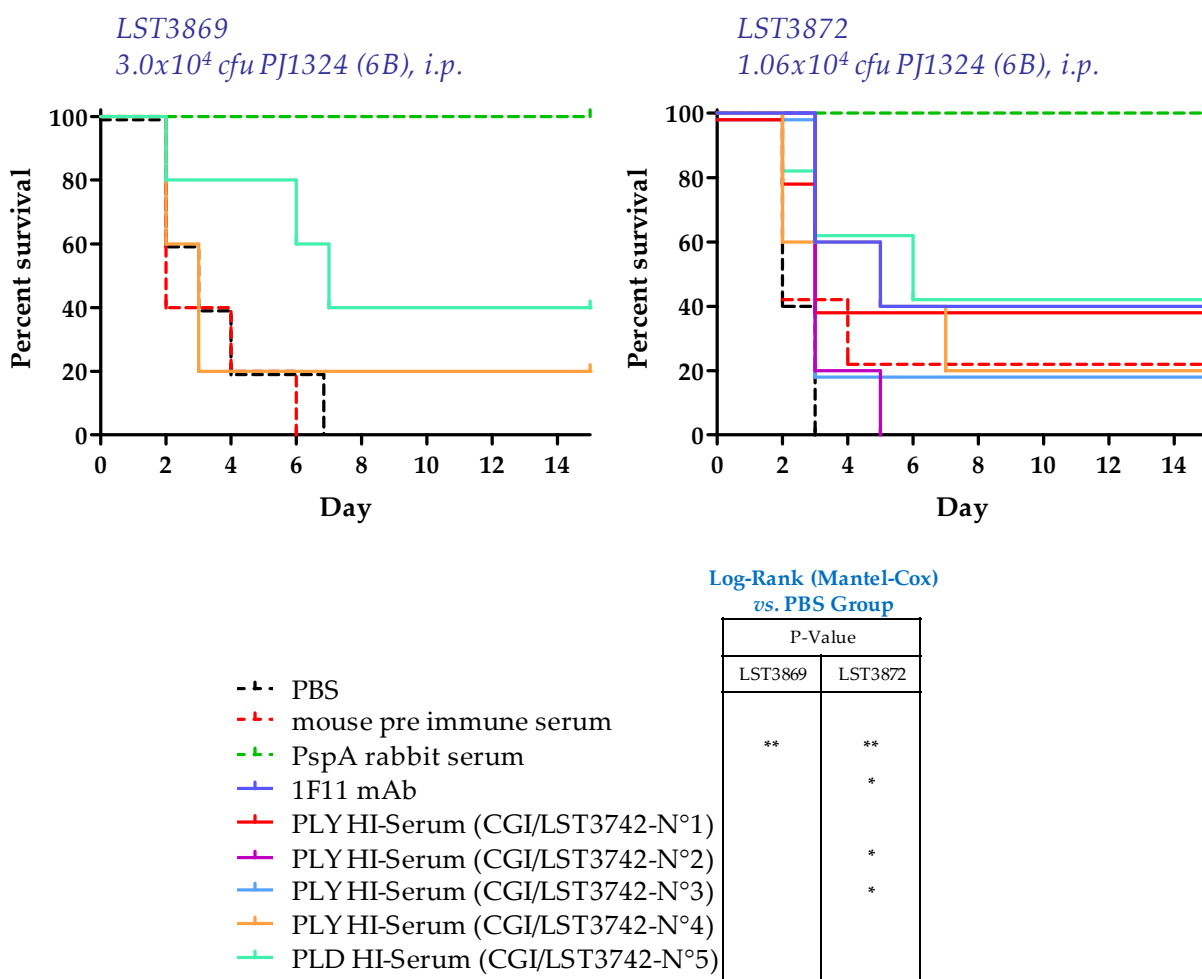


Figure 37: In vivo Efficacy of Polyclonal Anti-PLY Antibodies Against i.p. Challenge with the *S. pneumoniae* Strain PJ1324 (6B). The survival of individual mice was monitored for 15 days post challenge. All survival-curves were statistically compared to the mock-immunized PBS-control group, using the Log-rank (Mantel-Cox) test where $p < 0.01$ (**) and $p < 0.05$ (*) are indicated.

Data generated for subsequent testing of the hyperimmune-sera in an *i.p.* challenge with the pneumococcal strain D39 also showed high virulence of the strain (previously discussed in 4.2.2). Limited protection was only observed in one of the groups immunized with anti-PLY serum (CGI/LST3742-N°4; 20 % survival) and none of the sera was found to induce a statistically significant prolongation of the challenge-survival, compared to the mock-immunized PBS-control group (Figure 38).

The positive control – anti-PspA_FL_T4 mouse serum – was not protective in the given setting and the statistical evaluation of the data-sets indicates no significant difference in survival compared to the control-group.

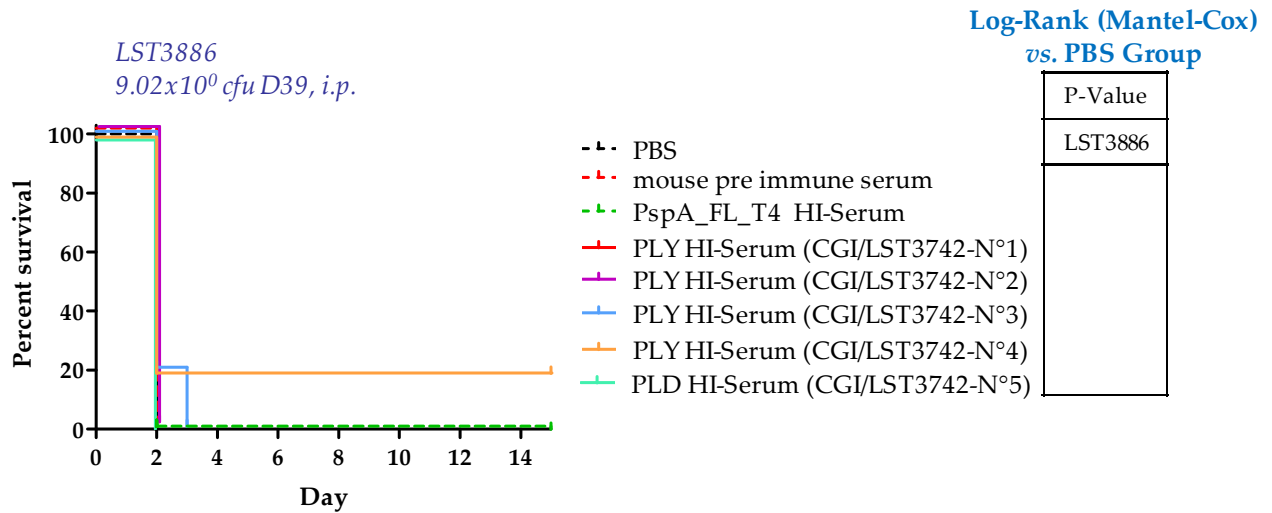


Figure 38: In vivo Efficacy of Polyclonal Anti-PLY Antibodies Against i.p. Challenge with the *S. pneumoniae* Strain D39. The survival of individual mice was monitored for 15 days post challenge. All survival-curves were statistically compared to the mock-immunized PBS-control group, using the Log-rank (Mantel-Cox) test.

In subsequent experiments, evaluating the protective potential of the polyclonal antibodies in a model of lethal pneumonia post *i.n.* infection with the pneumococcal strain A66.1 (serotype 3) no protection upon immunization with anti-PLY serum was observed (Figure 39). Neither antibodies generated against PLD, nor those generated against PLYΔD4 were protective. Statistical analysis did not reveal any extended survival through administration of polyclonal antibodies compared to the mock-immunized control group. The positive control, PspA_FL polyclonal rabbit serum, was found to be protective and significantly prolonged survival (p<0.01).

D – RESULTS

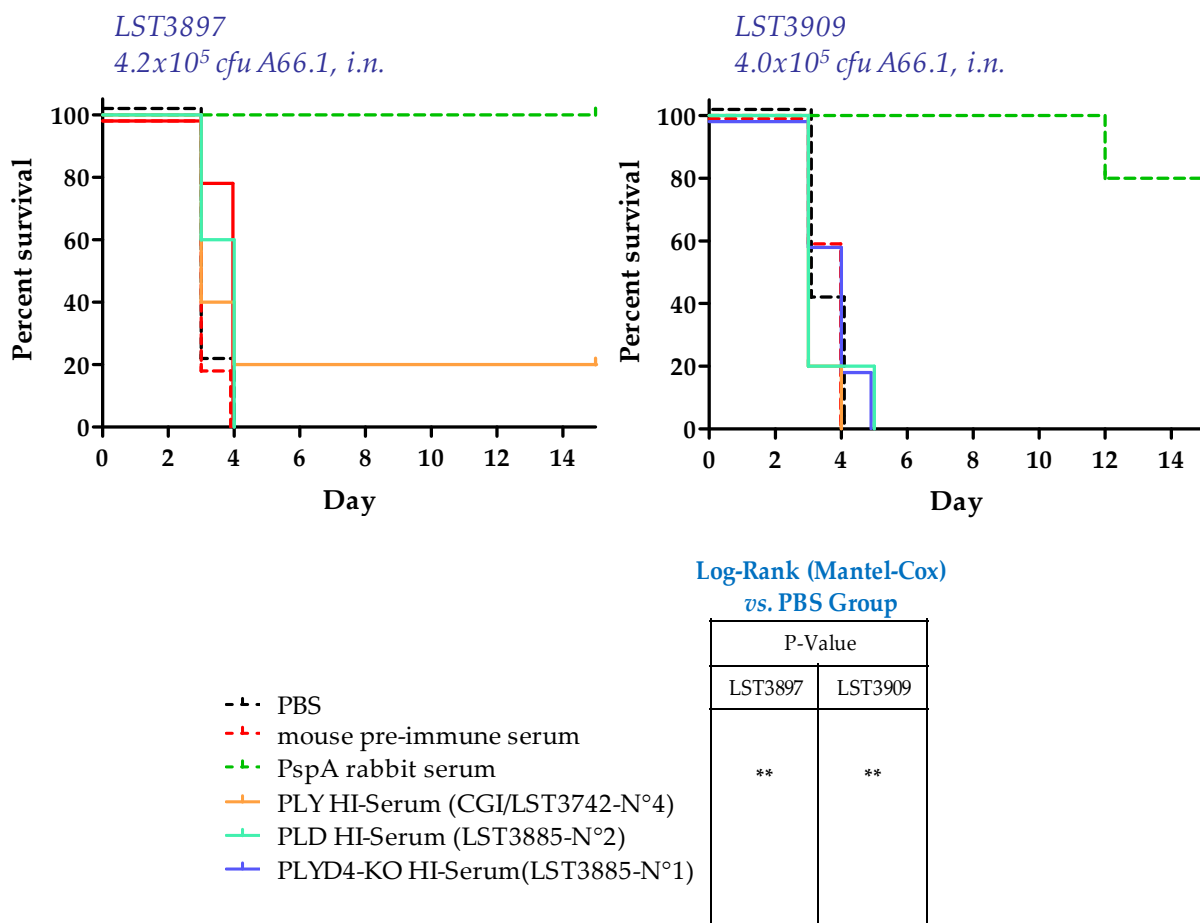


Figure 39: In vivo Efficacy of Polyclonal Anti-PLY Antibodies Against *i.n.* Challenge with the *S. pneumoniae* Strain A66.1. The survival of individual mice was monitored for 15 days post challenge. All survival-curves were statistically compared to the mock-immunized PBS-control group, using the Log-rank (Mantel-Cox) test where $p < 0.01$ is indicated with *.

Finally, protection was studied in an *i.v.* lethal sepsis model using the pneumococcal strains TIGR4 (serotype 4) and WU2 (serotype 3). Enhanced protection was just observed against WU2-challenge (Figure 40, right graph) using anti-PLD (40 % survival) and anti-PLY Δ D4 (60 % survival) sera generated in LST3885. As both negative control groups (PBS and mouse pre-immune serum) showed 20 % survival, the protection obtained for the group immunized with anti-PLY serum were not considered significant. Statistical analysis showed no significant difference between any of the groups challenged with TIGR4 but a significantly ($p < 0.05$) prolonged survival of the positive control group challenged with WU2.

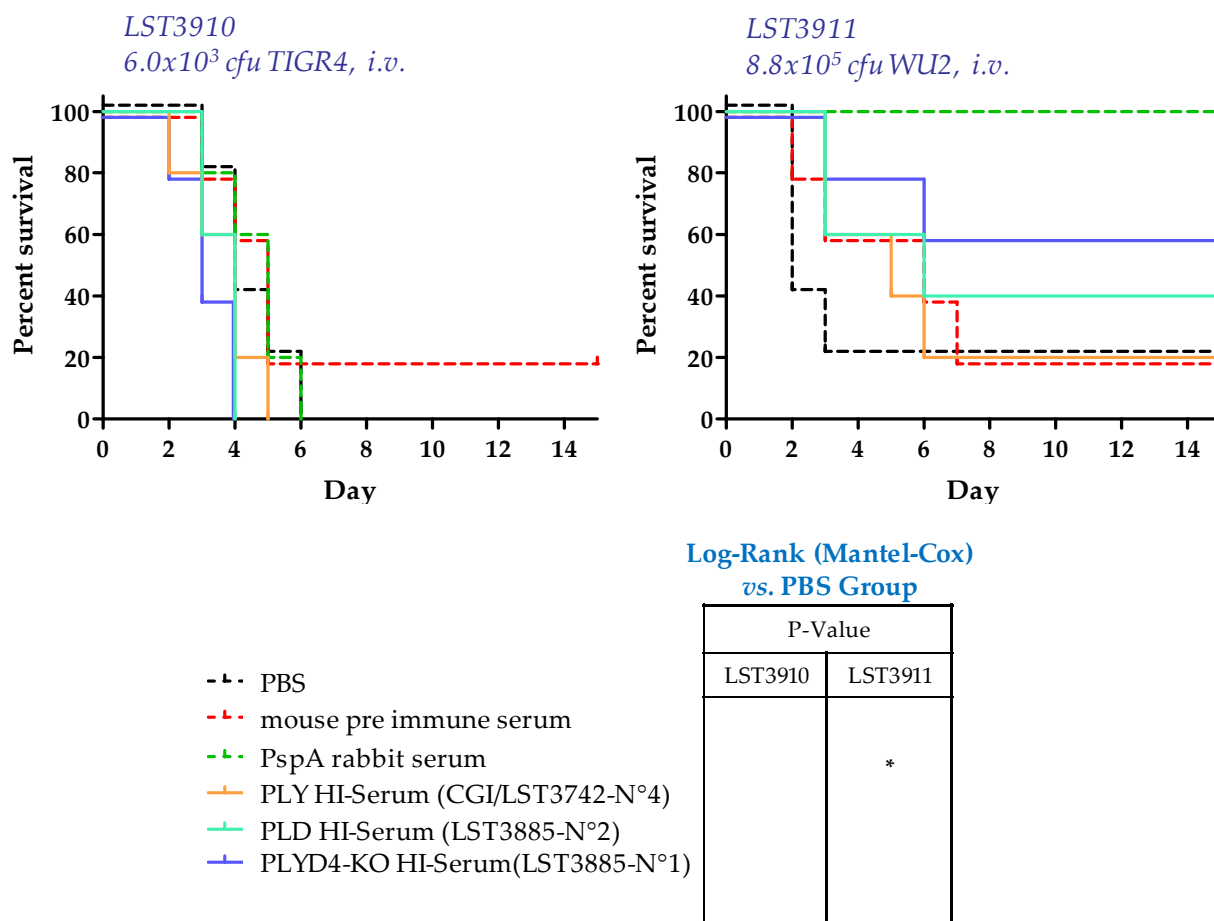


Figure 40: *In vivo* Efficacy of Polyclonal Anti-PLY Antibodies Against *i.v.* Challenge with the *S. pneumoniae* Strains TIGR4 and WU2 The survival of individual mice was monitored for 15 days post challenge. All survival-curves were statistically compared to the mock-immunized PBS-control group, using the Log-rank (Mantel-Cox) test where $p < 0.05$ is indicated with *.

4.3.2 IL-6 ELISA

As aforementioned, different levels of protection against a lethal challenge (*i.p.* with *S. pneumoniae* A66.1) between mice that were immunized *i.p.* with the same amounts of hyperimmune-sera generated against PLY (see data for LST3872 in the previous sections), were observed. Since PLY acts as a TLR4 agonist it was investigated if differences in the induced cytokine responses correlate with differences in survival.

In preliminary studies, changes in the cytokine-profile of mice infected with pneumococcus were determined in serum 24 h post *i.p.* challenge using a multiplex bead array. Significantly increased IL-6 and IFN- γ levels were observed, but all other cytokines were found to be expressed below the detection limit (unpublished data). Therefore the inflammatory IL-6 response of the mice (24 h post challenge) was determined by analyzing IL-6 levels in the serum in an ELISA-based assay and subsequent statistical comparison (t-test, 1-tailed, type 1) with the

D – RESULTS

corresponding pre-immune serum pools and the PBS control-group (LST3872-Group N°1). As animal health regulations did not permit to draw sufficient amounts of serum for analysis of single mice, pools of each group were tested (LST3872-Groups N° 4-6 and 13-16).

All IL-6 ELISA read-out data used for the following statistical calculations are represented in the appendix section.

The computed data (Figure 41 and Supplementary Table 15) indicate that, compared to the corresponding pre-immune sera, all groups except of the group immunized with hyperimmune-serum generated against PspA_FL_T4 (CGI/LST3742-N°9) showed a statistically significant increase ($p < 0.001$) in the IL-6 response 24 h post challenge.

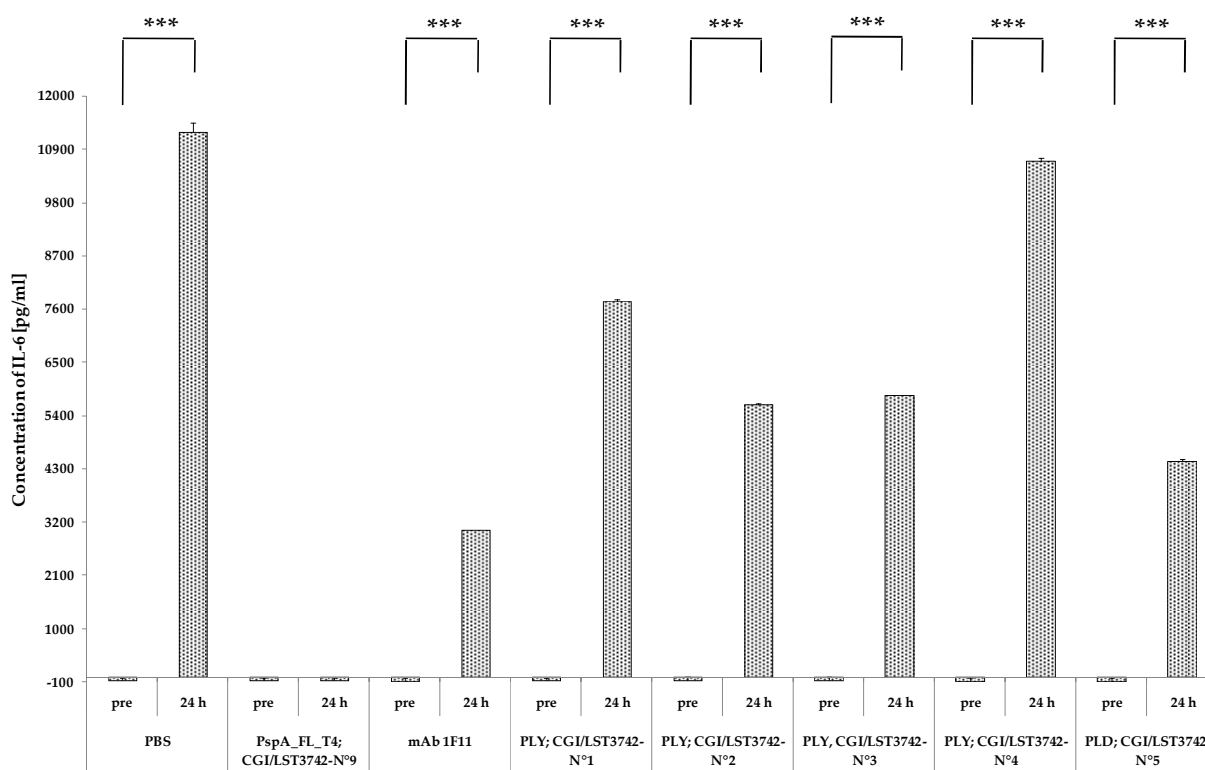


Figure 41: Results for the IL-6 ELISA. The concentration of IL-6 was determined in pooled pre-immune sera and pooled sera obtained 24 h post challenge. Results were statistically compared between the sera by means of a t-test (1-tailed, type 1) and significant fold-increases of $p < 0.001$ are depicted as ***.

Calculation of the average fold-changes in the IL-6 response and statistical analysis relative to the mock-immunized PBS-control group revealed that apart from one group (receiving hyperimmune-serum generated against PLY (CGI/LST3742-Group N°4)), all groups show statistically significant decreases in the IL-6 response compared to the control (Figure 42 and Supplementary Table 15).

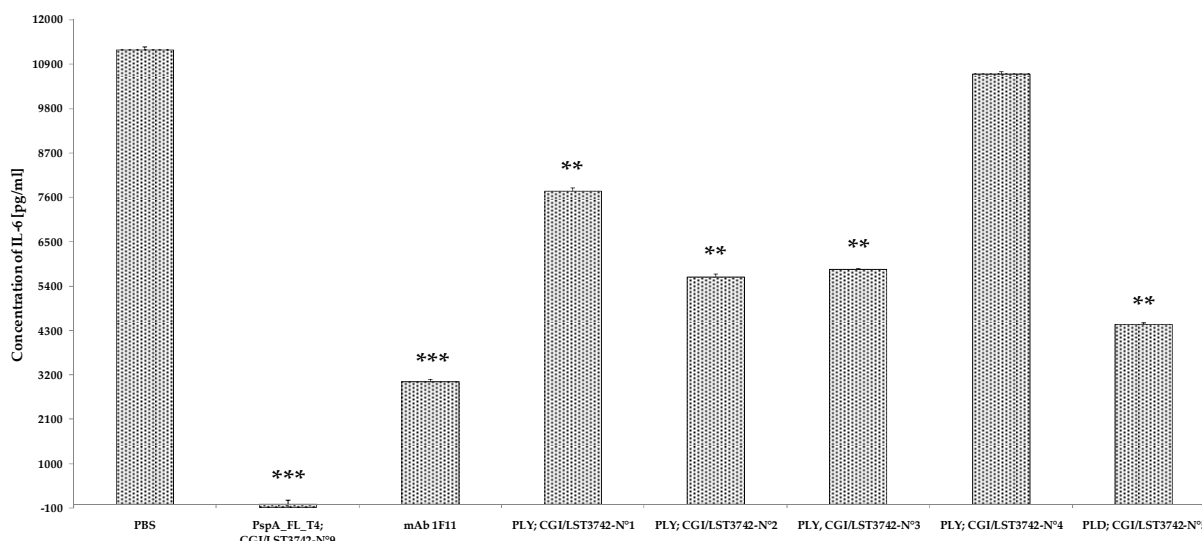


Figure 42: Statistical Comparison of Immunized Groups vs. PBS Control Group. The IL-6 response in the pooled sera obtained 24 h post challenge was statistically compared to the mock-immunized PBS-control group by means of a t-test (1-tailed, type 1), where $p < 0.001$ (***) and $p < 0.01$ (**) are indicated

Consequently passive immunization with anti-PspA-FL_T4 antibodies was shown to fully inhibit the inflammatory IL-6 immune response, and thus to protect mice from lethal pneumococcal infection (Supplementary Table 15). This correlation between decreased IL-6 response and the observed enhanced challenge-survival is also seen for groups immunized with mAb 1F11 (SantaCruz) and polyclonal anti-PLD antibodies.

4.3.3 Setup of Cell Based Functional *in vitro* Assays

Hemolysis-Inhibition Assay

Since lead mAbs against PLY and constructs thereof should be primarily selected based on their efficacy in inhibiting PLY-induced lysis of hRBCs prior to further evaluation in animal models of protection and inflammation, a **Hemolysis-Inhibition Assay** was set-up in the course of this project.

For the determination of one “Hemolytic Unit” [HU], which was defined as the amount of PLY being required and sufficient for the lysis of 50 % of hRBCs, freshly isolated hRBCs (5×10^8 cell/ml) were incubated with different concentrations of PLY. ddH₂O and DPBS served as controls for 100 % and 0 % hemolysis respectively. After separation of cells and supernatant, the OD at 541 nm was measured and the % hemolysis was calculated in reference to the controls. Based on titration-curves as exemplified in Figure 43 one HU was determined for each batch of freshly isolated hRBCs. It should be noted that during the assay-setup it was observed that dependent on the quality of the isolated hRBCs, the HU varied significantly (data not shown).

D – RESULTS

Based on this it was concluded that the HU has to be determined for each batch of isolated hRBCs before the following inhibition assays, whose results strongly depend on the correct determination of the hemolytic unit.

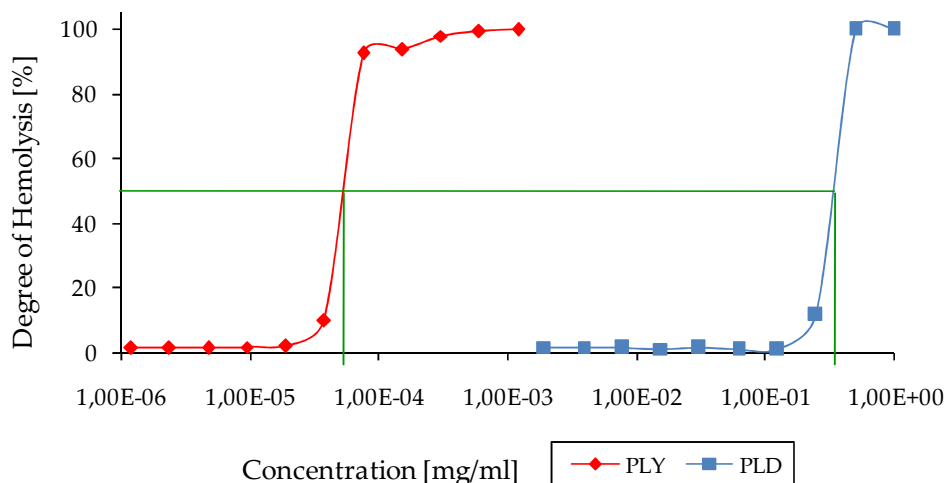


Figure 43: Determination of the Hemolytic Unit. Freshly isolated hRBCs were titrated with different concentrations of PLY and PLD. For both the Hemolytic Unit was determined at 50 % hemolysis, which is indicated by the green lines.

After determination of the HU, a hemolysis-inhibition assay was set-up (schematic representation in Figure 15). For this purpose different amounts of PLY representing 0.5, 1 or 2 HU were incubated with commercially available PLY specific mAbs (1F11, SantaCruz and PLY-4, Abcam) in order to inhibit PLY induced release of hemoglobin. An unrelated mAb (IC-N^o5, 5D12G4B8 PA-169-4) generated against a protein from *Borrelia burgdorferi* was used as the appropriate negative assay-control in all assays (data not shown).

The molar ratios of PLY to mAb (PLY:mAb) at 50 % hemolysis with an antigen concentration of 1.6×10^{-3} mg/ml (corresponding to 3.0×10^{-8} mmol/ml; 2 HU) were 1:50.6 and 1:135.3 for the mAbs 1F11 and PLY-4 respectively. Hence mAb 1F11 (SantaCruz) had an approximately 2.7-fold higher hemolysis-inhibition activity than the mAb PLY-4 (Abcam) (represented in Figure 44).

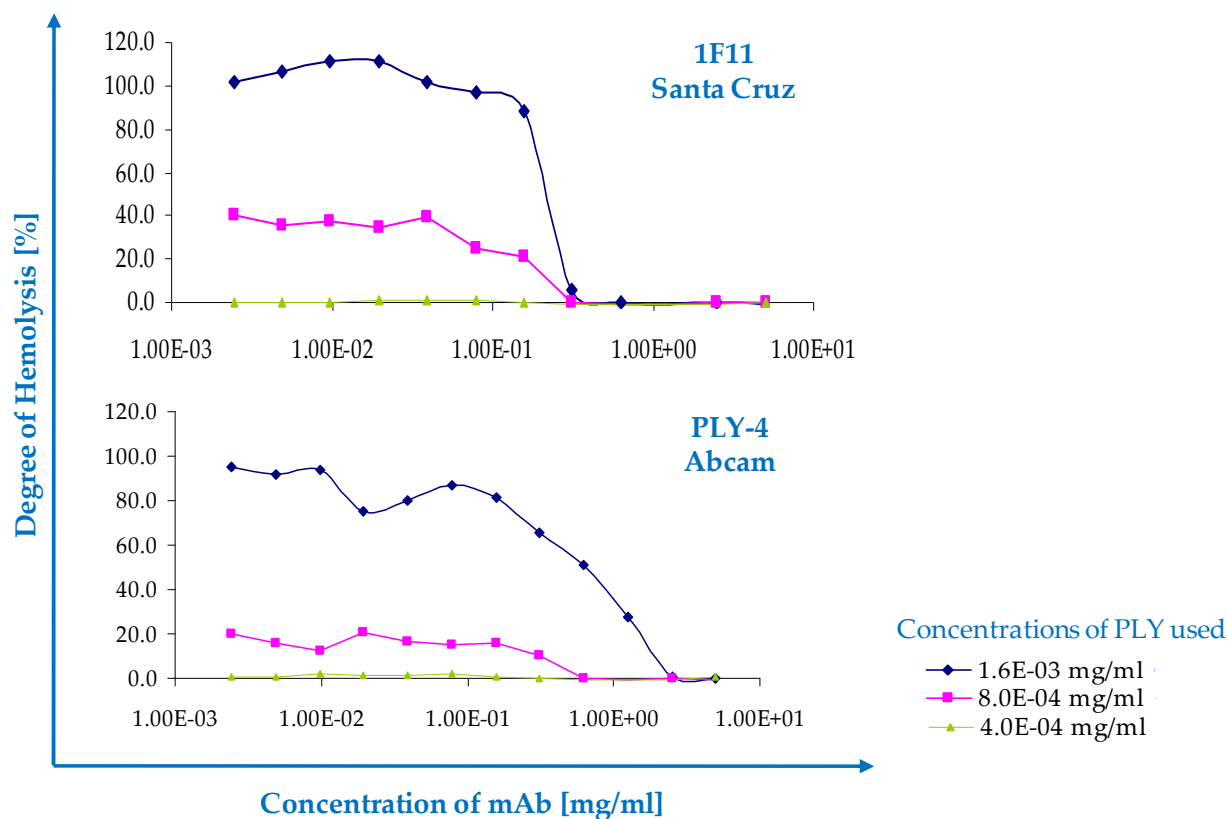


Figure 44: Hemolysis-Inhibition Assay using Commercial mAbs. Two commercially available mAbs - 1F11 (SantaCruz) and PLY-4 (Abcam) - were titrated for their hemolysis-inhibition potential against different concentrations of PLY.

The observed differences between the mAbs and the finding that the hemolysis of hRBCs by PLY can be fully inhibited, allowed the further establishment of the hemolysis-inhibition assay using the hyperimmune-sera generated in-house against PLY and constructs thereof.

Thus, hyperimmune-sera generated against PLY and PLD (CGI/LST3742 Group-N° 1-5), the corresponding pre-sera, as well as a positive control mAb (1F11, SantaCruz) and a negative-control pre- and hyperimmune-serum (CGI/LST3742 Group-N° 13) were used in an initial setup. The results obtained (Figure 45) indicate unspecific protection against the lysis of hRBCs by the sera, as also the pre-immune sera, as well as the unrelated-controls were protective against hemolysis.

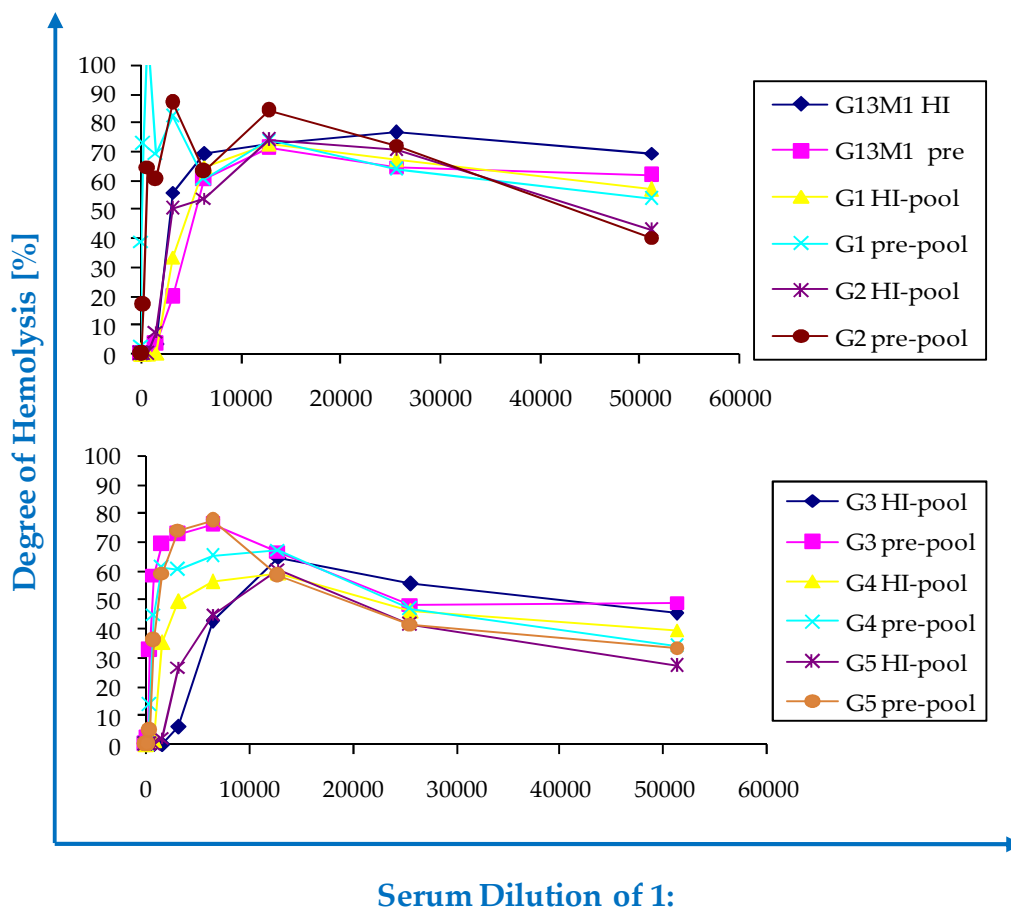


Figure 45: Hemolysis-Inhibition Assay using Hyperimmune-Sera. The anti-sera generated against PLY (G1-G4) and PLD (G5), the corresponding pre-immune sera, as well as a positive control mAb (1F11, SantaCruz) and a negative control pre- and hyperimmune-serum (G13) were tested in several dilutions for their hemolysis-inhibition potential of 2 HU of PLY.

In a repeating experiment the sera were pre-incubated with PLY (1 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$) in order to inhibit the unspecific binding of serum-components and hence enhancing the specific protection-potential of the polyclonal antibodies. Differences between the groups (pre- and hyperimmune sera) as well as the expected results for the controls could now be observed (data not shown). Based on these findings and indicative data found in literature, it was concluded that *inter alia* the relatively high cholesterol-levels in the sera – typically 0.97 mg/ml serum in healthy, female C3H/HeN mice ^[397] – lead to a significant binding of PLY to cholesterol – PLY is characterized as cholesterol-binding protein ^[266] – and hence inhibit the function of PLY, thus inducing an antibody independent inhibition of hemolysis.

To circumvent this, total IgG was purified from a hyperimmune-serum pool (CGI/LST3742 Group-N^o4) that showed the highest PLY titer in ELISA (Figure 24), as well as the

corresponding pre-immune serum-pool and commercial serum from naïve mice (PAA), via a protein G column-purification.

The purified IgGs were subsequently used for the setup of the hemolysis-inhibition assay as aimed before, using different concentrations of PLY. After removal of cholesterol and other serum-components by column-purification, no antibody-independent protection against the lysis of hRBCs remained, and a PLY-concentration dependent degree of hemolysis was observed (Figure 46).

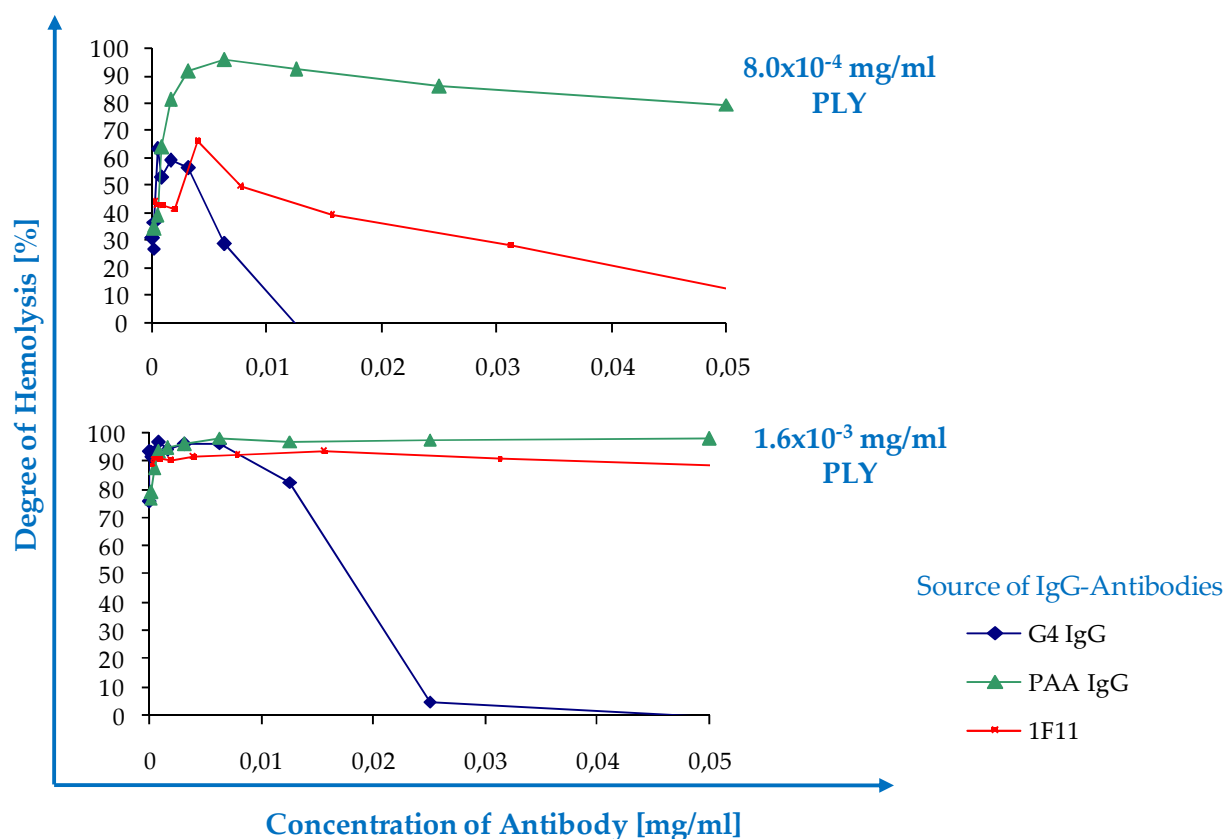


Figure 46: Hemolysis-Inhibition Assay using Purified IgGs. Polyclonal IgGs against PLY (CGI/LST3742 Group N^o4), the positive-control mAb 1F11 (SantaCruz) as well as the negative-control, purified IgGs from naïve mouse serum (PAA), were titrated for their inhibitory potential of hemolysis induced by 8.0×10^{-4} mg/ml and 1.6×10^{-3} mg/ml of PLY.

The molar ratios of PLY to antibody (PLY:HI-IgG or PLY:mAb) at 50 % hemolysis with an initially-used antigen concentration of 1.6×10^{-3} mg/ml (corresponding to 3.0×10^{-8} mmol/ml; 2 HU) were 1:3.9 and 1:51.2 for the purified IgGs (from CGI/LST3742-Group-N^o4) and mAb 1F11 (SantaCruz) respectively. Thus, the data obtained (Figure 46) allow the conclusion that the purified polyclonal IgGs generated against PLY are capable of inhibiting hemolysis, and are expressing an approximately 13.1-fold higher protection-potential than the commercial mAb 1F11 (SantaCruz) used as a positive control.

TLR4-Reporter Assay

In addition to their neutralizing activity on erythrocyte-hemolysis lead mAbs should also be capable to interfere with the PLY-induced activation of hTLR4. For this purpose, a “TLR4-Reporter Assay” based on the downstream-signaling due to the activation of hTLR4 *via* MyD88, I κ B and NF κ B leading to the secretion of SEAP, was set-up using commercially available, stably transfected HEK-Blue™ hTLR4 cells (InvivoGen).

In order to determine the optimal assay parameters, and to optimize the data-readout, an initial screening was set-up using different cell numbers for the assay. Therefore three different concentrations of HEK-Blue™ hTLR4 cells were incubated with varying concentrations of PLY, PLD and PLY Δ D4. Dilutions of LPS (K12) from *E. coli* were used as positive-controls and dilutions of an unrelated recombinant protein from *Borrelia burgdorferi*, being in the same buffer and containing approximately the same amounts of LPS as the three PLY constructs, were used.

In initial studies the reporter system was unintentionally activated in an unspecific manner due to standard pipetting of the cells during cultivation (data not shown). Therefore it is emphasized that the HEK-Blue™ hTLR4 cells have to be handled surpassingly careful, in order to avoid this unspecific activation.

In subsequent experiments (Figure 47) PLY was found to induce killing of HEK-Blue™ cells when used in higher concentration ranges (indicated by the decay of the hTLR4 activation when using concentrations higher than 0.05 mg/ml of PLY, Figure 47-C). However, all three PLY-constructs were capable of activating the hTLR4 in the tested concentration range of 2×10^{-2} mg/ml to 6.4×10^{-6} mg/ml (Figure 47-C to E).

The optimal cell-number for the assay was determined: 1.25×10^6 cells/ml were too many and 5×10^5 cells/ml were too few cells, leading to unspecific or insufficient activation of the reporter-system respectively. The microscopy-based evaluation of the cell morphology during cultivation showed confluent cells in the wells containing 1.25×10^6 cells/ml and cells in bad shape in both settings (data not shown). Based on this it was concluded that the cell number for the assay is optimal at about 1×10^6 cells/ml where (i) the most sufficient activation of the reporter system was observed, (ii) the unspecific activation of the reporter-system was not determinable and (iii) the controls – LPS and unrelated recombinant protein – gave the expected results (Figure 47). The slightly unspecific activation of the hTLR4-reporter system at high concentrations of unrelated protein (Figure 47-B), might arise from the LPS-content of the

protein-preparation. For screening purposes PLY would be used at a concentration of 4×10^{-3} mg/ml where no toxin-independent activation is expected.

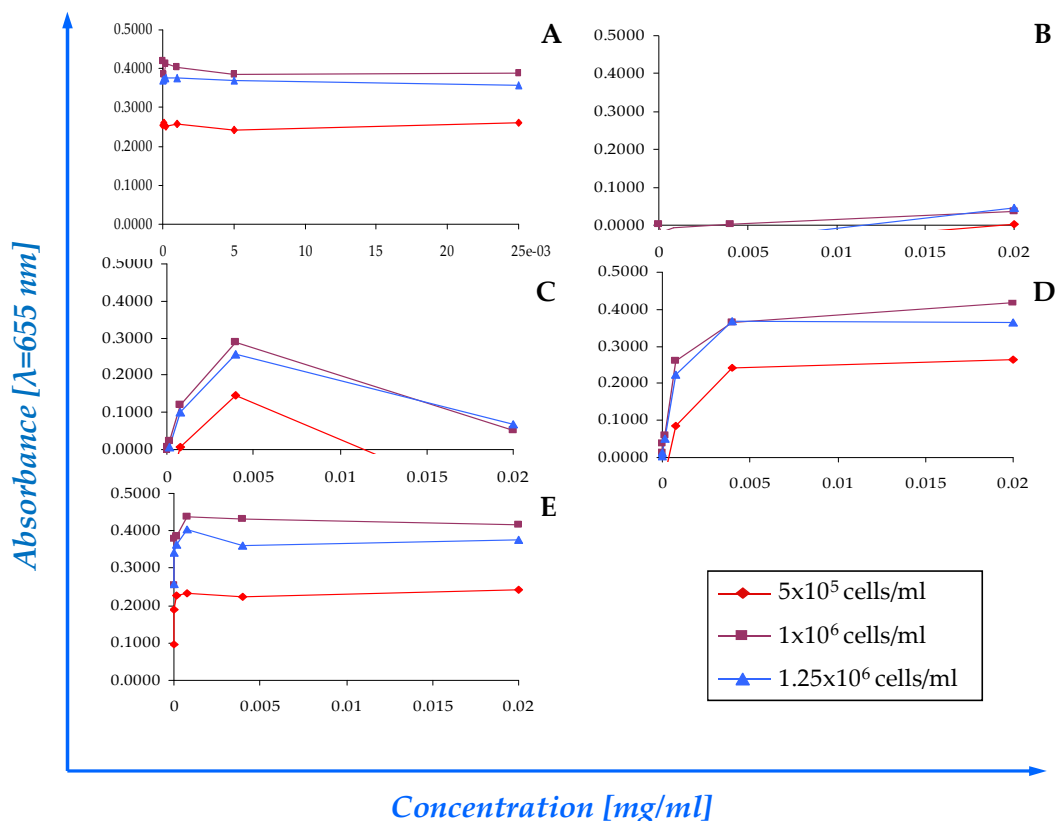


Figure 47: Initial Setup of the TLR4-Reporter Assay. For the optimization of the assay conditions, LPS (K12) from *E. coli* (A) and an unrelated recombinant protein from *B. burgorferi* (B) were used as positive- and negative-assay controls. The assay was set-up for the determination of the activation capability of the hTLR4-activation by PLY (C), PLD (D) and PLY Δ D4 (E) using three different concentrations of HEK-BlueTM hTLR4 cells.

Finally, the inhibitory effects of the commercial mAb 1F11 (SantaCruz) and the purified, polyclonal IgGs generated against PLY (pooled hyperimmune-serum of CGI/LST3742 Group-N^o4) on the activation of the hTLR4-reporter system were assessed.

Repeated experiments showed that both - mAb 1F11 and purified IgG – could inhibit the activation of hTLR4 through PLY and PLD in a concentration dependent manner (the results of one experiment are shown in Figure 48).

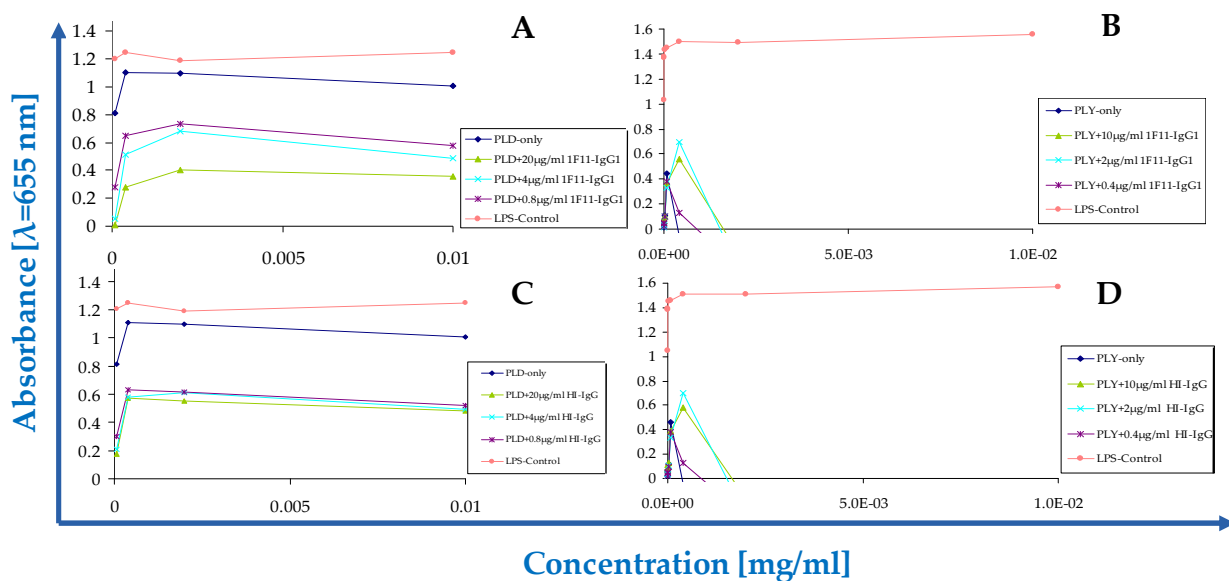


Figure 48: TLR4-Reporter Assay using Purified IgGs and the Commercial mAb 1F11. Different concentrations of mAb 1F11 ((A) and (B)) and purified, polyclonal IgGs ((C) and (D)) were tested for their inhibitory potential against the activation of hTLR4 by PLD ((A) and (C)) and PLY ((B) and (D)).

Without the use of inhibitory antibodies, PLY and PLD – compared to LPS, which at a concentration of 8×10^{-5} mg/ml achieved 100 % hTLR4-activation – were found to induce cell-killing and approximately 67 % activation of the hTLR4-reporter system respectively. Calculating the molar ratios of antigens to antibodies at 50 % hTLR4-activation – correlated to LPS being 100 % – with an initially-used antibody concentration of 2 μ g/ml – corresponding to 1.33×10^{-5} μ mol/ml – resulted in ratios of 1:1.6 (PLY:mAb), 1:1.7 (PLY:HI-IgG), 1:6.9 (PLD:mAb) and 1:7.2 (PLD:HI-IgG). Both – purified polyclonal IgG and the commercial mAb 1F11 – showed stronger inhibition of PLY than PLD.

4.3.4 Flow Cytometric Approaches for the Optimization of Sorting-Conditions for Human B-Cells

For the generation of human mAbs against PLY and other target antigens with the “Sindbis Virus Based Mammalian Cell Surface Display”, the staining conditions for eukaryotic cells needed optimization in order to use them subsequently for flow-cytometry based sorting of human B-cells.

The major limitation of this approach is that PLY specifically binds to all cholesterol containing cell membranes, hence to all eukaryotic cells, leading to antigen independent positive results. Additionally the toxin-like character of PLY, causing cell-lysis, remains problematic. Therefore the use of mutated forms of the toxin – non-cytolytic PLD and PLY Δ D4 lacking the cell binding domain 4 – could circumvent the problems associated with the wild-

type toxin. Due to patent restriction, PLD could not be used for the generation of human mAbs (Kirkham, *et al.*; “Mutant pneumolysin proteins” US-Patent N° 7,820,789; Mitchell, *et al.* “Novel Adjuvant Compounds”, US-Patent Application 20100166795) and as that the cell binding domain also represents an attractive mAb target the use of PLY Δ D4 was also limited.

Thus, the optimization of the sorting conditions for PLY-specific B-cells was explored by using different eukaryotic cells, such as HL-60, HEK-293T, hybridoma and hPBMCs, as well as applying several blocking-reagents and approaches. Recombinantly expressed and purified PLD and PLY Δ D4, were used as controls for the staining-optimization with the wild-type toxin.

Initially the cytolytic activity of PLY and PLD on HL-60 cells was evaluated and blocking was tested with the PLY-specific mAb 1F11 (SantaCruz). PLY and PLD bound to HL-60 cells in a concentration-dependent manner – represented for PLD (Figure 49-A). PLY killed the cells even at low concentrations of approximately 2 μ g/ml whereas PLD hardly affected cell viability at higher concentrations of up to 200 μ g/ml (Figure 49-B). Even without a specific incubation step 1 μ g/ml mAb 1F11 were capable to block the binding of PLY and PLD to HL-60 cells (Figure 49-B).

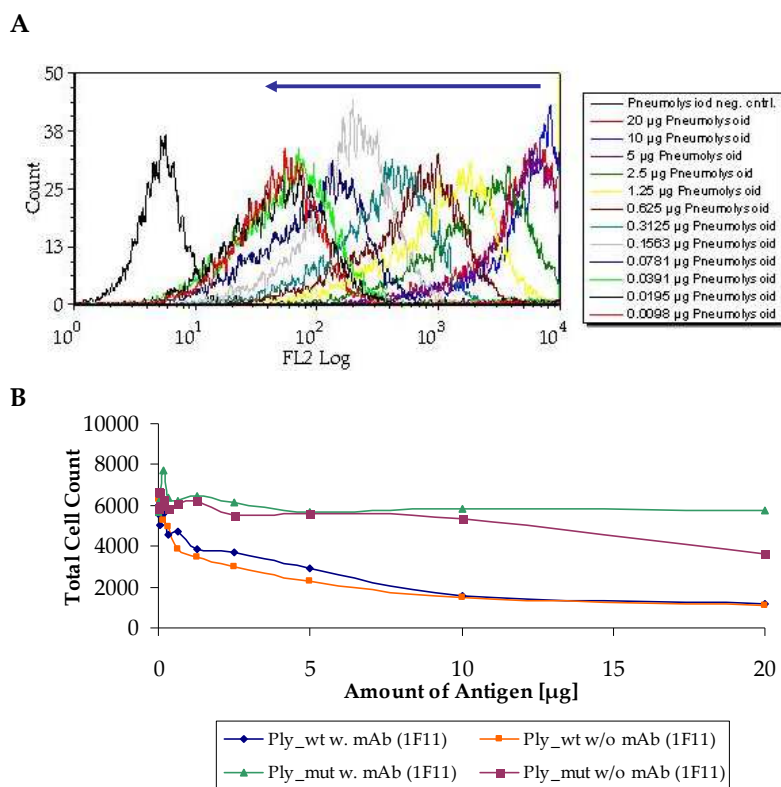


Figure 49: Binding Inhibition of PLY and PLD to HL-60 Cells using the mAb 1F11. A concentration-dependent binding of both antigens – represented for PLD (A) – was evaluated by flow cytometry, where the concentration-dependent effects of the antigens only and in combination with the mAb 1F11 (SantaCruz) on the total cell-count were determined (B).

D – RESULTS

Subsequently, different blocking reagents namely FCS, AmphotericinB, cholesterol in 100 % ethanol and a water soluble cholesterol-derivative were tested in different concentrations for their ability of blocking the PLY-specific signal (data not shown). In summary, it was found that none of the blocking reagents used was capable to inhibit the binding of PLY to eukaryotic cells completely.

Therefore a peptide (Oxpholipin 11D, peptide-sequence: ECTGLAWEWWRT), reported of binding to cholesterol [398], and hence aimed to block the binding of PLY to the cells, was synthesized (ThinkPeptides) and tested. Oxopholipin 11D did not show any concentration-dependent effects, but the signal was found to be slightly blocked when HL-60 cells were pre-incubated with the peptide and PLD – pre-incubated with mAb 1F11 or HI-serum specific for PLY (CGI/LST3742-N°4) – being subsequently added (data not shown). But no complete blocking of the signal could be achieved with the peptide based approach. Possibly through binding of the mAb and the polyclonal antibodies in the HI-serum to the peptide as well as to PLY and/or PLD the observed blocking might have been false-positive, which was difficult to prove.

In a final approach aiming to inhibit binding of PLY and PLD to HL-60 cells, both antigens were coupled to different FluoSpheres®. The coupling to all three FluoSpheres® tested was successful (data not shown). The amine-modified beads exhibit a concentration-dependent binding with an optimal range between 0.0063-0.0016 % beads per 100 µl (Figure 50). The pore-forming ability of PLY, and hence the induction of the lysis of HL-60 cells was inhibited when the toxin was coupled to the FluoSpheres® (Figure 50-A).

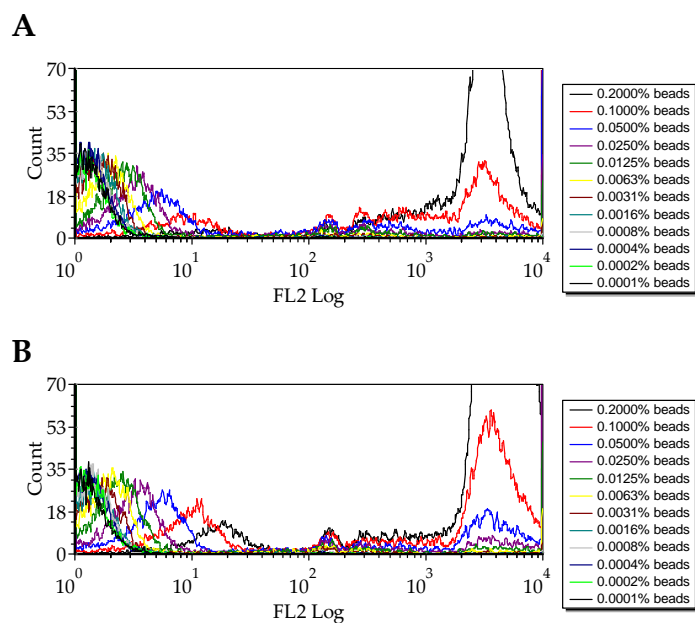


Figure 50: Signal Blocking of PLY and PLD Coupled to Amine-Modified FluoSpheres®. The inhibition of the unspecific binding of PLY and PLD to HL-60 cells was determined by coupling both antigens to amine-modified FluoSpheres® and evaluating the binding characteristics for bead-bound PLY (A) and PLD (B) by means of flow cytometry.

The results obtained for the use of the blocking reagents, Oxpholipin 11D and the antigen-coupled FluoSpheres® were subsequently checked with other eukaryotic cells than HL-60 cells, namely HEK-293T cells, hybridoma cells and isolated hPBMCs. All these approaches showed extremely low cell recovery when compared to HL-60 cells and hence no conclusion could be drawn at this stage (data not shown).

D – RESULTS

5 TITER-SCREENINGS OF SERA FROM HEALTHY HUMAN DONORS

In order to find suitable sources for the isolation of hPBMCs and subsequent B-cell sorting as required for the generation of human mAbs based on the mammalian cell surface display technology, thirty-five healthy human blood-donors were screened for their serum-antibody titers against PspA and PLY and constructs thereof.

For this purpose serum-dilutions (200-25,000-fold dilution) of each donor were tested in duplicates in ELISA-based screenings for antibodies against recombinant variants of PspA and PLY. Several high-titer donors were identified for each of the constructs tested (see Figure 51 representing the results obtained for the 1:1000 serum dilutions and the *Appendix* for all data obtained), which suggests potential sources for the aimed isolation and purification of hPBMCs and antigen-specific B-cells.

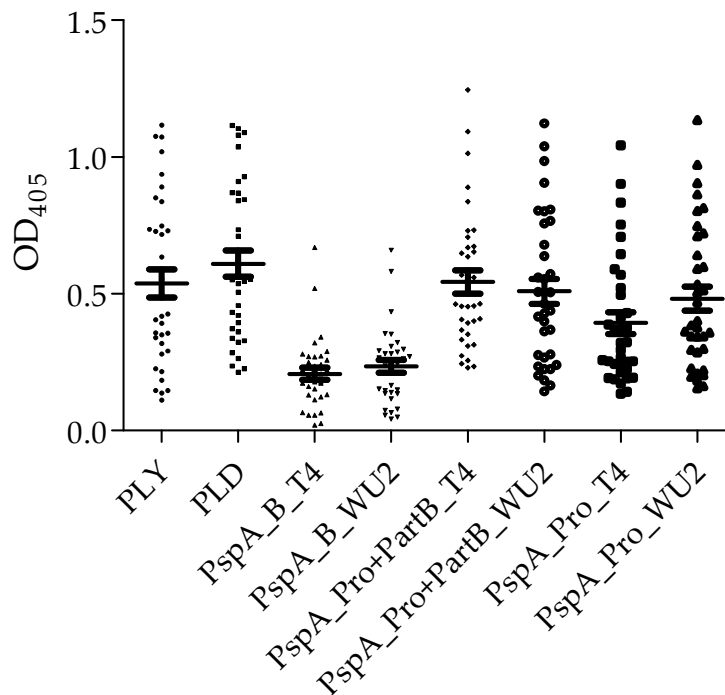


Figure 51: Human Serum-Titer Screening for Target Antigens. In total 35 healthy donors were tested for their serum-titer against eight pneumococcal antigen-constructs.

E - DISCUSSION AND CONCLUSION

The overall aim of the present study was to characterize three highly conserved and pneumococcal-specific target antigens – LTA, PspA and PLY – for their suitability as human mAb-based therapeutics. For this purpose these were expressed, purified and evaluated *in vitro*. Additionally the generation as well as the in-depth characterization of the *in vitro* functionality and *in vivo* efficacy of mouse polyclonal antibodies and human mAbs targeting the three antigens was envisaged.

The advantage in targeting LTA compared to other surface polysaccharides of *S. pneumoniae* would be the conservation of its chemical composition across the whole species [63, 108, 119-120] and interference with its complex biological nature *e.g.* interaction with host receptors or activation of inflammatory responses. [29-31, 33-36, 63, 128, 133-147, 150-170] Additionally antibodies against the PCho moiety as well as active immunization with protein conjugates were found to be protective against some pneumococcal strains in mice. [399-403] However, surface exposure of LTA and thus accessibility to antibodies as well as beneficial effects of anti-LTA antibodies during pneumococcal disease are still controversial. [404-406]

LTA was aimed to be isolated in its native form by means of an n-butanol extraction and subsequent HIC-purification from *in vitro* grown pneumococci. Therefore, this purification method described in the literature [126] was further optimized by successfully establishing buffer and gradient conditions for the elution of purified and commercially available LTA from *S. pyogenes* in pilot experiments.

One major problem for the LTA purification posed the need for a suitable detection and quantification method. First, the literature based phosphomolybdenum-blue assay [126] based on the reactivity of the LTA-phosphate backbone was explored using purified LTA from GAS and HIC fractions containing pneumococcal LTA. It was found that the detection-limit of this assay was 100 µg/ml for LTA from GAS and that the background in the samples was high. Thus this method was not suitable to screen and select the fractions for further processing.

As an alternative approach an ELISA based assay was set up. For this purpose several commercially available LTA-specific antibodies were screened and a mAb from GenWay detecting LTA from GAS at concentrations of 0.1 ng per ml at an antibody-dilution of 1:200 was selected. This antibody also showed reactivity with pneumococcal structures in the HIC fractions in several purification attempts, suggesting that this assay would be capable to detect

E - DISCUSSION AND CONCLUSION

pneumococcal LTA in a fast and easy way. Nevertheless it is important to stress that the extent of the (cross-) reactivity of the used primary mAb – raised against intact *Listeria monocytogenes* – with pneumococcal LTA remains unknown. Antibodies specific for pneumococcal LTA would be the ideal solution but these are currently not commercially available. It was attempted to generate hyperimmune-sera against pneumococcal LTA in house by immunizing mice with the pooled fractions from each of the main peaks from the HIC-run represented in Figure 30. However, none of the two groups raised detectable antibody titers against LTA (data not shown).

It was also attempted to assess the quality of purified LTA in terms of purity by separation on PAGE and subsequent Alcian-Blue and silver staining. This was successful for purified LTA from GAS resulting in a band-like pattern as described in the literature [389, 394-395], but due to the high amounts required this was not followed up in the course of this project.

In summary the isolation and purification of pneumococcal LTA require a profound knowledge in carbohydrate chemistry. Due to the time constraints of this project and low immunogenicity of the purified structures, LTA could not be followed up as a target. Future approaches could be based on MS/MS-analyses of the putatively purified LTA which will allow to gather full certainty about the isolated and purified product. In addition it should be emphasized that pneumococcal LTA was already fully chemically synthesized and subjected to structural analyses and biological studies [129-130] which presents a potential source for collaboration in the future.

The presence of PspA on all pneumococcal strains, its essentiality as virulence factor, and above all the effective protection against pneumococcal infections observed in animal models make PspA an attractive target for mAb development. [174-178, 180, 183, 186, 202-203, 221-223]

The expression and purification of recombinant PspA and several subconstructs thereof, was based on the PspA-sequences of the pneumococcal wild-type strains WU2 (PspA Family 1, Clade 2) and TIGR4 (PspA Family 2, Clade 3), as representatives for the two major PspA-families [176]. That way the potential for cross-family-reactive antibodies should be most efficiently explored.

Good antibody titers were raised by *s.c.* immunization which also recognized linear epitopes as determined by peptide ELISA. Primarily sera were screened for differences in surface staining on live pneumococci in order to correlate these findings with subsequent

protection *in vivo*. This would allow establishing a screening assay during human mAb development.

Surface staining of the wild-type strains TIGR4 and D39 indicated a clade-specific recognition of PspA with antibodies generated against the TIGR4- and WU2-constructs respectively. Using a TIGR4 Δ PspA strain showed cross-reactivity of the hyperimmune-sera generated against the recombinant WU2-constructs with another protein than PspA, as these sera showed the same profile on the TIGR4 wild-type and knock-out strain. This hypothesis is substantiated by data generated using a TIGR4 Δ PspC strain, which suggest that the antibodies rather recognize PspC, another choline binding protein of pneumococci that also contains a proline-rich region. [202]

The relatively low antibody binding to the strain EF3030 (PspA Family 1, Clade 1) by all hyperimmune-sera tested, indicated that independent of the background the antibodies were raised against, PspA was recognized in a very clade-specific manner. Thus, although reported in the literature [183, 220], no cross-clade reactivity was achieved for any of the generated and tested hyperimmune-sera.

For *in vivo* efficacy testing of antibodies in several different mouse models – challenging with PJ1324 (6B), TIGR4, A66.1, D39 and WU2 in distinct application routes (*i.n.*, *i.p.* and *i.v.*) – were employed.

i.v. challenge with TIGR4 and *i.p.* challenge with D39 was found to be too lethal allowing no significant survival in any of the groups, even not those receiving the positive control – polyclonal anti-PspA_FL_T4 rabbit serum, thus this data were excluded from further analyses. This is in accordance with literature data that suggest difficulties in obtaining complete protection of mice challenged with serotypes 2 and 4 although immunized with the homologous PspA variant. [407]

In a model of *i.p.* infection with a PspA Family 2 Clade 3 strain (PJ1324) a clade-specific protection was observed for the anti-PspA sera generated against TIGR4 variants. All antibodies generated against the TIGR4 constructs significantly protected mice with p-values below 0.01, whereas anti-WU2 sera showed no effect. Interestingly, antibodies against the proline-rich region could not protect mice from death consistently although recent reports indicated that the proline-rich region gave rise to cross-clade protection. [176, 193, 201-203, 232-233]

E - DISCUSSION AND CONCLUSION

Upon *i.v.* challenge with a PspA Family 1 Clade 2 strain (WU2) only a clade-specific protection with anti-Pro_WU2 and anti-Pro+PartB_WU2 sera was observed with p-values below 0.05. Although no cross-clade protection was observed, a protective effect of the proline-rich region was seen, which is in accordance to literature data. [176, 193, 201-203, 232-233]

In *i.n.* challenge with a PspA Family 1 Clade 2 strain (A66.1) no consistent cross-clade protection was observed with the sera specific for the TIGR4 variants, except for the positive control rabbit serum against PspA. Whereas in one experiment antibodies against the proline-rich region showed significant protection with a p-value below 0.01, in the other experiment – using a different hyperimmune serum batch – only antibodies against the WU2 constructs significantly protected in a clade-specific manner. As hyperimmune sera generated in different experiments – with the same protein batches and immunization protocol – had to be used for these studies, these were compared for *in vitro* differences. However, mice had no pre-existing antibodies against *S. pneumoniae* and hyperimmune sera did not show any differences in terms of recognition of PspA on the surface of live *S. pneumoniae* strains TIGR4 and A66.1 by means of a flow-cytometry based surface staining (data not shown). Therefore more in depth *in vitro* (e.g. OPK assays) and *in vivo* studies would be needed for the LST3885 hyperimmune sera in order to proof their protective-potential observed in initial studies.

In conclusion, hyperimmune sera generated against the different PspA-constructs conferred a clade-specific protection against lethal challenge. Although indicated in the literature [176, 193, 201-203, 232-233], none of the PspA sera tested showed significant and consistent protection across PspA clades in any of the lethal challenge-models tested. Based on these data it is likely that a mAb-based therapy would require a cocktail of at least two different mAbs – one recognizing Family 1 PspA and another one reacting with Family 2 PspA – in order to cover all pneumococcal strains.

In summary, surface staining data seemed to be predictable for the *in vivo* efficacy of the polyclonal antibodies against PspA: *in vitro* strains were stained in a clade-specific manner (Table 5) and also *in vivo* only the expected clade-specific protection was observed in several lethal challenge models.

PLY is a cytotoxic, pore-forming antigen that is conserved across all pneumococcal strains and also shares a high degree of sequence similarity with cytolysins from other bacterial species. [13, 38, 63, 249-263, 277-283] The multiple roles during pneumococcal infection based on its toxic

nature – *e.g.* the acceleration of bacterial transmission by the induction of tissue-damage and the inhibition of the proliferation of host immune cells [50, 63-64, 289-293] – and its manifold functions as an immunologic agonist – *e.g.* recognition and induction of inflammatory responses or interference with opsonophagocytosis [37-56, 63, 300, 322-323] – emphasize the potential of anti-PLY mAbs in adjunctive therapies. [284, 301, 313, 323, 338-340]

Passive immunization studies with antibodies against PLY and PLD showed intermediate levels of protection only against *i.p.* challenge with a serotype 6B strain (PJ1324). In all other studies using different strains and challenge routes no significant and consistent protection was observed with anti-PLY and anti-PLD sera.

Based on these findings and since it is expected that PLY-specific antibodies act primarily *via* interference with the inflammatory responses, the inflammatory IL-6 response – in pre-immune sera and sera obtained 24 h post *i.p.* challenge of mice challenged with the strain A66.1 – was determined in ELISA. A statistically significant ($p < 0.001$) increase of IL-6 levels in all groups – except in the PspA positive control group – was observed 24 h post challenge. Comparison to the mock-immunized PBS-control group revealed that all groups – except the one receiving anti-PLY serum (CGI/LST3742-group N°4) – had a significantly reduced IL-6 response. The inhibition of the inflammatory IL-6 immune-response in the group immunized with the PspA-construct also correlated to full protection of mice from lethal pneumococcal infection. This correlation was also seen for the groups immunized with a PLY-specific mAb and polyclonal anti-PLD antibodies. To obtain a more detailed picture of the induced cytokine-response nonetheless would require a multiplex cytokine-profile. Thus in addition to the IL-6-response also the determination and correlation of other inflammatory chemokines and cytokines such as macrophage inflammatory protein 2, IFN- γ , IL-5, IL-10, IL-12, IL13, monocyte chemoattractant protein 1/JE and TNF- α , as described for other mAb efficacy-screenings in the literature [408-412], might be necessary and useful in the future.

Taken together it is concluded that, independent of the route of challenge and pneumococcal strain, the generated polyclonal antibodies against PLY, PLD and PLY Δ D4 do not entail full protection against lethal challenge in the given settings.

Lead mAbs against PLY were aimed to be primarily selected on their efficacy in cell-based functional *in vitro* assays prior to *in vivo* studies for their protective and anti-inflammatory functionality. For this purpose two assays – a Hemolysis-Inhibition Assay and an hTLR4-Reporter Assay – were successfully setup and optimized for PLY-specific mouse mAbs and for

E - DISCUSSION AND CONCLUSION

polyclonal mouse IgGs, generated against different PLY constructs and purified in the course of this project.

Both assays were shown to work robust and yielded reproducible results. The Hemolysis-Inhibition Assay revealed a significantly higher activity of purified polyclonal IgGs, compared to the mouse mAb 1F11. In contrast the results obtained for the mAb 1F11 suggest a stronger inhibitory potential for the hTLR4-activation than the polyclonal antibodies. The hTLR4-Reporter Assay further indicated that both – purified polyclonal IgGs and the commercial mAb 1F11 – inhibit the reporter-activation by PLY more powerful than the activation by PLD. This can be deduced from the fact that both antibodies were generated against the toxin and not the toxoid, hence possess a higher specificity against PLY.

Since human mAbs against PLY are aimed to be generated with the “*Sindbis Virus Based Mammalian Cell Surface Display*” in the future, the staining-conditions for eukaryotic cells were optimized in order to allow subsequent FACS-based screenings of human memory B-cells. As the use of the mutant toxoids PLD and PLY Δ D4 is limited by the loss of essential structural features as well as patent-protections thereof, the sorting conditions for the wild-type toxin were optimized by interfering with the specific binding of PLY to the cholesterol containing membranes of eukaryotic cells. The most promising results were obtained when coupling PLY to amine-modified FluoSpheres[®] in order to inhibit the pore-forming and cell-binding ability of the antigen.

Although the bead-based blocking approach showed the most promising results, it was concluded that further optimizations of the assay parameters such as centrifugation-conditions, cell treatment, *etc.* will be required in order to obtain optimal conditions for the sorting of human memory B-cells in the future.

Because of the need of finding suitable sources for hPBMC-isolation and subsequent memory B-cell sorting – which is the basis of the generation of human mAbs by the “*Sindbis Virus Based Mammalian Cell Surface Display*” – sera from thirty-five healthy human blood-donors were screened for their antigen-specific antibody-titers against different PLY- and PspA-constructs. Summarizing several high-titer donors were identified for each of the constructs tested, which suggests potential sources for the prospective isolation of antigen-specific human memory B-cells.

Concluding, the differences that arose for all *in vivo* efficacy tests of polyclonal anti-PspA and anti-PLY antibodies suggest that more in-depth *in vitro* assays such as OPK would be required to compare the differences in the hyperimmune-serum batches. Furthermore future experiments should be set-up using a larger number of mice per group to strengthen the statistic read-out.

In addition to this it will be essential to test higher immunization doses for all of the hyperimmune sera in mouse experiments, as the applied amount of approximately 100 µg of total IgG per 23 g body-weight just represents a screening dose, which might significantly differ from the dose used in therapeutic settings. This is exemplified by the only FDA-approved anti-infective mAb (Palivizumab, brand-name Synagis®, MedImmune) that targets the respiratory syncytial virus (RSV) and is administered at doses of up to 15 mg per kilogram body-weight in humans, which reflects approximately 345 µg mAb per 23 g body-weight in mice. ^[413]

In conclusion, this work demonstrates the successful expression, purification and in-depth *in vitro* and *in vivo* characterization of several constructs of the pneumococcal target antigens PspA and PLY and polyclonal mouse antibodies generated against each. For the third target antigen – LTA, which was shown to be difficult to be isolated and purified – several qualification and quantification approaches were accomplished and optimized. The foundations for the prospective generation of human mAbs – targeting different constructs of PspA and PLY – were successfully laid by identifying potential human blood-donors for the isolation of antigen-specific memory B-cells and by the optimization of the sorting-conditions for PLY in the course of this project. Two functional cell-based assays were effectively setup and optimized and are thus ready to be used for the in-depth characterization and primary screening for the functionality of human mAbs to be generated in the future.

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G - INDEX OF FIGURES AND TABLES

1 TABLE OF FIGURES

Figure 1: The Morphology of <i>S. pneumoniae</i>	10
Figure 2: Pneumococcal Deaths in Children.....	11
Figure 3: Nasal Colonization by <i>S. pneumoniae</i>	13
Figure 4: Statistical Surveillance of <i>S. pneumoniae</i>	14
Figure 5: Advertisement for a Pneumococcal Serum Therapy in 1933.....	17
Figure 6: Important Pneumococcal Virulence Factors	18
Figure 7: Schematic Drawing of the Gram-Positive Cell Wall Depicting WTA and LTA	19
Figure 8: The Structural Model for Pneumococcal LTA	20
Figure 9: The Several Roles of WTA and LTA in Gram-Positive Bacteria	21
Figure 10: The Interactions of WTA and LTA with the Eukaryotic Host.....	22
Figure 11: Protein Structure of PspA.	23
Figure 12: The Families and Clades of PspA	24
Figure 13: The Structure of PLY	27
Figure 14: Schematic of the <i>Sindbis Virus Based Mammalian Cell Surface Display</i> Technology.....	32
Figure 15: Schematic Representation of the Hemolysis-Inhibition Assay.....	51
Figure 16: Amino Acid Sequences of Recombinant PspA_FL_T4 (A) and PspA_FL_WU2 (B)...	52
Figure 17: Recombinant Constructs of PspA (SP0117).....	53
Figure 18: Amino Acid Sequences of Recombinant PLY and Constructs Thereof.....	54
Figure 19: Colony PCR.....	55
Figure 20: Plasmid Purification and Double-Restriction Digest.....	55
Figure 21: Example for a Small Scale Expression - PspA_B_T4.....	56
Figure 22: Monitoring of the IMAC-Purification	56
Figure 23: Quality Control Post Dialysis.....	57
Figure 24: Results of the Titer-Screening of Hyperimmune Sera	58
Figure 25: Dose Titration for the <i>i.v.</i> and <i>i.p.</i> Challenge with <i>S. pneumoniae</i>	60
Figure 26: ELISA-Read-Outs of the HIC-Optimization with GAS-LTA.....	61
Figure 27: Chromatogram of a HIC-Run	62
Figure 28: Standard Calibration for the Phosphomolybdenum-Blue Reaction.....	63
Figure 29: Result for the Phosphomolybdenum-Blue Reaction of Pneumococcal LTA.....	63

G – INDEX OF FIGURES AND TABLES

Figure 30: ELISA-Based Screening of HIC-Fractions of Pneumococcal LTA.....	64
Figure 31: PAGE-Separation (A) and Silver Staining Approach (B) for GAS-LTA.....	65
Figure 32: Results for the Peptide ELISA	67
Figure 33: <i>In vivo</i> Efficacy of Polyclonal Anti-PspA Antibodies Against <i>i.p.</i> Challenge with the <i>S. pneumoniae</i> Strain PJ1324 (6B).....	69
Figure 34: <i>In vivo</i> Efficacy of Polyclonal Anti-PspA Antibodies Against <i>i.p.</i> Challenge with the <i>S. pneumoniae</i> Strain D39.....	70
Figure 35: <i>In vivo</i> Efficacy of Polyclonal Anti-PspA Antibodies Against <i>i.n.</i> Challenge with the <i>S. pneumoniae</i> Strain A66.1.....	71
Figure 36: <i>In vivo</i> Efficacy of Polyclonal Anti-PspA Antibodies Against <i>i.v.</i> Challenge with the <i>S. pneumoniae</i> Strains TIGR4 and WU2.....	72
Figure 37: <i>In vivo</i> Efficacy of Polyclonal Anti-PLY Antibodies Against <i>i.p.</i> Challenge with the <i>S. pneumoniae</i> Strain PJ1324 (6B).....	74
Figure 38: <i>In vivo</i> Efficacy of Polyclonal Anti-PLY Antibodies Against <i>i.p.</i> Challenge with the <i>S. pneumoniae</i> Strain D39.....	75
Figure 39: <i>In vivo</i> Efficacy of Polyclonal Anti-PLY Antibodies Against <i>i.n.</i> Challenge with the <i>S. pneumoniae</i> Strain A66.1.....	76
Figure 40: <i>In vivo</i> Efficacy of Polyclonal Anti-PLY Antibodies Against <i>i.v.</i> Challenge with the <i>S. pneumoniae</i> Strains TIGR4 and WU2.....	77
Figure 41: Results for the IL-6 ELISA.....	78
Figure 42: Statistical Comparison of Immunized Groups <i>vs.</i> PBS Control Group.....	79
Figure 43: Determination of the Hemolytic Unit	80
Figure 44: Hemolysis-Inhibition Assay using Commercial mAbs	81
Figure 45: Hemolysis-Inhibition Assay using Hyperimmune-Sera.	82
Figure 46: Hemolysis-Inhibition Assay using Purified IgGs.....	83
Figure 47: Initial Setup of the TLR4-Reporter Assay.....	85
Figure 48: TLR4-Reporter Assay using Purified IgGs and the Commercial mAb 1F11	86
Figure 49: Binding Inhibition of PLY and PLD to HL-60 Cells using the mAb 1F11.....	87
Figure 50: Signal Blocking of PLY and PLD Coupled to Amine-Modified FluoSpheres®	89
Figure 51: Human Serum-Titer Screening for Target Antigens.....	90

2 LIST OF TABLES

Table 1: Examples of the Most Promising Vaccination Approaches Based on PspA	26
Table 2: <i>S. pneumoniae</i> Strains used in this Study	36
Table 3: Gradient for IMAC-Purification of Recombinant Proteins.....	41
Table 4: Overview of All Proteins Purified in Recombinant Form.	57
Table 5: Results for the Surface Staining of Live <i>S. pneumoniae</i> using HI-Sera (CGI/LST3742)....	68

3 *TABLE OF SUPPLEMENTARY FIGURES*

Supplementary Figure 1: Sequence Alignments of the B-Region of PspA.....A-4
Supplementary Figure 2: Sequence Alignments of the Pro-Rich Region of PspA (aa 1–180).....A-5
Supplementary Figure 3: Sequence Alignments of the Pro-Rich Region of PspA (aa 180-335) .A-5
Supplementary Figure 4: EC50-Values for the Titer-Screening of all PLY-Groups.A-13
Supplementary Figure 5: EC50-Values for the Titer-Screening of all PspA-GroupsA-14
Supplementary Figure 6: Statistical Comparison of Hyperimmune Serum TitersA-14

4 LIST OF SUPPLEMENTARY TABLES

Supplementary Table 1: Overview of all Primers Used.....	A-4
Supplementary Table 2: Results for the Human Serum-Titer Screening (Donor-N° 1 to 5).....	A-6
Supplementary Table 3: Results for the Human Serum-Titer Screening (Donor-N° 6 to 10).....	A-7
Supplementary Table 4: Results for the Human Serum-Titer Screening (Donor-N° 11 to 15)...	A-8
Supplementary Table 5: Results for the Human Serum-Titer Screening (Donor-N° 16 to 20)...	A-9
Supplementary Table 6: Results for the Human Serum-Titer Screening (Donor-N° 21 to 25).	A-10
Supplementary Table 7: Results for the Human Serum-Titer Screening (Donor-N° 26 to 30).	A-11
Supplementary Table 8: Results for the Human Serum-Titer Screening (Donor-N° 31 to 35).	A-12
Supplementary Table 9: Immunization Scheme for CGI/LST3742 and LST3885	A-13
Supplementary Table 10: Overview of All Dose-Titrations with <i>S. pneumoniae</i> Strains	A-15
Supplementary Table 11: Overview of All <i>in vivo</i> Efficacy Experiments with HI-Sera	A-15
Supplementary Table 12: Log-Rank (Mantel-Cox) Test for the <i>in vivo</i> Efficacy of Polyclonal Anti-PspA Antibodies.....	A-16
Supplementary Table 13: Log-Rank (Mantel-Cox) Test for the <i>in vivo</i> Efficacy of Polyclonal Anti-PLY Antibodies.....	A-16
Supplementary Table 14: Results for the IL-6 ELISA	A-17
Supplementary Table 15: Results for the IL-6 ELISA and Correlation to Subsequent Challenge Survival.....	A-18

H - APPENDICES**A-1 TABLE OF ABBREVIATIONS**

Ab	antibody
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
BSA	bovine serum albumin
CD(14)	cluster of differentiation (14)
cfu	colony forming unit
dH ₂ O	distilled/ultrapure water
DMEM-medium	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbant assay
Fab	antigen-binding fragment of an antibody
FACS	fluorescence activated cell sorting
Fc	crystallizable fragment of an antibody
FCS	fetal calf serum
FL	full-length
GAS	group A streptococcus, <i>Streptococcus pyogenes</i>
gDNA	genomic DNA
HBSS	Hank's Buffered Salt Solution
HEK293 cells	human embryonic kidney 293 cells
HI (-serum)	hyperimmune (-serum)
HIC	hydrophobic interaction chromatography
His-tag	histidine-tag
HL-60 cell(s)	human promyelocytic leukemia cell(s)
hPBMC(s)	human peripheral blood mononuclear cell(s)
HPH	high-pressure homogenization
hRBC(s)	human red blood cell(s)
HRP	horse-reddish peroxidase

HU	hemolytic unit
IFN- γ	interferon- γ
Ig (G)	immunoglobulin (G)
IL (-6)	interleukin (-6)
IMAC	immobilized metal ion affinity chromatography
<i>i.n.</i>	intranasal
<i>i.p.</i>	intraperitoneal
IPTG	isopropyl β -D-thiogalacto-pyranoside
<i>i.v.</i>	intravenous
LB-medium	Luria-Bertani medium
LPS	lipopolysaccharide
LTA	lipoteichoic acid
mAb	monoclonal antibody
MHC	major histocompatibility complex
MWCO	molecular weight cut-off
MyD88	myeloid differentiation primary response gene (88)
NCBI	National Center for Biotechnology Information
OD	optical density
o/n	over night
OPK	opsonophagocytic killing
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with 0.1 % Tween TM -20
PCR	polymerase chain reaction
PLY	pneumolysin
PMNL(s)	polymorphonuclear leukocyte(s)
PR (-region)	proline rich (-region)
PspA	pneumococcal surface protein A
RPMI 1640-medium	Roswell Park Memorial Institute 1640-medium
RT	room temperature
s.c.	subcutaneous

H – APPENDICES

scFV	single-chain variable fragment
SDS	sodium dodecyl sulfate
TAE-buffer	Tris-acetate-EDTA buffer
Taq polymerase	polymerase from <i>Thermus aquaticus</i>
Th1/2/17	T-helper cell type 1, 2 or 17
THB	Todd Hewitt Broth
THY	Todd Hewitt Broth with 0.5 % yeast extract
TLR	toll like receptor
WTA	wall teichoic acid

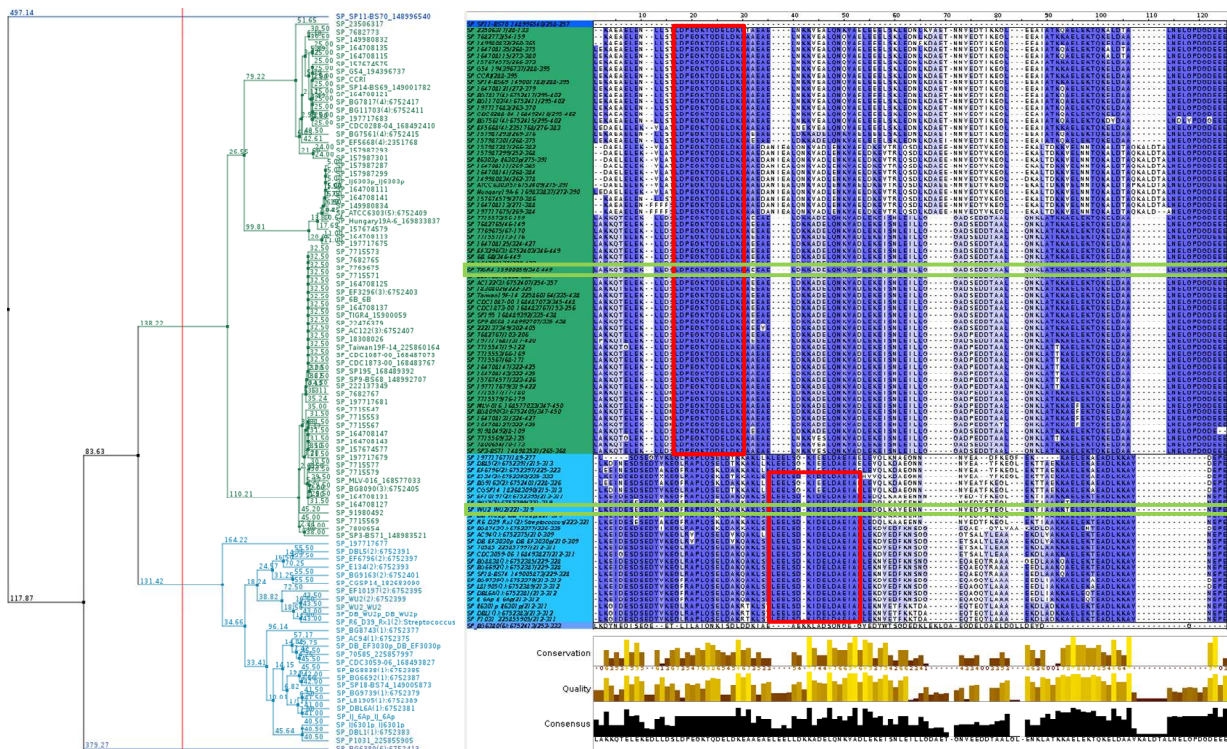
A-2 PRIMER SEQUENCES

Supplementary Table 1: Overview of all Primers Used

Primer ID	Specification	5'-Sequence-3'	Orientation
102	pET 16b T7 promoter, in MCS	TAATACGACTCACTATAGGG	S
103	pET 16b T7 terminator, in MCS	GCTAGTATTGCTCAGCGG	AS
210-8769	Ply_wt/pet28b+	ATATATCCATGGCAAATAAAGCAGTAAATGACTTTATACTAG	S
210-8770	Ply_wt/pet28b+	ATATATCTCGAGTCAITTTTCTACCTTATCTTCTACCTGAGGATAG	AS
210-8771	Ply_mut/pet28b+	CATACTGCATTCTGGGACATTATTGAC	S
210-8772	Ply_mut/pet28b+	GTCATAATGTCCAAGAATGCAGTATG	AS
210-8773	PspA_B_T4	ATATATCCATGGCTTTAGCAAAAAACAAACAGAACTTGAAAAAC	S
210-8774	PspA_B_T4	ATATATCTCGAGTTCCTTCTCATCTCCATCAGGGCC	AS
210-8775	PspA_B_WU2	ATATATCCATGGCTCTCAAAGAGATTGATGAGTCTGAATCAG	S
210-8776	PspA_B_WU2	ATATATCTCGAGTTCGGCTCATAAAGTCTTCTTAAGG	AS
210-8779	PspA_FL_T4	ATATATCCATGGAAAGTCTCCACAAGTTGTCGAAAAATC	S
210-8780	PspA_FL_T4	ATATATCTCGAGAACCCATTCACCATGGCATTGAC	AS
210-8781	PspA_FL_WU2	ATATATCCATGGAAAGTCTCCCGTAGCTAGTCACTAAAGC	S
210-8782	PspA_FL_WU2	ATATATCTCGAGAACCCATTCACCGTTTTCATTGAC	AS

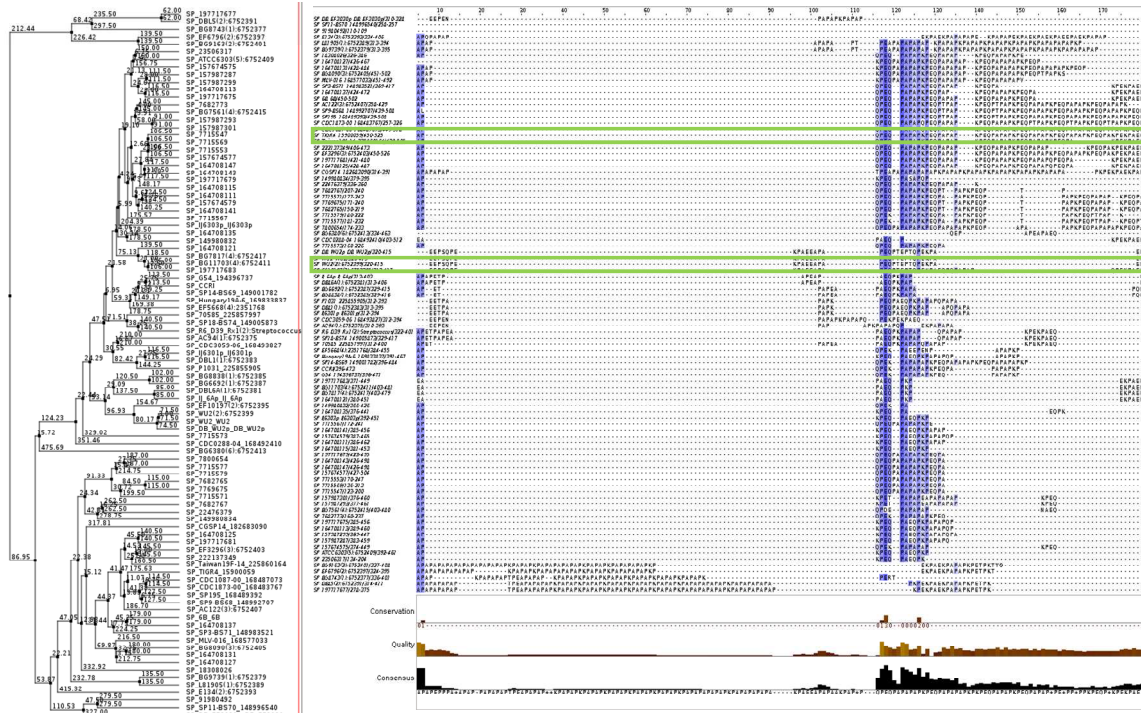
N.B.: S = sense; AS = anti-sense

A-3 PROTEIN SEQUENCE ALIGNMENTS OF PspA FROM SEVERAL PNEUMOCOCCAL STRAINS

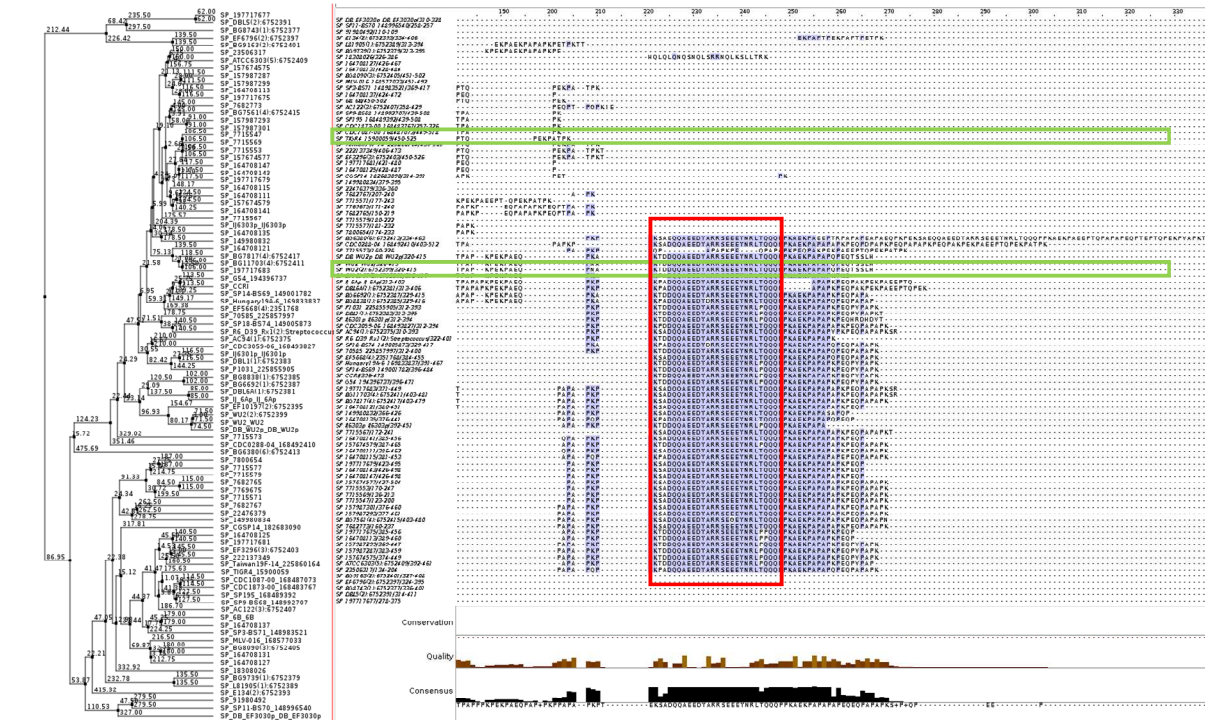


Supplementary Figure 1: Sequence Alignments of the B-Region of PspA. The alignments were calculated using all protein-sequence information published for PspA from different *S. pneumoniae* strains by the NCBI, which are represented by the cladogram to the left of the alignments. The B-regions of PspA from TIGR4 (upper green box) and WU2 (lower green box) are indicated within the red boxes. (Sequence alignments by Wolfgang Schüler, Intercell AG)

H – APPENDICES



Supplementary Figure 2: Sequence Alignments of the Pro-Rich Region of PspA (aa 1–180). The alignments were calculated using all protein-sequence information published for PspA from different *S. pneumoniae* strains by the NCBI, which are represented by the cladogram to the left of the alignments. The proline-rich regions of PspA from TIGR4 (upper green box) and WU2 (lower green box) are indicated. (Sequence alignments by Wolfgang Schüler, Intercell AG)



Supplementary Figure 3: Sequence Alignments of the Pro-Rich Region of PspA (aa 180–335). The alignments were calculated using all protein-sequence information published for PspA from different *S. pneumoniae* strains by the NCBI, which are represented by the cladogram to the left of the alignments. The proline-rich regions of PspA from TIGR4 (upper green box) and WU2 (lower green box) are indicated. The red box highlights the non-proline block within the proline-rich region of PspA from WU2. (Sequence alignments by Wolfgang Schüler, Intercell AG)

A-4 SERUM-SCREENINGS OF HUMAN BLOOD DONORS

ELISA-Results for all Donors Screened:

The tables in this section (Supplementary Table 2 to

Supplementary Table 8) represent the results (OD₄₀₅) obtained for all human donor sera screened in duplicates for the stated antigens.

Supplementary Table 2: Results for the Human Serum-Titer Screening (Donor-N° 1 to 5)

Antigen	Dilution	Donor-N° 1		Donor-N° 2		Donor-N° 3		Donor-N° 4		Donor-N° 5	
		average	stdev	average	stdev	average	stdev	average	stdev	average	stdev
Ply_wt	200	0.514	0.013	1.108	0.034	0.421	0.067	0.962	0.023	0.924	0.001
	1000	0.145	0.002	0.850	0.108	0.110	0.005	0.350	0.018	0.357	0.012
	5000	0.038	0.013	0.327	0.071	0.025	0.004	0.110	0.019	0.090	0.002
	25000	0.005	0.001	0.086	0.018	0.002	0.000	0.031	0.008	0.015	0.004
Ply_mut	200	0.800	0.008	1.196	0.035	0.720	0.078	1.101	0.002	1.114	0.004
	1000	0.235	0.006	1.116	0.019	0.214	0.026	0.537	0.003	0.588	0.017
	5000	0.052	0.003	0.557	0.073	0.044	0.004	0.132	0.001	0.147	0.004
	25000	0.008	0.004	0.208	0.052	0.007	0.002	0.029	0.003	0.027	0.000
PspA_B_T4	200	0.486	0.083	1.004	0.037	0.653	0.062	0.772	0.026	0.796	0.027
	1000	0.123	0.013	0.279	0.068	0.131	0.018	0.202	0.001	0.204	0.015
	5000	0.017	0.001	0.031	0.008	0.018	0.002	0.036	0.000	0.035	0.002
	25000	-0.004	0.005	0.000	0.009	-0.006	0.002	0.004	0.003	-0.003	0.001
PspA_B_WU2	200	0.624	0.039	0.889	0.065	0.999	0.040	0.936	0.011	1.031	0.009
	1000	0.135	0.006	0.266	0.045	0.322	0.051	0.281	0.006	0.355	0.004
	5000	0.020	0.000	0.053	0.006	0.062	0.018	0.050	0.000	0.063	0.002
	25000	-0.003	0.003	0.009	0.001	0.002	0.005	0.008	0.004	0.005	0.001
PspA_Pro+PartB_T4	200	0.867	0.081	1.111	0.114	0.942	0.013	1.135	0.109	0.980	0.000
	1000	0.234	0.011	0.367	0.069	0.273	0.023	0.673	0.032	0.313	0.019
	5000	0.042	0.001	0.068	0.016	0.051	0.008	0.174	0.018	0.062	0.004
	25000	0.006	0.003	0.009	0.004	0.005	0.003	0.032	0.001	0.007	0.000
Pro+PartB_WU2	200	0.992	0.009	1.202	0.034	0.819	0.073	1.269	0.014	1.092	0.021
	1000	0.275	0.013	0.505	0.125	0.201	0.011	0.639	0.023	0.507	0.036
	5000	0.050	0.005	0.108	0.037	0.208	0.013	0.193	0.007	0.246	0.036
	25000	0.032	0.037	0.020	0.002	0.136	0.019	0.050	0.047	0.153	0.013
PspA_Pro_T4	200	0.888	0.026	1.012	0.033	0.833	0.038	1.165	0.013	0.900	0.058
	1000	0.225	0.006	0.320	0.050	0.214	0.010	0.644	0.025	0.253	0.021
	5000	0.050	0.005	0.063	0.008	0.044	0.006	0.167	0.013	0.056	0.008
	25000	0.005	0.004	0.015	0.004	0.003	0.001	0.043	0.004	0.004	0.001
PapA_Pro_WU2	200	1.009	0.062	1.223	0.119	0.754	0.107	1.285	0.003	1.149	0.033
	1000	0.343	0.073	0.598	0.042	0.344	0.010	0.590	0.016	0.640	0.042
	5000	0.200	0.060	0.205	0.035	0.213	0.006	0.298	0.003	0.254	0.045
	25000	0.107	0.139	0.019	0.005	0.172	0.009	0.020	0.003	0.095	0.079

Color-Code

OD ₄₀₅
>1
0.5-1
<0.5

Supplementary Table 3: Results for the Human Serum-Titer Screening (Donor-N° 6 to 10)

Antigen	Dilution	Donor-N° 6		Donor-N° 7		Donor-N° 8		Donor-N° 9		Donor-N° 10	
		average	stdev	average	stdev	average	stdev	average	stdev	average	stdev
Ply_wt	200	0.676	0.002	0.538	0.001	0.512	0.071	0.962	0.020	0.487	0.051
	1000	0.215	0.021	0.183	0.018	0.146	0.027	0.404	0.005	0.137	0.022
	5000	0.047	0.002	0.057	0.003	0.021	0.014	0.127	0.035	0.027	0.002
	25000	0.010	0.001	0.012	0.001	0.016	0.004	0.019	0.001	0.002	0.002
Ply_mut	200	1.048	0.007	0.777	0.006	0.951	0.072	1.162	0.030	0.759	0.049
	1000	0.457	0.015	0.263	0.007	0.323	0.058	0.841	0.028	0.226	0.030
	5000	0.101	0.013	0.065	0.001	0.065	0.009	0.242	0.005	0.047	0.006
	25000	0.023	0.000	0.012	0.000	0.019	0.002	0.053	0.001	0.009	0.001
PspA_B_T4	200	0.356	0.012	0.941	0.002	0.316	0.056	0.178	0.020	0.984	0.069
	1000	0.056	0.004	0.269	0.008	0.055	0.007	0.025	0.000	0.271	0.043
	5000	-0.001	0.001	0.047	0.001	0.004	0.001	-0.003	0.001	0.048	0.011
	25000	-0.006	0.000	0.000	0.001	-0.004	0.001	-0.011	0.000	0.004	0.004
PspA_B_WU2	200	0.964	0.016	1.136	0.019	0.454	0.084	0.969	0.043	0.228	0.033
	1000	0.292	0.005	0.582	0.018	0.078	0.016	0.271	0.001	0.042	0.008
	5000	0.060	0.012	0.127	0.003	0.005	0.003	0.046	0.001	0.001	0.004
	25000	0.007	0.000	0.020	0.002	0.001	0.001	0.000	0.001	-0.002	0.004
PspA_Pro+PartB_T4	200	0.843	0.004	1.085	0.012	1.058	0.054	0.928	0.001	0.902	0.045
	1000	0.230	0.010	0.408	0.002	0.406	0.056	0.256	0.002	0.245	0.031
	5000	0.042	0.004	0.085	0.001	0.084	0.014	0.043	0.001	0.051	0.003
	25000	0.007	0.001	0.012	0.001	0.020	0.006	0.003	0.000	0.254	0.308
Pro+PartB_WU2	200	1.176	0.044	1.135	0.040	0.748	0.114	1.116	0.035	1.001	0.036
	1000	0.463	0.024	0.767	0.040	0.278	0.014	0.553	0.007	0.363	0.012
	5000	0.143	0.032	0.308	0.018	0.154	0.012	0.210	0.037	0.144	0.006
	25000	0.044	0.003	0.162	0.023	0.010	0.001	0.151	0.002	0.007	0.006
PspA_Pro_T4	200	0.861	0.009	1.121	0.004	0.978	0.079	0.951	0.016	0.793	0.076
	1000	0.238	0.005	0.387	0.001	0.267	0.045	0.243	0.001	0.193	0.030
	5000	0.054	0.011	0.097	0.003	0.050	0.004	0.066	0.028	0.042	0.002
	25000	0.013	0.005	0.017	0.004	0.013	0.008	0.005	0.002	0.008	0.000
PapA_Pro_WU2	200	1.178	0.004	1.167	0.039	0.669	0.097	1.081	0.002	0.995	0.042
	1000	0.505	0.028	0.972	0.115	0.295	0.018	0.533	0.056	0.377	0.069
	5000	0.265	0.006	0.358	0.010	0.203	0.040	0.224	0.009	0.186	0.097
	25000	0.021	0.001	0.165	0.009	0.009	0.004	0.138	0.008	0.007	0.002

Color-Code

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>1
0.5-1
<0.5

Supplementary Table 4: Results for the Human Serum-Titer Screening (Donor-N° 11 to 15)

Antigen	Dilution	Donor-N° 11		Donor-N° 12		Donor-N° 13		Donor-N° 14		Donor-N° 15	
		average	stdev	average	stdev	average	stdev	average	stdev	average	stdev
Ply_wt	200	0.784	0.024	1.263	0.042	0.945	0.035	1.129	0.002	1.139	0.026
	1000	0.225	0.021	1.117	0.016	0.356	0.002	0.731	0.138	0.937	0.014
	5000	0.066	0.010	0.522	0.006	0.090	0.001	0.200	0.028	0.337	0.002
	25000	0.009	0.004	0.127	0.005	0.032	0.002	0.038	0.005	0.095	0.008
Ply_mut	200	1.043	0.007	1.175	0.028	0.897	0.030	1.070	0.003	1.164	0.021
	1000	0.420	0.016	1.089	0.004	0.337	0.011	0.553	0.004	0.911	0.042
	5000	0.097	0.000	0.523	0.028	0.078	0.004	0.142	0.004	0.364	0.001
	25000	0.019	0.004	0.124	0.006	0.021	0.002	0.027	0.003	0.097	0.000
PspA_B_T4	200	1.023	0.008	0.778	0.037	0.810	0.006	1.140	0.006	0.218	0.012
	1000	0.257	0.013	0.178	0.008	0.235	0.022	0.669	0.019	0.065	0.001
	5000	0.053	0.001	0.033	0.013	0.055	0.033	0.193	0.039	0.007	0.001
	25000	0.003	0.002	-0.011	0.004	0.005	0.019	0.045	0.016	-0.012	0.001
PspA_B_WU2	200	0.880	0.074	1.147	0.055	0.915	0.045	1.027	0.006	0.515	0.023
	1000	0.237	0.015	0.660	0.041	0.280	0.011	0.353	0.018	0.136	0.013
	5000	0.067	0.016	0.228	0.052	0.057	0.003	0.139	0.094	0.033	0.016
	25000	0.003	0.005	0.077	0.032	0.022	0.013	0.011	0.003	0.024	0.029
PspA_Pro+PartB_T4	200	1.207	0.011	1.252	0.012	1.201	0.153	1.191	0.003	1.007	0.013
	1000	0.654	0.048	1.245	0.170	0.568	0.139	0.889	0.003	0.462	0.018
	5000	0.145	0.023	0.686	0.178	0.174	0.112	0.291	0.018	0.144	0.016
	25000	0.035	0.019	0.289	0.210	0.087	0.078	0.097	0.058	0.088	0.025
Pro+PartB_WU2	200	1.220	0.011	1.166	0.017	1.279	0.144	1.131	0.004	0.794	0.039
	1000	0.808	0.047	1.040	0.006	0.571	0.151	0.506	0.026	0.224	0.013
	5000	0.194	0.026	0.340	0.001	0.095	0.016	0.119	0.017	0.042	0.001
	25000	0.043	0.002	0.225	0.030	0.021	0.005	0.029	0.004	0.009	0.001
PspA_Pro_T4	200	1.163	0.017	1.190	0.107	0.864	0.063	1.130	0.029	0.819	0.018
	1000	0.589	0.036	1.043	0.117	0.252	0.001	0.753	0.017	0.254	0.016
	5000	0.137	0.015	0.485	0.054	0.046	0.002	0.232	0.007	0.056	0.006
	25000	0.032	0.004	0.130	0.024	0.033	0.018	0.053	0.001	0.021	0.001
PapA_Pro_WU2	200	1.179	0.057	1.233	0.007	0.998	0.018	1.049	0.011	0.724	0.019
	1000	0.813	0.030	0.904	0.021	0.345	0.021	0.400	0.028	0.196	0.007
	5000	0.260	0.071	0.247	0.016	0.081	0.001	0.110	0.014	0.046	0.003
	25000	0.114	0.098	0.052	0.005	0.021	0.003	0.021	0.005	0.017	0.006

Color-Code

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>1
0.5-1
<0.5

Supplementary Table 5: Results for the Human Serum-Titer Screening (Donor-N° 16 to 20)

Antigen	Dilution	Donor-N° 16		Donor-N° 17		Donor-N° 18		Donor-N° 19		Donor-N° 20	
		average	stdev	average	stdev	average	stdev	average	stdev	average	stdev
Ply_wt	200	1.020	0.009	1.209	0.012	1.019	0.003	1.136	0.042	0.963	0.076
	1000	0.419	0.030	1.073	0.018	0.554	0.003	0.717	0.033	0.426	0.059
	5000	0.110	0.001	0.497	0.002	0.159	0.003	0.224	0.018	0.118	0.002
	25000	0.020	0.002	0.127	0.002	0.034	0.001	0.069	0.001	0.024	0.000
Ply_mut	200	1.055	0.003	1.184	0.013	1.069	0.015	1.118	0.073	1.125	0.049
	1000	0.506	0.027	1.037	0.006	0.551	0.004	0.655	0.049	0.432	0.004
	5000	0.126	0.001	0.242	0.330	0.142	0.003	0.188	0.016	0.102	0.003
	25000	0.024	0.001	0.064	0.076	0.030	0.001	0.051	0.009	0.019	0.005
PspA_B_T4	200	0.849	0.002	0.083	0.004	0.508	0.027	0.734	0.027	0.307	0.003
	1000	0.207	0.008	0.019	0.005	0.112	0.001	0.220	0.016	0.066	0.003
	5000	0.034	0.010	-0.016	0.001	0.021	0.013	0.056	0.006	0.007	0.004
	25000	0.006	0.014	-0.021	0.000	-0.005	0.012	0.028	0.010	0.000	0.019
PspA_B_WU2	200	0.846	0.010	0.462	0.019	0.961	0.016	0.775	0.011	0.610	0.005
	1000	0.248	0.008	0.139	0.009	0.297	0.016	0.255	0.013	0.146	0.004
	5000	0.058	0.001	0.023	0.001	0.072	0.005	0.056	0.000	0.036	0.006
	25000	0.027	0.004	0.002	0.002	0.024	0.011	0.013	0.003	0.008	0.002
PspA_Pro+PartB_T4	200	0.898	0.019	1.074	0.006	1.030	0.206	1.111	0.047	1.179	0.005
	1000	0.454	0.274	0.463	0.006	0.648	0.001	0.560	0.131	0.635	0.017
	5000	0.067	0.017	0.120	0.008	0.121	0.093	0.146	0.029	0.171	0.023
	25000	0.011	0.010	0.026	0.004	0.025	0.029	0.040	0.024	0.051	0.008
Pro+PartB_WU2	200	0.808	0.013	0.999	0.008	1.198	0.006	1.210	0.007	1.141	0.020
	1000	0.143	0.006	0.368	0.005	0.680	0.028	0.906	0.011	0.428	0.030
	5000	0.028	0.002	0.093	0.025	0.155	0.010	0.260	0.001	0.083	0.015
	25000	0.001	0.006	0.031	0.004	0.041	0.006	0.069	0.003	0.014	0.002
PspA_Pro_T4	200	0.757	0.022	1.019	0.053	1.130	0.004	1.120	0.039	0.993	0.014
	1000	0.191	0.012	0.369	0.008	0.521	0.025	0.496	0.030	0.295	0.003
	5000	0.079	0.034	0.077	0.001	0.134	0.011	0.113	0.008	0.062	0.001
	25000	0.017	0.012	0.024	0.006	0.036	0.006	0.024	0.002	0.017	0.011
PapA_Pro_WU2	200	0.746	0.018	0.907	0.013	1.134	0.025	1.182	0.002	0.950	0.025
	1000	0.155	0.001	0.298	0.006	0.512	0.031	0.721	0.005	0.227	0.011
	5000	0.033	0.004	0.077	0.021	0.119	0.012	0.231	0.176	0.041	0.001
	25000	0.004	0.003	0.066	0.057	0.023	0.001	0.134	0.170	0.006	0.001

Color-Code

OD ₄₀₅
>1
0.5-1
<0.5

Supplementary Table 6: Results for the Human Serum-Titer Screening (Donor-N° 21 to 25)

Antigen	Dilution	Donor-N° 21		Donor-N° 22		Donor-N° 23		Donor-N° 24		Donor-N° 25	
		average	stdev	average	stdev	average	stdev	average	stdev	average	stdev
Ply_wt	200	1.149	0.021	0.782	0.256	0.844	0.039	0.627	0.238	1.139	0.025
	1000	0.736	0.014	0.392	0.029	0.339	0.004	0.279	0.002	0.728	0.011
	5000	0.233	0.012	0.124	0.010	0.090	0.001	0.082	0.004	0.221	0.005
	25000	0.058	0.009	0.044	0.008	0.018	0.001	0.027	0.000	0.042	0.004
Ply_mut	200	1.149	0.018	0.911	0.002	0.965	0.020	0.885	0.035	1.169	0.002
	1000	0.735	0.001	0.372	0.066	0.361	0.021	0.328	0.003	0.710	0.004
	5000	0.223	0.022	0.111	0.030	0.112	0.013	0.089	0.001	0.231	0.006
	25000	0.057	0.006	0.028	0.008	0.016	0.004	0.023	0.001	0.043	0.004
PspA_B_T4	200	0.586	0.030	0.659	0.057	0.602	0.031	0.690	0.238	0.487	0.005
	1000	0.174	0.006	0.250	0.011	0.240	0.022	0.342	0.002	0.161	0.008
	5000	0.042	0.006	0.086	0.008	0.075	0.020	0.145	0.004	0.047	0.008
	25000	0.012	0.005	0.040	0.008	0.010	0.002	0.089	0.000	0.038	0.047
PspA_B_WU2	200	0.820	0.008	1.027	0.076	0.561	0.032	0.146	0.006	0.246	0.018
	1000	0.249	0.011	0.434	0.032	0.164	0.007	0.049	0.011	0.066	0.002
	5000	0.055	0.004	0.101	0.005	0.036	0.005	0.017	0.019	0.010	0.001
	25000	0.011	0.008	0.023	0.004	0.005	0.001	-0.005	0.004	0.007	0.014
PspA_Pro+PartB_T4	200	1.292	0.004	1.208	0.032	0.902	0.021	0.823	0.006	1.156	0.040
	1000	1.093	0.040	0.670	0.015	0.400	0.004	0.309	0.003	0.707	0.009
	5000	0.436	0.017	0.227	0.004	0.146	0.004	0.130	0.004	0.228	0.001
	25000	0.152	0.001	0.104	0.003	0.083	0.004	0.086	0.008	0.100	0.001
Pro+PartB_WU2	200	1.195	0.021	1.088	0.016	0.645	0.037	0.776	0.023	1.097	0.004
	1000	0.802	0.050	0.417	0.006	0.165	0.004	0.225	0.000	0.560	0.021
	5000	0.230	0.006	0.085	0.002	0.037	0.001	0.042	0.004	0.134	0.004
	25000	0.046	0.004	0.015	0.004	-0.001	0.004	-0.003	0.002	0.016	0.003
PspA_Pro_T4	200	1.158	0.036	0.812	0.326	0.549	0.044	0.544	0.045	0.923	0.006
	1000	0.833	0.026	0.415	0.016	0.192	0.003	0.142	0.001	0.430	0.010
	5000	0.272	0.001	0.096	0.001	0.048	0.001	0.031	0.002	0.111	0.004
	25000	0.063	0.008	0.024	0.001	0.010	0.001	0.007	0.000	0.021	0.001
PapA_Pro_WU2	200	1.058	0.055	1.079	0.029	0.397	0.003	0.786	0.013	0.689	0.004
	1000	0.711	0.030	0.469	0.004	0.201	0.000	0.220	0.004	0.384	0.001
	5000	0.199	0.003	0.089	0.004	0.034	0.001	0.035	0.004	0.086	0.004
	25000	0.035	0.006	0.010	0.003	-0.009	0.001	-0.006	0.000	0.007	0.001

Color-Code

OD ₄₀₅
>1
0.5-1
<0.5

Supplementary Table 7: Results for the Human Serum-Titer Screening (Donor-N° 26 to 30)

Antigen	Dilution	Donor-N° 26		Donor-N° 27		Donor-N° 28		Donor-N° 29		Donor-N° 30	
		average	stdev	average	stdev	average	stdev	average	stdev	average	stdev
Ply_wt	200	0.791	0.011	1.197	0.018	1.208	0.025	1.104	0.006	1.095	0.013
	1000	0.291	0.003	0.890	0.022	1.076	0.011	0.588	0.006	0.498	0.016
	5000	0.081	0.006	0.281	0.001	0.520	0.025	0.164	0.008	0.161	0.006
	25000	0.026	0.011	0.059	0.004	0.135	0.008	0.028	0.002	0.036	0.000
Ply_mut	200	0.806	0.003	1.185	0.021	1.195	0.008	1.136	0.004	1.105	0.018
	1000	0.285	0.001	0.868	0.023	1.080	0.018	0.627	0.023	0.545	0.016
	5000	0.086	0.010	0.281	0.003	0.567	0.041	0.187	0.016	0.152	0.002
	25000	0.011	0.004	0.060	0.007	0.145	0.006	0.036	0.001	0.030	0.001
PspA_B_T4	200	0.907	0.012	0.798	0.054	0.678	0.019	0.599	0.033	0.734	0.027
	1000	0.272	0.008	0.290	0.004	0.173	0.002	0.198	0.002	0.220	0.016
	5000	0.204	0.192	0.082	0.010	0.043	0.009	0.051	0.002	0.056	0.006
	25000	0.012	0.005	0.019	0.006	0.001	0.000	0.015	0.017	0.028	0.010
PspA_B_WU2	200	0.670	0.000	0.528	0.019	0.775	0.008	0.890	0.024	0.560	0.008
	1000	0.242	0.031	0.152	0.013	0.221	0.014	0.286	0.006	0.148	0.013
	5000	0.059	0.012	0.029	0.002	0.057	0.007	0.064	0.007	0.040	0.010
	25000	0.015	0.006	0.011	0.016	0.014	0.001	0.012	0.001	0.011	0.005
PspA_Pro+PartB_T4	200	1.117	0.011	0.948	0.009	0.954	0.023	1.214	0.018	0.955	0.023
	1000	0.455	0.001	0.393	0.011	0.332	0.006	0.837	0.004	0.352	0.006
	5000	0.151	0.003	0.151	0.010	0.120	0.006	0.247	0.004	0.127	0.006
	25000	0.075	0.001	0.077	0.005	0.066	0.002	0.092	0.006	0.068	0.001
Pro+PartB_WU2	200	0.837	0.021	0.822	0.027	0.820	0.000	1.168	0.005	0.907	0.023
	1000	0.240	0.000	0.234	0.007	0.183	0.001	0.804	0.034	0.267	0.014
	5000	0.047	0.008	0.043	0.003	0.026	0.003	0.235	0.010	0.060	0.020
	25000	-0.005	0.002	-0.005	0.004	-0.010	0.001	0.041	0.006	-0.002	0.004
PspA_Pro_T4	200	0.702	0.006	0.379	0.001	0.668	0.001	1.010	0.004	0.829	0.003
	1000	0.187	0.008	0.134	0.004	0.172	0.016	0.570	0.021	0.256	0.012
	5000	0.037	0.001	0.032	0.004	0.029	0.000	0.148	0.008	0.055	0.002
	25000	0.009	0.001	0.005	0.000	0.006	0.000	0.030	0.003	0.013	0.000
PapA_Pro_WU2	200	0.673	0.004	0.388	0.001	0.746	0.033	0.919	0.007	0.836	0.010
	1000	0.162	0.004	0.208	0.001	0.182	0.001	0.803	0.027	0.285	0.010
	5000	0.041	0.020	0.053	0.005	0.021	0.003	0.245	0.016	0.071	0.001
	25000	-0.010	0.000	-0.004	0.001	-0.009	0.001	0.055	0.003	-0.005	0.002

Color-Code

OD ₄₀₅
>1
0.5-1
<0.5

Supplementary Table 8: Results for the Human Serum-Titer Screening (Donor-N° 31 to 35)

Antigen	Dilution	Donor-N° 31		Donor-N° 32		Donor-N° 33		Donor-N° 34		Donor-N° 35	
		average	stdev	average	stdev	average	stdev	average	stdev	average	stdev
Ply_wt	200	1.168	0.030	1.217	0.013	1.202	0.060	0.876	0.053	1.112	0.105
	1000	0.836	0.047	1.019	0.017	0.748	0.005	0.318	0.022	0.634	0.023
	5000	0.306	0.005	0.398	0.018	0.233	0.017	0.096	0.006	0.186	0.011
	25000	0.063	0.003	0.114	0.010	0.058	0.008	0.023	0.007	0.048	0.006
Ply_mut	200	1.178	0.006	1.238	0.064	1.250	0.022	0.977	0.071	1.067	0.054
	1000	0.870	0.016	1.105	0.062	0.929	0.033	0.395	0.013	0.845	0.011
	5000	0.306	0.005	0.503	0.009	0.289	0.012	0.119	0.018	0.264	0.018
	25000	0.067	0.001	0.130	0.004	0.076	0.008	0.016	0.002	0.075	0.030
PspA_B_T4	200	1.072	0.033	0.445	0.045	0.982	0.028	0.427	0.001	0.543	0.009
	1000	0.519	0.040	0.152	0.007	0.320	0.009	0.178	0.021	0.129	0.002
	5000	0.234	0.091	0.047	0.011	0.066	0.000	0.073	0.021	0.023	0.000
	25000	0.037	0.023	0.006	0.007	0.012	0.000	0.003	0.001	0.003	0.000
PspA_B_WU2	200	0.280	0.003	0.765	0.025	0.875	0.022	0.258	0.001	0.447	0.008
	1000	0.073	0.004	0.308	0.025	0.262	0.004	0.055	0.002	0.115	0.004
	5000	0.043	0.033	0.073	0.004	0.059	0.021	0.016	0.004	0.008	0.002
	25000	0.009	0.000	0.019	0.006	0.004	0.005	0.005	0.013	-0.011	0.001
PspA_Pro+PartB_T4	200	1.149	0.025	1.173	0.069	1.230	0.053	0.980	0.035	1.125	0.090
	1000	0.731	0.020	0.734	0.035	1.014	0.005	0.453	0.007	0.552	0.004
	5000	0.244	0.011	0.251	0.005	0.368	0.001	0.155	0.001	0.173	0.001
	25000	0.087	0.001	0.095	0.004	0.128	0.001	0.071	0.000	0.079	0.002
Pro+PartB_WU2	200	0.988	0.012	1.190	0.080	1.186	0.083	1.134	0.025	1.091	0.032
	1000	0.399	0.008	0.756	0.076	0.985	0.023	1.123	0.025	0.439	0.021
	5000	0.084	0.005	0.207	0.024	0.318	0.024	0.665	0.054	0.091	0.011
	25000	0.009	0.008	0.046	0.011	0.076	0.008	0.186	0.013	0.025	0.008
PspA_Pro_T4	200	0.653	0.035	1.179	0.017	1.215	0.033	0.954	0.057	0.964	0.036
	1000	0.323	0.004	0.708	0.008	0.902	0.041	0.365	0.000	0.376	0.018
	5000	0.082	0.001	0.192	0.006	0.277	0.032	0.095	0.007	0.065	0.004
	25000	0.018	0.001	0.026	0.000	0.046	0.007	0.001	0.002	-0.004	0.002
PapA_Pro_WU2	200	0.550	0.028	1.189	0.030	1.160	0.028	1.151	0.051	1.017	0.041
	1000	0.358	0.006	0.749	0.026	0.863	0.087	1.135	0.027	0.362	0.019
	5000	0.105	0.004	0.193	0.006	0.228	0.038	0.698	0.151	0.062	0.005
	25000	0.014	0.011	0.035	0.005	0.043	0.010	0.116	0.006	0.000	0.001

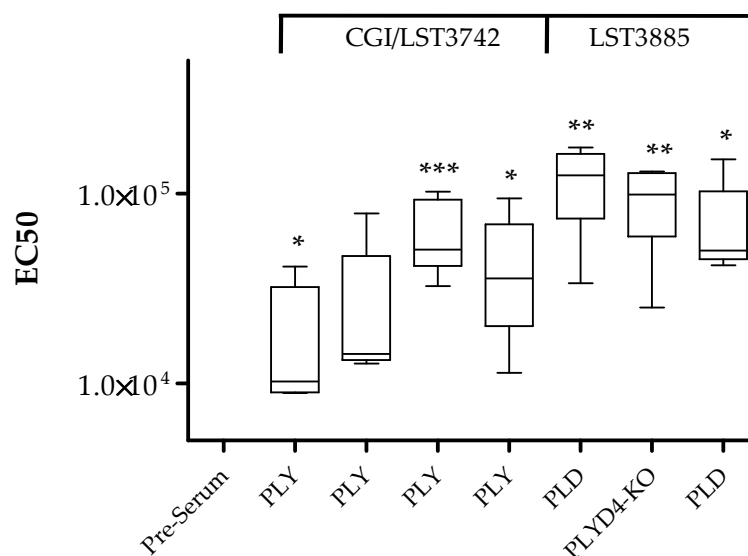
Color-Code

OD ₄₀₅
>1
0.5-1
<0.5

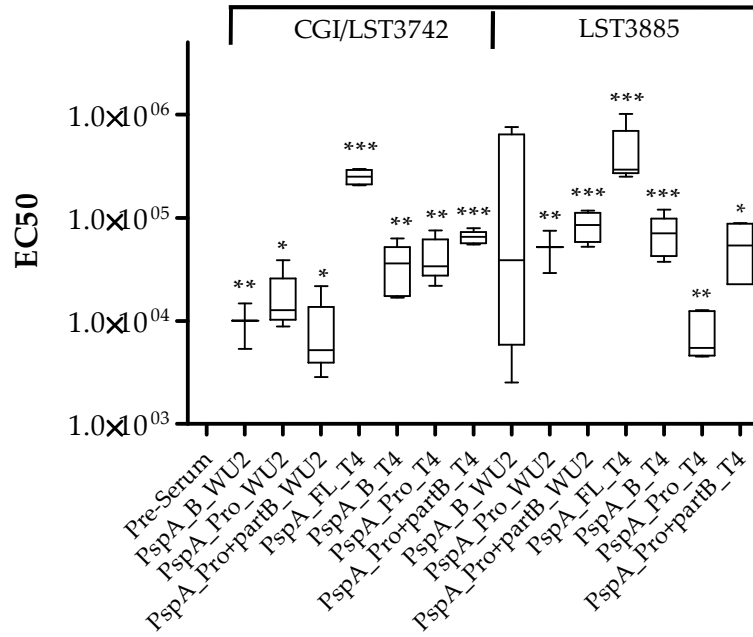
A-5 TITER-SCREENINGS OF MOUSE HYPERIMMUNE SERA

Supplementary Table 9: Immunization Scheme for CGI/LST3742 and LST3885

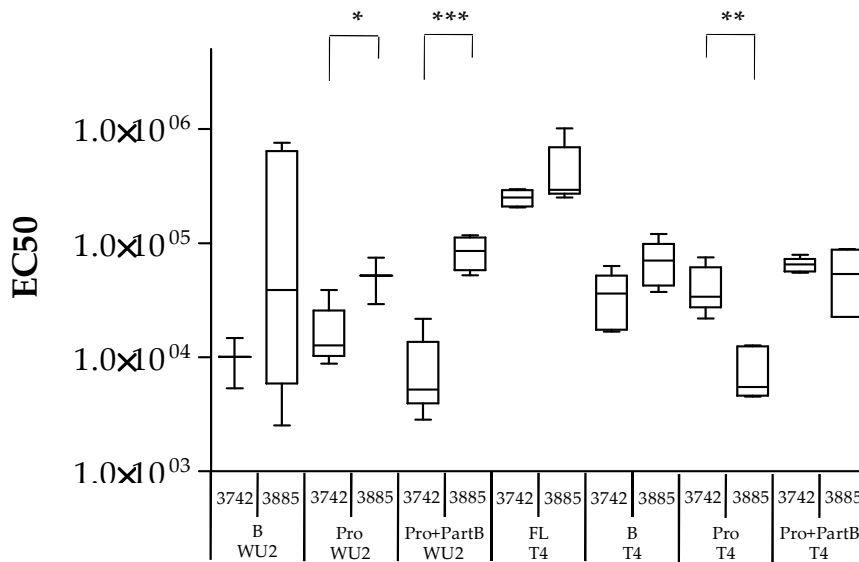
Immunization	Group-N°	Antigen	Amount of Antigen per Injection [µg/mouse]	Adjuvant	N° of Surviving Mice
CGI/LST3742	1	PLY	10	CFA/IFA	5
	2	PLY	10	Alum	5
	3	PLY	50	CFA/IFA	5
	4	PLY	50	Alum	5
	5	PLD	50	Alum	5
	6	PspA_B_WU2	50	Alum	2
	7	PspA_Pro_WU2	50	Alum	5
	8	PspA_Pro+PartB_WU2	50	Alum	5
	9	PspA_FL_T4	50	Alum	5
	10	PspA_B_T4	50	Alum	5
	11	PspA_Pro_T4	50	Alum	5
	12	PspA_Pro+PartB_T4	50	Alum	5
LST3885	1	PLYΔD4	50	Alum	5
	2	PLD	50	Alum	5
	3	PspA_B_WU2	50	Alum	5
	4	PspA_Pro_WU2	50	Alum	3
	5	PspA_Pro+PartB_WU2	50	Alum	4
	6	PspA_FL_T4	50	Alum	5
	7	PspA_B_T4	50	Alum	5
	8	PspA_Pro_T4	50	Alum	5
	9	PspA_Pro+PartB_T4	50	Alum	4



Supplementary Figure 4: EC50-Values for the Titer-Screening of all PLY-Groups. The results were statistically evaluated vs. pre-immune serum using a t-test (2-tailed, type 2) and the obtained values for $p < 0.001$ (***) , $p < 0.01$ (**) and $p < 0.05$ (*) are depicted.



Supplementary Figure 5: EC50-Values for the Titer-Screening of all PspA-Groups. The results were statistically evaluated vs. pre-immune serum using a t-test (2-tailed, type 2) and the obtained values for $p < 0.001$ (***) , $p < 0.01$ (**) and $p < 0.05$ (*) are depicted.



Supplementary Figure 6: Statistical Comparison of Hyperimmune Serum Titers. The antibody-titers obtained by immunizing mice with the same constructs are compared for the experiments CGI/LST3742 and LST3885 by means of a t-test (2-tailed, type 2) and the obtained values for $p < 0.001$ (***) , $p < 0.01$ (**) and $p < 0.05$ (*) are depicted.

A-6 IN VIVO VIRULENCE OF DIFFERENT *S. PNEUMONIAE* STRAINSSupplementary Table 10: Overview of All Dose-Titrations with *S. pneumoniae* Strains

Experiment-N°	<i>S. pneumoniae</i> Strain	Route of Application	Starting Bacterial Count [cfu]	
			Expected	Real
LST3869	D39	<i>i.p.</i>	1.00x10 ⁶	1.01x10 ⁶
LST3898	PJ1324 (6B)	<i>i.v.</i>	1.00x10 ⁶	8.14x10 ⁵
LST3898	TIGR4	<i>i.v.</i>	1.00x10 ⁶	1.58x10 ⁶
LST3898	D39	<i>i.v.</i>	1.00x10 ⁵	7.24x10 ⁴
LST3898	WU2	<i>i.v.</i>	1.00x10 ⁶	1.63x10 ⁶

A-7 SUMMARIZED DATA OF THE IN VIVO EFFICACY TESTING OF POLYCLONAL ANTIBODIES

Experimental Setup

Supplementary Table 11: Overview of All in vivo Efficacy Experiments with HI-Sera

Experiment-N°	<i>S. pneumoniae</i> Strain	Route of Application	Starting Bacterial Count [cfu]	
			Expected	Real
LST3869	PJ1324 (6B)	<i>i.p.</i>	1.0x10 ⁴	3.0x10 ⁴
LST3872	PJ1324 (6B)	<i>i.p.</i>	1.0x10 ⁴	1.06x10 ⁴
LST3886	D39	<i>i.p.</i>	1.0x10 ¹	9.02x10 ⁰
LST3897	A66.1	<i>i.n.</i>	5.0x10 ⁵	4.2x10 ⁵
LST3909	A66.1	<i>i.n.</i>	5.0x10 ⁵	4.0x10 ⁵
LST3910	TIGR4	<i>i.v.</i>	5.0x10 ³	6.0x10 ³
LST3911	WU2	<i>i.v.</i>	5.0x10 ⁵	8.8x10 ⁵

Statistical Evaluation of Challenge Survival (Mantel-Cox)

The tables in this section represent the calculated, statistical p-values (Log-rank (Mantel-Cox) test, using SE) in the course of challenge survival of each group tested against the mock-immunized PBS control-group used in each experiment. All statistical calculations were carried out using GraphPad Prism®, Version 5.01.

Supplementary Table 12: Log-Rank (Mantel-Cox) Test for the *in vivo* Efficacy of Polyclonal Anti-PspA Antibodies

Immunization with Serum <i>vs.</i>	Log-rank (Mantel-Cox) Test, p-value <i>vs.</i> PBS Group						
	LST3869	LST3872	LST3886	LST3897	LST3909	LST3910	LST3911
mouse pre-immune (neg. cntrl.)	0.6262	0.3173	1.0000	1.0000	0.5485	0.5258	0.5308
PspA (rabbit) (pos. cntrl.)	0.0017	0.0023	<i>n.d.</i>	0.0016	0.0023	0.7955	0.0126
PspA_FL_T4	0.0017	0.0023	0.3173	1.0000	0.5127	0.5571	0.5308
PspA_B_T4	0.0017	0.0023	1.0000	0.2207	0.8527	0.4477	0.9141
PspA_Pro+PartB_T4	0.0017	0.0023	0.3173	0.3532	0.0978	0.1729	0.1336
PspA_Pro_T4	0.2289	0.0023	0.3173	0.0016	0.2055	0.4473	0.6015
PspA_B_WU2	0.1151	0.0993	1.0000	0.0659	0.0023	0.1597	0.9141
PspA_Pro+PartB_WU2	0.2666	0.0993	0.1336	0.1762	0.1573	0.2845	0.0126
PspA_Pro_WU2	0.1151	0.3518	1.0000	0.3532	0.0179	0.3255	0.0126

n.d. ... serum was not used in this experiment**Supplementary Table 13: Log-Rank (Mantel-Cox) Test for the *in vivo* Efficacy of Polyclonal Anti-PLY Antibodies**

Immunization with Serum <i>vs.</i>	Log-rank (Mantel-Cox) Test, p-value <i>vs.</i> PBS Group						
	LST3869	LST3872	LST3886	LST3897	LST3909	LST3910	LST3911
mouse pre-immune (neg. cntrl.)	0.6262	0.3173	1.0000	1.0000	0.5485	0.5258	0.5308
PspA (rabbit) (pos. cntrl.)	0.0017	0.0023	<i>n.d.</i>	0.0016	0.0023	0.7955	0.0126
<i>mAb</i> 1F11 (SantaCruz)	<i>n.d.</i>	0.0179	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
PLY (CGI/LST3742/N°1)	<i>n.d.</i>	0.0993	1.0000	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
PLY (CGI/LST3742/N°2)	<i>n.d.</i>	0.0442	1.0000	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
PLY (CGI/LST3742/N°3)	0.7914	0.0442	1.0000	0.3532	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
PLY (CGI/LST3742/N°4)	<i>n.d.</i>	0.0978	0.3173	<i>n.d.</i>	0.5127	0.2975	0.3707
PLD (CGI/LST3742/N°5)	0.0917	0.0528	1.0000	0.2207	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
PLYΔD4 (LST3885-N°1)	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	0.0719	0.3518	0.0889	0.0968
PLD (LST3885-N°2)	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	0.8527	0.1729	0.1996

n.d. ... serum was not used in this experiment

A-8 DATA READ-OUTS OF THE IL-6 ELISA

The following tables (Supplementary Table 14 and Supplementary Table 15) represent the results of the IL-6 ELISA carried out in triplicates for determining differences in the cytokine response of naïve mice (pre) compared to mock-immunized mice and mice immunized with different hyperimmune-sera (d1). The quantification was facilitated by a standard-calibration using mouse IL-6 in different concentrations (data not shown).

Supplementary Table 14: Results for the IL-6 ELISA

Immunization	Type of Serum	Average Absorbance [$\lambda=450$ nm]	Concentration of IL-6 [pg/ml]	Standard Deviation [pg/ml]
PBS	pre	0.000	-86.12	-69.61
	d1	2.058	11265.22	188.77
PspA_FL_T4; CGI/LST7342-N°9	pre	0.000	-84.28	-71.87
	d1	0.000	-86.12	-72.29
mAb 1F11	pre	-0.002	-95.31	-73.42
	d1	0.565	3028.92	-16.65
PLY; CGI/LST7342-N°1	pre	0.001	-82.44	-70.88
	d1	1.422	7760.33	40.77
PLY; CGI/LST7342-N°1	pre	0.002	-71.41	-70.88
	d1	1.036	5629.08	14.57
PLY; CGI/LST7342-N°3	pre	0.001	-78.76	-70.88
	d1	1.070	5816.65	5.50
PLY; CGI/LST7342-N°4	pre	-0.001	-91.64	-67.89
	d1	1.948	10660.23	-55.69
PLD; CGI/LST7342-N°5	pre	-0.001	-89.80	-73.42
	d1	0.823	4455.89	-46.14

Supplementary Table 15: Results for the IL-6 ELISA and Correlation to Subsequent Challenge Survival.
 The computed fold-changes including the statistical comparisons carried out for the groups are illustrated.

Immunization with	Level of IL-6 (24 h [pg/ml])	Fold-Increase vs. Pre-Serum	t-Test p-Value for Fold-Increase vs. Pre-Serum	Fold-Decrease vs. PBS-Group	t-Test p-Value for Fold-Decrease vs. PBS-Group	Challenge Survival (Day 15) [%]
<i>PBS</i>	11265.22	130.8	3.7E-04			0
<i>PspA_FL_T4; CGI/LST3742-N°9</i>	-86.1191	1.0	2.1E-01	130.8	3.9E-04	100
<i>mAb 1F11 (SantaCruz)</i>	3028.924	31.8	2.5E-04	3.7	8.7E-04	40
<i>PLY; CGI/LST3742-N°1</i>	7760.332	94.1	1.5E-04	1.5	2.8E-03	40
<i>PLY; CGI/LST3742-N°2</i>	5629.084	78.8	1.7E-04	2.0	2.7E-03	0
<i>PLY, CGI/LST3742-N°3</i>	5816.648	73.9	1.3E-04	1.9	2.5E-03	20
<i>PLY; CGI/LST3742-N°4</i>	10660.23	116.3	3.3E-06	1.1	1.1E-01	20
<i>PLD; CGI/LST3742-N°5</i>	4455.886	49.6	2.7E-05	2.5	1.2E-03	40

SUPPLEMENTAL

Abstract

Streptococcus pneumoniae is a common human pathogen that causes a variety of life-threatening invasive diseases such as pneumonia, bacteremia and meningitis. Despite the availability of licensed vaccines and antibiotic treatments, morbidity and mortality attributed to this bacterium remain significant in developing and developed countries. Due to increasing antibiotic resistance and limited efficacy of existing vaccines in at-risk populations, there is a need for new treatment strategies such as passive immunotherapy using human monoclonal antibodies (mAbs).

In this study, three conserved antigens of *S. pneumoniae* – lipoteichoic acid (LTA), pneumococcal surface protein A (PspA) and pneumolysin (PLY) – were characterized for their suitability as targets for a mAb-based anti-infective therapy.

Although isolation and purification procedures could be optimized for LTA from *Streptococcus pyogenes*, native LTA could not be extracted from *S. pneumoniae* in sufficient quantity and quality, thus limiting more in-depth studies of this antigen.

Recombinant full-length PspA and PLY as well as domains thereof were expressed, purified and subsequently proven to be highly immunogenic in naïve C3H/HeN mice. These antisera were characterized in-depth *in vitro*: in surface staining and in ELISA, antibodies were shown to recognize PspA in a clade-specific manner. Polyclonal antibodies against Family 1 PspA also reacted with other Proline-rich cell-surface proteins – presumably PspC – but not with Family 2 PspA.

Consequently these antisera were tested *in vivo* by passive transfer and subsequent lethal challenge with different *S. pneumoniae* strains in mice. The results correlated with surface staining data: anti-PspA hyperimmune sera were only effective against pneumococci expressing homologous PspA but not against those with a heterologous variant. Anti-PLY sera were not fully protective although conferring prolonged survival. Interestingly the observed protection correlated with the level of inflammatory IL-6, induced in mice.

Two *in vitro* assays exploiting the function of PLY were set up to allow a detailed characterization of selected antibodies: a *Hemolysis-Inhibition Assay* and an *hTLR4-Reporter Assay*. PLY-specific murine polyclonal and monoclonal antibodies reduced the hemolytic activity of PLY on erythrocytes and interfered with the activation of TLR4 through PLY.

ABSTRACT

Since human mAbs against PLY will be generated from B-cells based on the “*Sindbis Virus Based Mammalian Cell Surface Display*” technology, healthy human donors were identified based on their antibody titers in ELISA. In addition PMBC staining conditions that are required for the selection of antigen-specific memory B cells were optimized.

In conclusion, a deeper insight into the mode of action of PspA- and PLY-specific antibodies could be gained with this work and analytical methods that are required for the selection and validation of human mAbs were developed. This way a basis for the development of a mAb-based therapy for the prevention and treatment of life-threatening pneumococcal diseases was established.

Keywords: *Streptococcus pneumoniae*, monoclonal antibodies, LTA, PLY, PspA

Kurzfassung

Streptococcus pneumoniae ist ein weitverbreiteter Erreger verschiedenster lebensbedrohlicher Krankheiten wie Pneumonien, Bakteriämien oder Meningitis. Obgleich lizenzierte Impfstoffe und Antibiotika zur Verfügung stehen, bleibt die Morbidität und Mortalität, nicht nur in Entwicklungsländern, sondern auch in Industrieländern auf einem hohen Niveau. Aufgrund zunehmender Antibiotikaresistenzen und der suboptimalen Wirksamkeit verfügbarer Impfstoffe in Risikogruppen, steigt der Bedarf für neue Therapien, wie beispielsweise einer passiven Immunisierung mit monoklonalen Antikörpern.

In dieser Arbeit wurden drei konservierte Antigene von *S. pneumoniae* – Lipoteichonsäure (LTA), pneumococcal surface protein A (PspA) und Pneumolysin (PLY) – auf ihre Eignung als Zielmoleküle für die Entwicklung einer anti-infektiösen Antikörper-Therapie hin untersucht.

Obwohl die Isolierung und Reinigung nativer LTA für den Erreger *Streptococcus pyogenes* erfolgreich optimiert wurde, konnte LTA nicht in ausreichender Menge und Reinheit aus *S. pneumoniae* gewonnen und somit keine weiterführenden Studien mit diesem Antigen durchgeführt werden.

Verschiedenste Konstrukte von PspA und PLY wurden rekombinant exprimiert, gereinigt und nachfolgend deren immunogene Wirkung in naiven C3H/HeN Mäusen demonstriert. Die gewonnenen Antiseren wurden in Folge genauestens *in vitro* analysiert. Oberflächenfärbungen lebender Pneumokokken zeigten, dass PspA spezifische Antikörper nicht alle PspA-Varianten detektieren konnten, sondern nur solche des selben PspA-Typs. Polyklonale Antikörper gegen Typ 1 PspA reagierten auch mit anderen Prolin-reichen Oberflächenproteinen, vermutlich PspC, jedoch nicht mit PspA Typ 2.

Des Weiteren wurden die generierten Antiseren in zuvor etablierten Mausmodellen durch passiven Transfer und nachfolgender letaler Infektion mit verschiedenen *S. pneumoniae* Stämmen auf ihre Wirksamkeit getestet. Die Ergebnisse korrelierten mit den Daten der Oberflächenfärbung: Anti-PspA Hyperimmunseren induzierten eine Protektion gegen Pneumokokken mit homologen jedoch nicht heterologen PspA. Anti-PLY Seren hingegen konnten keine vollständig protektive Wirkung erzielen, bewirkten jedoch eine erhöhte Überlebensrate. Die beobachtete Protektion korrelierte mit der Stärke der durch die Infektion induzierte inflammatorischen IL-6 Reaktion in den Tieren.

KURZFASSUNG

Im Hinblick auf die Wirkungsweise von PLY wurden zwei *in vitro* Analysen zur Antikörper-Charakterisierung entwickelt: ein Hämolyse-Inhibitions Assay und ein hTLR4-Reporter Assay. Pneumolysin spezifische murine poly- und monoklonale Antikörper bewirkten eine Reduktion der hämolytischen Aktivität von PLY auf Erythrozyten und interferierten auch mit der Aktivierung von TLR4 durch PLY.

Humane monoklonale Antikörper sollen in weiterer Folge aus humanen B-Zellen basierend auf der „*Sindbis Virus Based Mammalian Cell Surface Display*“ Technologie generiert werden. Zu diesem Zweck wurden gesunde Spender aufgrund ihres Antigen-spezifischen Titors im ELISA identifiziert. Zudem wurden die für die B-Zell-Selektion notwendigen PBMC-Färbetechniken optimiert.

Zusammenfassend demonstriert diese Arbeit einen vertiefenden Einblick in die Wirkungsweise von PspA und PLY-spezifischen Antikörpern sowie die Entwicklung von für die Selektion und Validierung humaner monoklonaler Antikörper notwendigen Analyseverfahren. Somit konnte die Grundlage für eine Antikörper-basierende Therapie zur Prävention und Behandlung von lebensbedrohlichen Pneumokokkeninfektionen geschaffen werden.

Schlagwörter: *Streptococcus pneumoniae*, monoklonale Antikörper, LTA, PLY, PspA

Curriculum Vitae

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Education	
since Oct-2008	Master Studies at the University of Vienna in " <i>Biological Chemistry</i> "
Sep-2007 to Jun-2008	Bachelor Studies at the Dublin Institute of Technology in " <i>Physical and Life Sciences, Chemical Sciences</i> "; Graduation with Distinction
Sep-2002 to Jun-2007	College of Chemistry, Specializing in " <i>Biochemistry, Biotechnology and Genetic Engineering</i> "; Reifeprüfung-Certificate and VET-Diploma awarded with Distinction
Sep-1998 to Jun-2002	Bundesgymnasium und Bundesrealgymnasium St. Pölten, Department of Life Sciences (Secondary School)
Sep-1994 to Jun-1998	Franz Jonas Volksschule St. Pölten (Elementary School)

Working Experience	
01-Apr-2010 to 31-Mar-2011:	<p>Master Thesis at Intercell AG <i>"Development of human monoclonal antibodies against conserved antigens from Streptococcus pneumoniae"</i> Supervision: O. Univ.-Prof. Dr. Alexander von Gabain and DI Dr. Carmen Giefing-Kröll</p>
01-Mar-2010 to 26-Mar-2010:	<p>University of Vienna <i>"The Cloning for Studying the Translocation of Glut4 in mdx Mouse Myoblasts, Protein Expression Profiling of Ubiquitin, Glut4, PKC-δ and pPKC-δ and Histopathologic Evaluation of Fiber Types in Dystrophic Mice"</i> Supervision: O. Univ.-Prof. Dr. Gerhard Wiche and Mag. Marianne Raith</p>

CURRICULUM VITAE

07-Dec-2009 to 21-Dec-2009	Medical University of Vienna, Clinical Institute of Medical and Chemical Laboratory Diagnostics (CIMCL) <i>"A Shotgun-MS-Approach for the Analysis of Coronary and Peripheral Human Plasma Samples of Very Late Stent Induced Thrombosis"</i> Supervision: Mag. Dr. Markus Kubicek
01-Jul-2009 to 31-Aug-2009	Intercell AG, Department for Serology and Immune Assays <i>"Functional Activity of Antibodies against Streptococcus pneumoniae Antigens – Influence of Capsule Thickness on the Efficiency of Opsonization and Phagocytic Killing"</i> Supervision: Dr. Sanja Selak
31-Mar-2008 to 02-May-2008	Bachelor Thesis at the Dublin Institute of Technology and FOCAS-Institute <i>"Evaluation of Small Molecule Interactions with DNA using Spectrophotometric Techniques"</i> Supervision: Dr. Sarah L. Rawe
03-Jul-2006 to 18-May-2007	Medical University of Vienna, Clinical Institute of Medical and Chemical Laboratory Diagnostics (CIMCL) <i>"The Identification of Complement-Regulated Proteins via Biochemical Identification-Methods like 2D Gel-Electrophoresis and Metabolic Labelling"</i> Supervision: Mag. Dr. Markus Kubicek and DI Dr. Bibiana Meixner
01-Aug-2005 to 31-Aug-2005	Donau Chemie AG <i>"Optimization of the Catalytic Capacity and Absorption-Efficiency of Activated Charcoal"</i> Supervision: DI Dr. Rainer Wiesböck and Dipl-Chem. Dr. Krunoslav Vojinovic

Awards and Honors	
since 2010	Member of the Austrian <i>"Students4Excellence"</i> -Network
2009	<i>"Leistungsstipendium"</i> -University of Vienna (Studienförderungsgesetz der Universität Wien, 05.10.2009-23.10.2009)
2009	<i>"Top-Stipendium"</i> of the Government of Lower Austria
2008	<i>"Brian O'Keeffe Award"</i> for Exceptional & Best Overall Performance in BSc Physical and Life Sciences (Chemistry) 2008
2008	<i>"Henkel Ireland Ltd. Award"</i> for Best Overall Performance in BSc Physical and Life Sciences (Chemistry) 2008
2008	<i>"Sigma-Aldrich (Ireland) Award"</i> for Exceptional & Best Project Performance in Final Year of the BSc Physical and Life Sciences (Chemistry) 2008
2001	Participant at the <i>"Summer-Academy for the Advancement of (Highly) Skilled Students"</i> , granted by the Government of Lower Austria