Aus der Klinik für Infektiologie und Pneumologie
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DISSERTATION

A semi-synthetic glycoconjugate vaccine for *Streptococcus pneumoniae* serotype 3 confers short-term protection against pneumonia in mice.

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Foreword

This thesis is based on data collected in pre-clinical *in-vitro* and *in-vivo* evaluations of a novel semi-synthetic glykoconjugate vaccine candidate against *Streptococcus pneumoniae* serotype-3. The study is the product of a collaborative effort between the working groups of Prof. Dr. Peter H. Seeberger, Director at the Max-Planck Institute of Colloids and Interfaces in Potsdam, and the laboratories of Prof. Dr. Leif Erik Sander and Prof. Dr. Martin Witzenrath at the Department of Internal Medicine, Infectious Diseases and Pulmonary Medicine (Clinical Director: Prof. Dr. Norbert Suttorp) at the Charité - Universitätsmedizin in Berlin, Germany. Development and production of the vaccine was undertaken by Prof. Dr. Seeberger's group, the pre-clinical evaluations described in this thesis were conducted at the laboratories at Charité – Universitätsmedizin in Berlin.

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Specific contributions: Figure 5- Page 1412, Figure 6-Page 1413, Figure 7-Seite 1414, and Figures S5 & S6 in ‚Supplemental Figures'.

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Tables and Figures

I. Abstract

Introduction: *Streptococcus pneumoniae* represents a significant cause of global morbidity and mortality in both children and adults. While there are vaccines currently available that can convey immunity to some of the major serotypes of *S. pneumoniae*, limited access to vaccines in low-income countries leads to insufficient coverage of those populations, and the shifting prevalence of serotypes coupled with bacterial resistance to antibiotics is a serious development in the US and Europe. The rational design of semi-synthetic glycoconjugate vaccines represents a novel method for effective and economical vaccine production, and is therefore a viable strategy for addressing these problems. A new oligosaccharide-conjugate vaccine against *S. pneumoniae* serotype 3 (SP3), produced by glycan synthesis developed by the working group of Prof. Dr. Peter H. Seeberger (Max Planck Institute für Kolloid- und Grenzflächenforschung, MPIKG) has shown promise as one being able to induce serological responses. Here, we tested the ability of this vaccine candidate to confer immunity in a mouse model of *S. pneumoniae* infection. **Methods:** In the current study, we conducted pre-clinical *in-vitro* and *in-vivo* experiments, in accordance with WHO-guidelines for the assessment of novel vaccines, to examine the relative efficacy and safety of a novel, semi-synthetic glycoconjugate vaccine for *S. pneumoniae* serotype 3 (SP3). *In vitro* experiments encompassed opsonophagocytosis assays implementing either differentiated HL-60 cells or peripheral human neutrophils. *In vivo* experiments employed a mouse-model of pneumococcal pneumonia to assess vaccine protection, based on clinical parameters, bacterial clearance from the lungs, bacteremia, local and systemic cellular immune-responses to SP3 challenge, pulmonary function tests, and pulmonary endothelium barrier integrity. **Results:** The vaccine candidate showed a robust protective effect in mice challenged with SP3 35 days following vaccination, but waning effect with no significant protection after 116 days, indicating a potential failure to convey long-term protection. **Conclusions:** These data show at once the promise of this approach of using semisynthetic oligosaccharides in vaccines, and support the need to expand the efforts of inducing long-lasting immunity through optimized vaccine-design.

Einleitung: *Streptococcus pneumoniae*–Infektionen stellen weltweit eine wichtige Ursache für Morbidität und Mortalität bei Kindern und älteren Erwachsenen dar. Obgleich Impfungen für viele der häufigsten Serotypen von *S. pneumoniae* existieren, verhindert eine beschränkte Verfügbarkeit der Impfstoffe in Entwicklungsländern die flächendeckende Immunisierung dieser Bevölkerungen. Gleichzeitig wird in den USA und Europa eine Verschiebung der beobachteten Serotyp-Prävalenzen bei zunehmender Antibiotika-Resistenz zu einer ernsten Herausforderung. Eine effektive und ökonomisch effiziente Lösung dieser Probleme stellt die Methode des rationalen Entwurfs synthetischer Polysaccharide-Impfungen dar. Vorläufige Daten konnten zeigen, dass ein semisynthetischer Oligosaccharid -Konjugatimpfstoff für *S. pneumoniae* Serotyp 3 (SP3), hergestellt mittels automatisierter Glykansynthese, die durch die Arbeitsgruppe von Prof. Dr. Seeberger (Max Planck Institute für Kolloid- und Grenzflächenforschung, MPIKG) entwickelt wurde, eine serologische Immunantwort in Mäusen erzeugen kann. In dem vorliegenden Projekt sollte nun die Fähigkeit des semisynthetischen SP3 Impfstoffs Protektive Immunität in einem Mausmodell zu induzieren. **Methoden:** In dieser präklinischen Studie wurden i*n-vitro* - und *in-vivo*-Experimente durchgeführt, die nach den WHO-Leitlinien für die Auswertung der Effektivität und Sicherheit neuer Impfstoffe gestaltet wurden. In den *in-vitro*-Experimenten wurden ‚Opsonophagocytosis Assays' (OPA) eingesetzt, welche Opsonophagozytose vermittelt durch spezifische Antikörper messen. Zu diesem Zweck wurden entweder ausdifferenzierte HL-60 Zellen, oder primäre menschliche Neutrophile Granulozyten verwendet. Die *in-vivo*-Experimente setzten ein Mausmodell der Pneumokokken-Pneumonie ein, um den Impfschutz gegen SP3-Infektion zu messen. Dazu wurden Daten zu klinischen Parametern, der Bakterienlast in Lunge und Blut, sowie zur Immunantwort erhoben. **Ergebnisse:** Der SP3-Impfstoff zeigte einen robusten Schutz in Mäusen, bei denen eine experimentelle Pneumonie mit SP3 35 Tage nach Impfung durchgeführt wurde, nicht jedoch in Mäusen, die 116 Tage nach Impfung infiziert wurden. Der Impfschutz war somit nicht langlebig. **Schlussfolgerung:** Die Studie zeigt, dass semi-synthetische Konjugatimpfstoffe protektive Immunität induzieren können, sie zeigt aber auch auf die Notwendigkeit weiterer Studien zur Erforschung der Langzeit-Immunität mit diesen neuartigen Impfstoffen auf.

II. Introduction

II. A. Epidemiology and the Implications for Public Health

II. A. i. Pneumococcal Infections Globally

S. pneumoniae is a gram-positive, alpha-hemolytic bacterium that transiently colonizes the nasopharynx. Children, and especially infants make up the primary carrier population, of which between 30-96% have shown to be affected. The adult population shows decidedly lower carrier rates ranging between 3 and 9 %[1-6]. *S. pneumoniae* usually exists in a diplococcal form and is characterized by its large polysaccharide-rich capsule. Based on serum reactivity to capsule components, some 90 serotypes of *S. pneumoniae* have been identified to date, of which approximately 13 have been implicated in roughly 70% of invasive pneumococcal disease (IPD) cases[2]. The prevalence of the 21 most common serotypes in IPD is summarized in Figure 1.
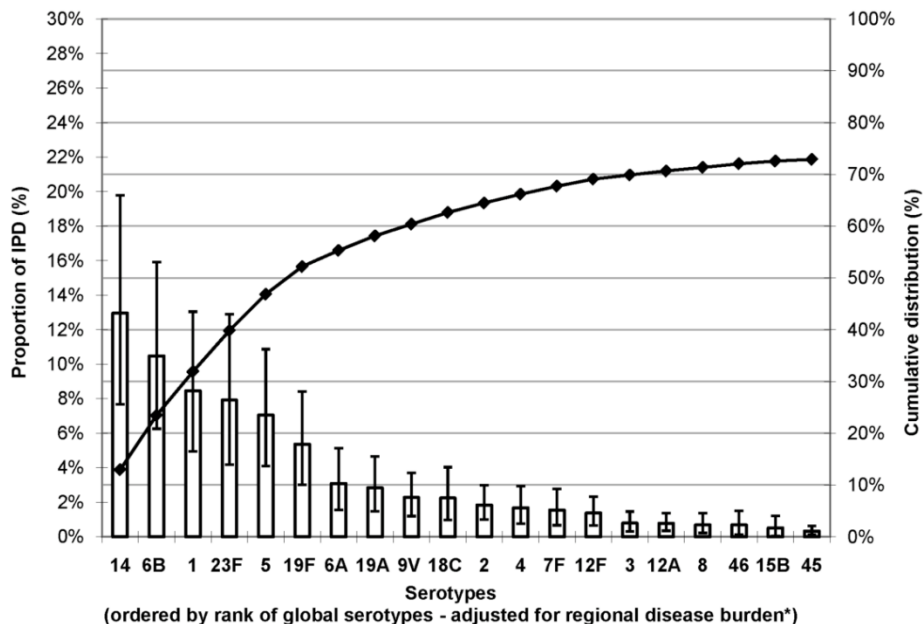
Figure 1. Global prevalence of the most 21 common serotypes of S pneumoniae in children under the age of 5. Error bars indicate 95% confidence interval for each serotype. Line indicates cumulative proportion of IPD caused by the serotypes. Figure taken from Johnson, et al., 2010[2].

Diseases caused by *S. pneumoniae* include infections of the ears (otitis), nose and throat, meningitis, and most notably pneumonia [3,4]. These diseases are leading causes of morbidity and mortality world-wide. Pneumonia is a leading cause of death in children under the age of 5 [5,6], accounting for approximately 15% of global deaths in this age-group[7,8]. *S. pneumoniae* remains the most significant cause of community acquired pneumonia (CAP), killing over 500,000 children in this age-group each year[9,10]. Pneumococcal pneumonia in the elderly and immune-compromised represent secondary, yet still highly significant sources of disease burden and death[11-12]. Indeed, *S. pneumoniae* is a leading causative agent of CAP in persons 65 years of age or older, which is in itself the fifth leading cause of death in this population[13].

II. A. ii. Disease Burden in Low-Income Countries

As with most infectious diseases, pneumococcal infections take their greatest toll in areas of the world with low income and resources[14], particularly in Africa and Asia, where between 48% and 88% of IPD cases are accounted for by serotypes included in currently available vaccines. Cases in this region account for 95% of global pneumococcal disease, amounting annually to 10 million cases and over 600,000 deaths of children under the age of five[3,6,12,15,2]. This disproportionate geographic distribution in pneumococcal disease prevalence reflects limited access to health-care and related resources in those regions[16]. Inadequate access to antibiotic treatments for acute infection and insufficient vaccine coverage constitute the two major obstacles to stemming the tide of illnesses caused by this pathogen[8]. Indeed, while rates of IPD in the United States plummeted, and remained relatively low with the advent of the first pneumococcal vaccines[17,15] (Figure 2), the associated disease burden in developing countries remains high, and is caused by serotypes included in current vaccines[15,18,2,19] (Figure 3), a clear indication of poor vaccine coverage in these populations.
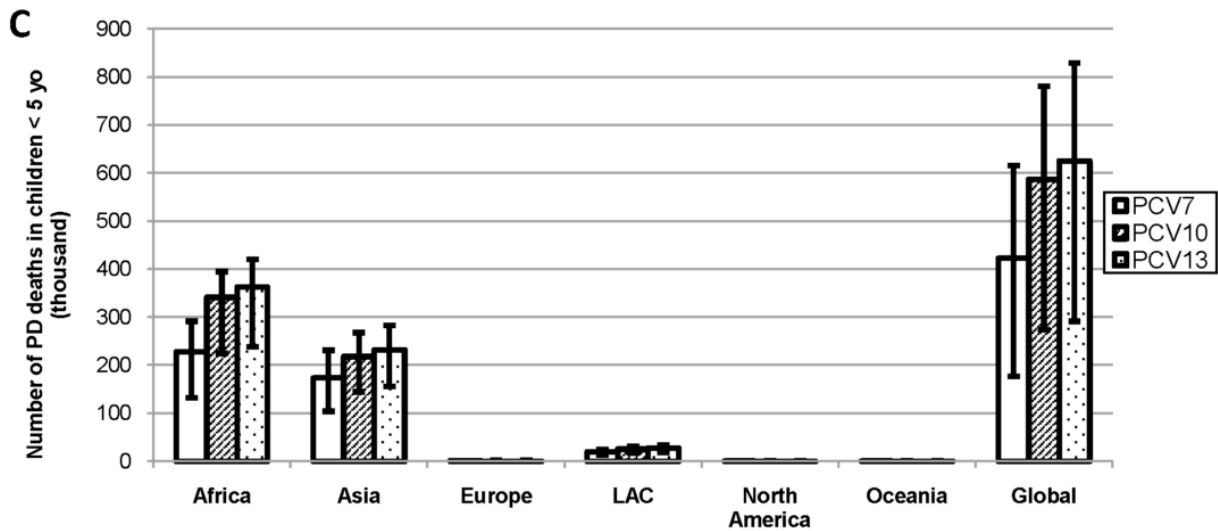
Figure 2. Mortality caused by serotypes of S. pneumoniae covered by licensed pneumococcal conjugate vaccines (PCVs). The graph shows absolute mortality attributable to IPD in children under the age of 5 by geographical region. The different bars indicate absolute number of deaths caused by serotypes covered by each of the respective pneumococcal vaccines. Figure taken from Johnson, et al., 2010[2].
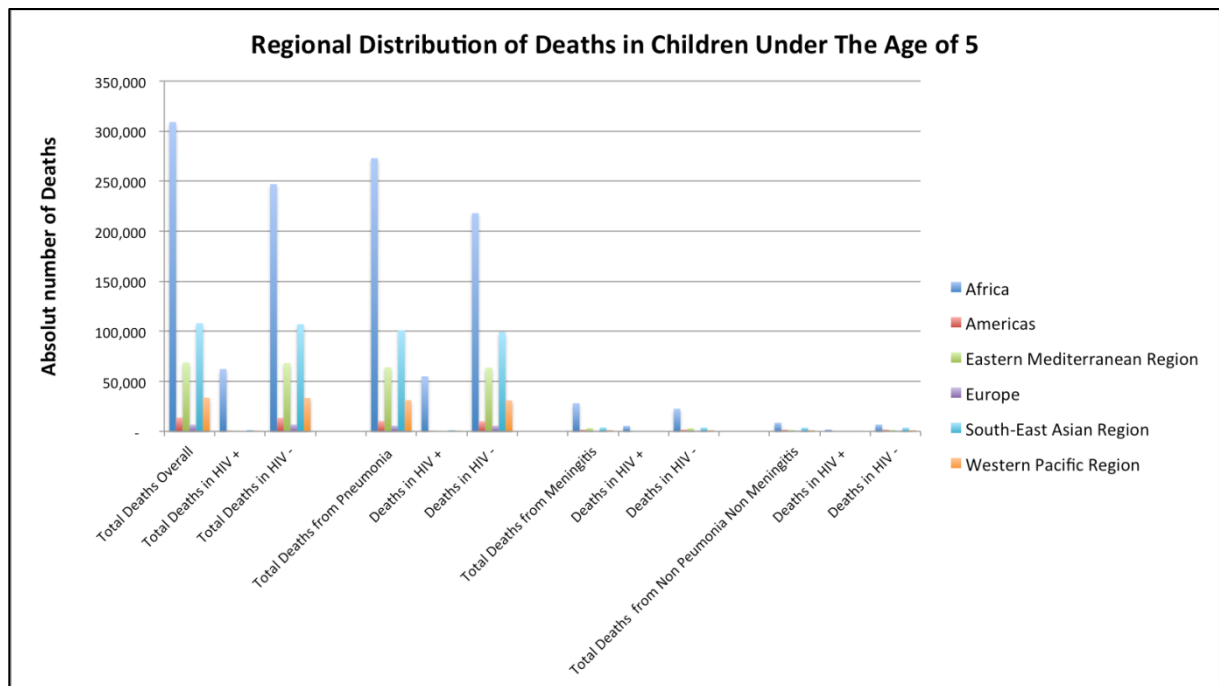
Figure 3. geographical distribution of deaths attributable to pneumococcal disease in children under the age of 5 (based on data obtained from the WHO: 'Programme for Immunization, Vaccines and Biologicals', 2008[9])

II. A. iii. Disease Burden in Europe and the United States

Higher income countries face different problems related to IPD. Increasing bacterial resistance to both first- and second- line antibiotics[15,20,21] and shifts in seroprevalence away

from types covered by currently available vaccines[22,19] (Figure 4) are increasing concerns both in the United States and Europe.

Management of pneumococcal disease is costly. The financial costs have been calculated to reach over 3.5 billion USD in the US and some 5 billion Euros in Europe annually. Thousands of hospital-days and physician working-hours are committed to this task each year[13,23,24].

Figure 1. Penicillin resistance in *Streptococcus pneumoniae*, United States, 1979–2004
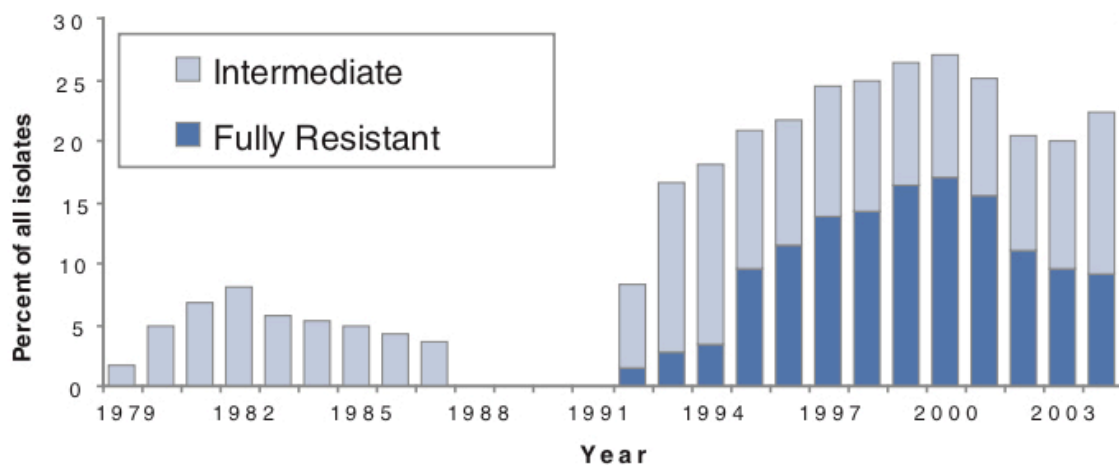
1979–1994: CDC Sentinel Surveillance System
1995–2004: CDC Active Bacterial Core Surveillance (ABCs) System, Emerging Infections Program[28]

Figure 4. Frequency of Penicillin resistance in S. pneumoniae isolates in the United States until the introduction of PCV7 (Prevnar7®)
Figure taken from Pilishvili, et al., 2012[15].

II. B. Vaccination as an approach to pneumococcal disease-control and prevention.

Several vaccines for *S. pneumoniae* have been developed to date (Table 1). The first widely implemented vaccine against *S. pneumoniae* was the 23-valent polysaccharide vaccine (PSV) Pneomovax23® (Merck™). For reasons discussed in the next section, PSVs confer limited immunity to young children. The introduction of the polysaccharide conjugate vaccine (PCV), Prevnar7® for *S. pneumoniae* in 2000 can be regarded as a breakthrough, since it reduced the incidence of pneumococcal disease in the United States in children under the age of five from 80 cases in 100,000 to less than 1 case in 100,000 within 7 years of its implementation[15] (Figure 5).

Trade Name	Manufacturer	PSV/PCV	Year Licensed	Serotypes incl.
Pneumovax23®	Merck™	PSV23	1983	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 33F
Prevnar7®	Pfizer™	PCV7	2000	4, 6B, 9V, 14, 18C, 19F, 23F
Prevnar9®	Pfizer™	PCV9	Unlicensed	4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5
Prevnar10®	Pfizer™	PCV10	Unlicensed	4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5,7
Prevnar13®	Pfizer™	PCV13	2010	4, 6B, 9V, 14, 18C, 19F, 23F 1, 3, 5, 6A, 7F and 19A

Table 1. [15]
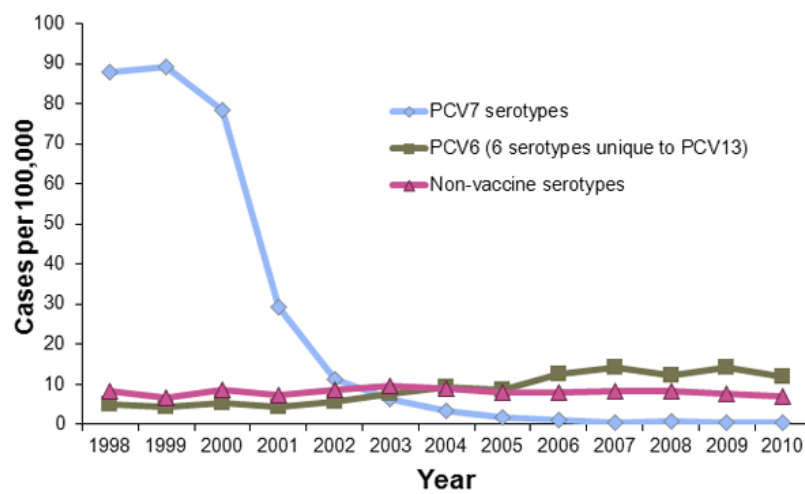
Figure 5. Incidence of IPD caused by S. pneumoniae PCV7-serotypes in children under the age of 5 before and after the release of the vaccine in 2001. (Figure modified from VPD Surveillance Manual, 5th Edition, 2012. Pneumococcal Disease: Chapter 11-13)[15].

Based on this evidence, it can be asserted that vaccination is a safe and efficient measure to reduce the problem of pneumoccal disease in populations where it is readily accessible. Development of cost-effective methods for vaccine production would greatly improve vaccine coverage in low-income countries and allow for a quicker adaptation of vaccine serotypes in response to changes in serotype distribution[25,26]. As such, developing new strategies for more efficient, defined and cost-effective vaccine production has been a priority of several organizations, including the WHO and UNICEF, which jointly put forth the Global Action Plan for Pneumonia and Diarrhea (GAPPD). This initiative sets specific goals for the eradication of IPD in children by 2025[4].

II. B. i. Current vaccines and mechanisms of inducing immunity to *S. pneumoniae*.

A critical feature of acquired immunity against *S. pneumoniae*, and many other capsulated bacterial pathogens, is the production of specific antibodies to capsular polysaccharides (CPS) found in the bacterial cell wall[27] (Figures 6 & 7). Humoral immunity in general is an extremely potent defense mechanism against extracellular bacterial pathogens. Pathogen-specific antibodies offer protection by opsonizing bacteria and thus enhancing phagocytic clearance. In addition, antibodies promote complement-dependent killing of pathogens[28,29,30]. Protection afforded by pneumococcal vaccines is based on their ability to elicit humoral immunity to capsular antigens, thereby promoting opsono-phagocytosis and complementary killing of invading pneumococci[31,32]. Each of the licensed vaccines contains native capsular polysaccharides purified from fermented bacterial cultures[31,32]. In the case of PSVs, these polysaccharides are administered as a suspension without modification[32]; whereas in PCVs, CPS are chemically bound, or conjugated, to a carrier protein[31].
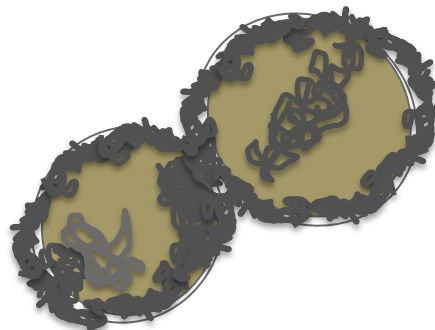
Figure 6. Author's rendering of S. pneumoniae showing the diplococcal morphology and dense capsule. Based on electron-micrographs depicted in Rockefeller.edu[33].

Phospholipid

Capsular Polysaccharide (Serogroup)

Outer Membrane Proteins

Outer Membrane

Pilus

Lipo-Oligosaccharide

Cytoplasmic Membrane Proteins

Cytoplasmic Membrane

Periplasmic Membrane

Outer Membrane
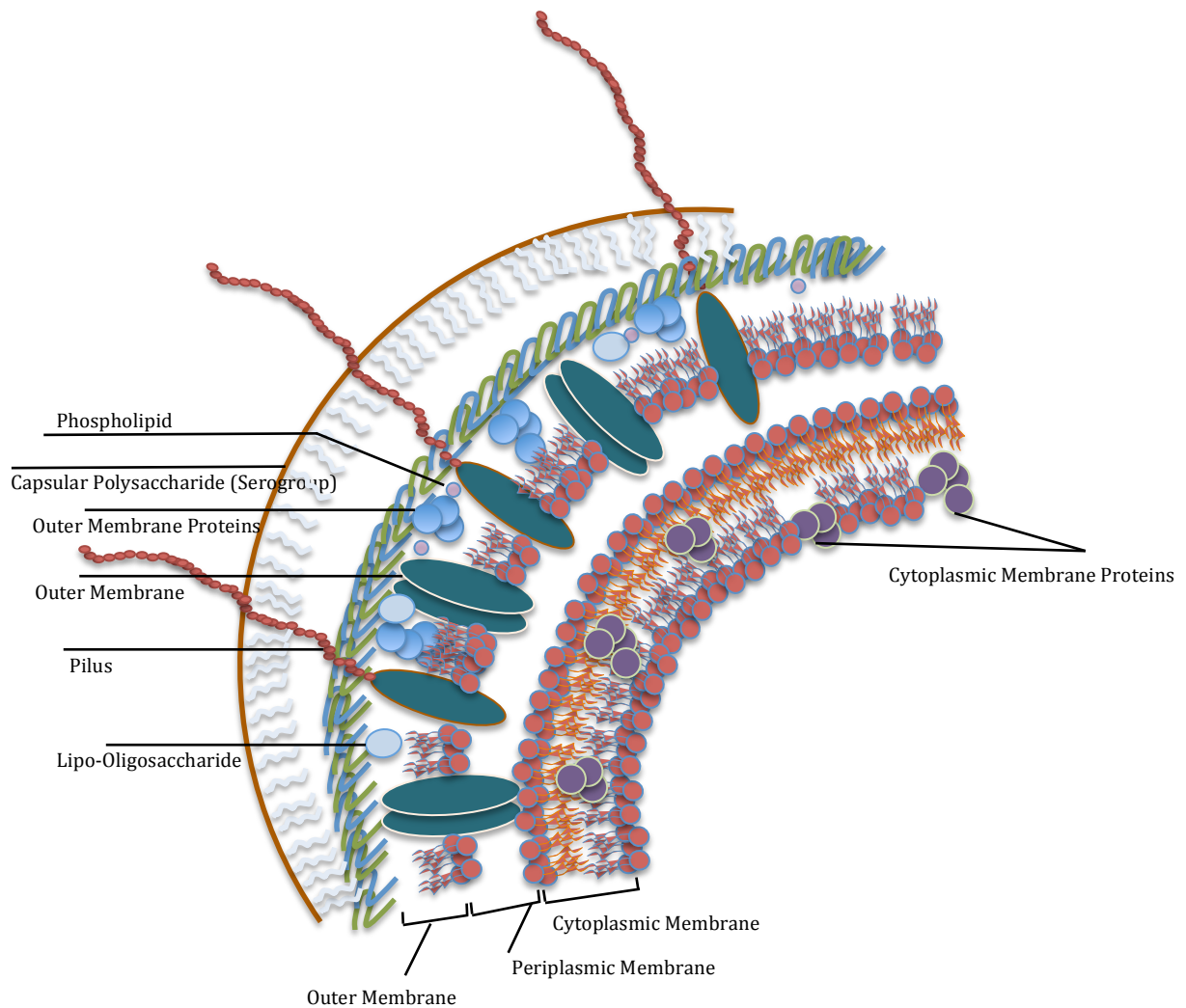
Figure 7. Author's rendering (modified from Stephens et. al, 2007) of the bacterial cell-wall cross section. The capsular polysaccharide contains the antigenic sequences that determine the serogroup[34].

Polysaccharides on their own elicit an immune response that is largely T-Helper-cell independent[27] and fail to induce long-lived B cell memory[35]. Multiple B-cell receptors bind to the epitopes within a polysaccharide (glycotopes) and are subsequently cross-linked, resulting in a cascade reaction that eventually leads to antibody production and B-Cell differentiation into short-lived plasma-cells[27,36]. This mechanism is not supported in children younger than 18-24 months, probably because they have not yet undergone the B-Cell maturation necessary to convey immunity to carbohydrates[37,38]. Consequently, polysaccharide vaccines such as PSV23 are ineffective in this very vulnerable age-group. The immune systems in small children can, however, form immunological memory via mechanisms belonging to the

thymus-dependent pathway, for which peptides are required in the activation process. In this case, antigen presenting cells, such as dendritic cells and B-cells present peptide antigens on MHC-II molecules to CD4^{+} T-helper cells, which in turn stimulate B-cell activation, proliferation, antibody class-switching and recombination (CSR) and the generation of B-cell memory[36,39,40](Figure 8). PCVs take advantage of this mechanism by coupling CPS to carrier-proteins, which provide peptide antigens to stimulate sufficient T-cell help to trigger the germinal center reaction resulting in B cell memory. Notably, most conjugate vaccines use the well-characterized detoxified diphtheria toxin derivative CRM-197 as a carrier protein, thereby mobilizing CRM-197-specific T-helper cells into the CPS-specific humoral immune response[41,39,42]. MHC II appears to play a critical role in this process of carrier-protein facilitated B-Cell activation[43,44,45],probably by direct MHC II Binding of carrier proteins and subsequent presentation to B-cell and release of IL 2&4[45]. PCVs allow for safe and successful vaccination of infants and children under the age of two[31].

T-helper cell-independent response as seen with PSV:
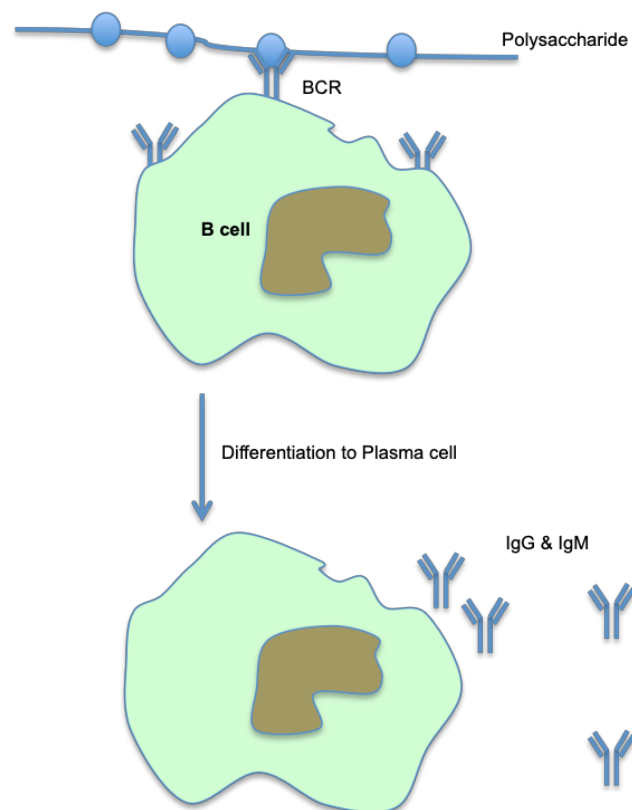
T-helper cell-dependent response as seen with PCV:
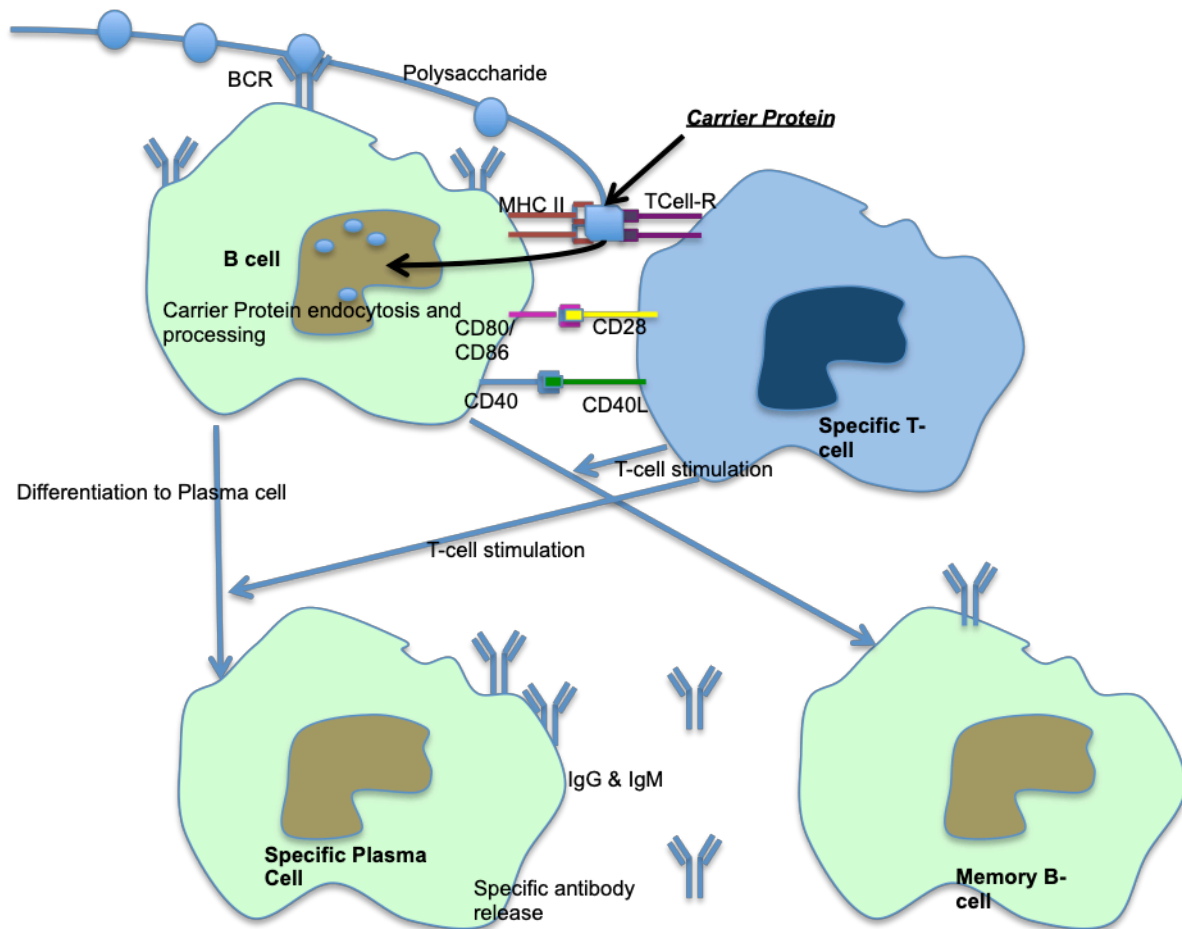
*Figure 8. Two pathways in the response to PSV (a) and PCV (b): Author's rendering (modified from Avci et al, 2011) of **a** T-Helper cell-independent response antibody production to PSV and **b** T-Helper cell-dependent response and antibody production to PCV[44–46,47].*

II. B. ii. Challenges in Vaccine Production

Implications for global accessibility and coverage.

The production of CPS for both of these vaccines and, indeed for all vaccines utilizing CPS as antigen, is very resource intensive and often hampered by technical difficulties in providing stable and pure antigens[48]. The production process requires large-scale cultivation of the specific bacterial strains, and subsequent isolation of the desired CPS[49,50]. There are many technical and logistical challenges in this process. To name only a few of the problems encountered in CPS production by fermentation and purification, cultivation of certain strains

is often difficult[51,52]; isolation and purification of CPS is invariably never completely effective, resulting in the inclusion of impurities, which may disrupt vaccine responses or cause side-effects[51,53]; and CPS may become unstable during this process[54,55]. Naturally, this process must also be tightly controlled and regulated at each step[49,50]. In sum, the production of natural CPS antigens incorporated into these vaccines represents one major hurdle to overcoming the prohibitively high costs of making such vaccines available globally.

II. B. iii. Rational Vaccine-Design

Approaches for vaccine development and application in developing a novel vaccine for *S. pneumoniae* serotype 3.

One promising approach to reducing production costs lies in the rational design of carbohydrate antigens and their synthetic production. Such a method allows for consistent and reliable production of carbohydrate antigens that could be employed in vaccine production with a comparatively insignificant window of chance for the inclusion of impurities[48]. Furthermore, this process would eliminate the problem of instability in the antigens produced, and circumvent the need for such rigorous inspection and regulation in the production process described above[48,56,57].

One significant challenge to this approach is identifying the carbohydrate sequences within the CPS that are sufficiently antigenic. Several methods have been developed to this end in the past few years, however, paving the way for advances in this field and expanding the currently available library on carbohydrate antigens[48,57].

S. pneumoniae serotype-3 (SP3) represents a previously well-characterized and clinically relevant pathogen. SP3 is known to cause IPD in both children and adults[58,59]. Several studies have made progress in describing the makeup of the CPS as well as identifying viable glycan hapten-candidates for use in vaccines[60]. In addition, PCV13 is known to be only weakly protective against SP3, making the latter a problematic serotype with a high medical need for better vaccines[61,62].

Recently developed glycan microarrays[63] were employed to identify glycotopes in the CPS of SP3 that could potentially provide protective immunity against SP3. Using this method, Prof. Peter H. Seeberger's group (MPIKG) generated a catalog of synthetic glycans fixed to a solid

surface, known as a microarray chip and then exposed to commercially available, well-characterized monoclonal antibodies known to opsonize SP3 bacteria. Following rinsing steps, secondary antibodies coupled to fluorophores were then applied to mark the fixed antibodies. Quantification of fluorescence on the glycan microarray allows for the identification of oligosaccharides (i.e. glycotopes) that best bind the primary antibodies[63] and therefore represent promising candidates for utilization in vaccines. Using this method, Prof. Seeberger's group confirmed that SP3-specific antibodies bind both native SP3 CPS, as well as a newly synthesized tetrasaccharide-sequence with high affinity. The synthetic tetrasaccharide sequence was subsequently used as the SP3-glycotope in the synthetic PCV tested in this study[64].

As discussed previously, conjugation to a carrier-protein is necessary for the elicitation of a thymus-dependent response in children under the age of 24 months.

CRM-197, an inactivated form of diphtheria toxin[65,66] and the carrier employed in PCV13, was selected as carrier protein in this vaccine as one that is well-characterized and licensed for this purpose[67]. A coupling process using a linking compound was undertaken to conjugate the hapten to its carrier protein. This linker and the bonds involved can also play a role in immunogenicity[48], which is why this constituent of the vaccine must be carefully selected so as not to negatively affect vaccine-response. Successful conjugation was verified by mass-spectroscopy, which showed that, on average, 6 to 7 of tetrasaccharide haptens were conjugated to each CRM-197 molecule employed for this purpose[68]. This newly generated conjugate vaccine is referred to as 'SP3-Tetrasaccharide' from hereon.

II. B. iv. Innate Immunity and Vaccines

The role of the innate immune system in the conference of an adaptive response to threats, and the implications for vaccination.

A necessary step in eliciting immune memory conferred by T- and B-cells involves antigen presentation by antigen-presenting-cells (APC) such as B-Cells, dendritic cells and macrophages[36,69,70]. PCVs on their own have a very limited capacity for evoking activation of antigen presenting cells. However, appropriate activation signals are required for the maturation of antigen presenting cells and the expression co-stimulatory molecules as well as

efficient processing and presentation of antigen. Thus, PCV application alone leads only to poor presentation of the antigens to immune cells and the resulting adaptive immune responses are limited[36,70,71]. The potential for eliciting immune-memory and a secondary immune-response is therefore reduced as well. The process of antigen presentation and sufficient activation takes place at the interface of the innate and adaptive immune-systems[72,70,73]. The innate response to a potential threat, or an inflammatory stimulus such as an adjuvant, is prerequisite for the initiation of a sustained adaptive immune-response[72,70,73].

To facilitate this interface-reaction, vaccines must be supplemented with a component that adequately alerts the innate immune system to a potential threat[36,71]. Such supplementary substances are termed 'adjuvants.' There are many such substances in use today; an aluminum-containing solution (Alum) such as Alyhdrogel® (InvivoGen™) employed in this study, is one of the oldest and most widely used adjuvants, which has a consistent track-record of being effective and very safe [74–76]. For the purposes of this study, Complete-Freund's-Adjuvant (CFA), an oil emulsion containing heat-inactivated Mycobacterium bovis components[77] was also employed. This latter adjuvant, while highly-effective at producing local-reactions and conferring immune-memory, is not approved for use in humans as it has shown to produce severe local and systemic side-effects[78]. The mechanisms by which each of these adjuvants elicits local-responses and subsequent activation of the adaptive immune-system are, as yet, not fully understood. Some evidence suggests that aluminum directly activates macrophages and dendritic cells, which subsequently go on to present antigens within secondary lymph organs[74–76]. CFA appears to elicit a similar reaction[78], but tends to generally induce immune-memory more effectively than aluminum-containing agents[79,71].

II. B. v. Preliminary Results from Studies in Mice.

Mice treated with SP3-Tetrasaccharide and CFA or Alum showed a significant immune response as determined by glycan microarray.

Small-scale, preliminary experiments were conducted by our collaborators at MPIKG to establish whether the SP3-Tetrasaccharide combined with either the Alum-adjuvant or CFA would elicit an effective humoral immune response in mice. Groups of 6 mice were immunized either with PBS, SP3-Tetrasaccharide, SP3-Tetrasaccharide + Alum or SP3-

Tetrasaccharide + CFA, followed by boosting at 14 and 28 days. Serum was then analyzed using glycan microarrays in a fashion similar to the one described above. Results from this analysis showed that mice from the SP3-Tetrasaccharide, SP3-Tetrasaccharide + Alum or SP3-Tetrasaccharide + CFA-groups produced immunoglobulin G (IgG) specific to both the SP3-Tetrasaccharide antigen, as well as to the native SP3-CPS, demonstrating that the vaccine could induce an immune response. A comparison of the results from these groups also showed that vaccines supplemented with either Alum or CFA elicited higher IgG titers compared to SP3-Tetrasaccharide alone. It was also shown that a significantly greater response was elicited when CFA was employed as an adjuvant when compared to Alum. These findings are also reflected in the literature wherein more robust responses to CFA were observed when compared directly with Alum[80]. Those findings confirmed robust immune-responsiveness to SP3-Tetrasaccharide when combined with an adjuvant.

II. B. vi. WHO Guidelines for Vaccine Development

II. B. vi. 1. Overview

While these studies provided evidence for the immunogenic nature of SP3-Tetrasaccharide antigen, proof of functional protection in an animal model must be provided before clinical trials in humans can be conducted. The WHO has established guidelines for non-clinical evaluation of vaccines, which are intended to standardize the establishment of risk-benefit analysis[50,81,82]. These guidelines encompass parameters for toxicity assessment, immunogenicity and protective effects of the vaccine in an animal model to assess for the benefits of treatment and its relative safety before proceeding with human trials[50,81].

Toxicity studies monitor both clinical data that may reflect illness in animals (appearance, behavior and body-weight), hematologic and serologic examinations (complete blood count, electrolytes, enzyme studies and electrolytes) and pathology-studies (complete necropsy, examination of immune-organs including the spleen, lymph nodes, bone-marrow—and pivotal organs—brain, kidney and liver)[82,81].

Establishing effectivity involves both *in-vivo* and *in-vitro/ex vivo* studies. *In-vitro/ex vivo* studies encompass measures of antibody-titers, mean antibody concentration, seroconversion rates and antibody functionality. The latter is the capacity of specific antibodies to promote

opsono-phagocytosis and killing of the target pathogen in a standardized *in-vitro* assay[82]. *In-vivo* studies combine both the toxicity studies described above as well as parameters adhering to established animal-models of pneumococcal disease, in the form of a challenge-experiment in which vaccinated animals are subjected to inoculation with the respective pathogen[81,83,84].

II. B. vi. 2. Application to the Current Study

Applying a mouse-model of pneumococcal pneumonia to the current study

A mouse model of pneumococcal pneumonia has been established in the working-group of Prof. Witzenrath Charité - Universitätsmedizin Berlin, Department of Infectious Diseases and Pulmonary Medicine[81,83,84,85,86]. This model encompasses direct monitoring of infected- mice and controls at 12 h intervals for clinical signs of morbidity (appearance of the fur and skin, lacrimation, behavior indicating stress etc.), bodyweight and temperature. Later, sacrificed animals are dissected and intubated for gathering spirometric data; BAL-Fluid and blood samples are collected for conduction FACS-studies and lung-permeability assessment; and lung tissue is obtained for histological examination.

In this study we tested the protective effects of a novel, semi-synthetic PCV against SP3 using the *in-vitro/ex vivo* and *in-vivo* methods described above. We were able to show that SP3-Tetrasaccharide induces protective humoral immunity as confirmed by positive OPA-titers in immunized mice 5 weeks after immunization, but not after 16 weeks. The protective nature of the vaccine in the short-term, but not in the long-term groups was indicated by: the absence of clinical signs and symptoms of infection, maintenance of body–weight and temperature, pulmonary bacterial load and bacteremia, and pulmonary endothelial permeability. Cell counts of bronchio-alveolar-lavage fluid (BALF) showed increased Neutrophil recruitment to lungs in non-vaccinated mice compared with vaccinated mice, and increases in Lymphocyte recruitment in vaccinated mice, supporting the conjecture of a specific immune response in vaccinated animals. Cell counts performed in whole-blood showed lower leukocyte counts in non-vaccinated groups than in vaccinated animals and uninfected controls. In sum, we were able to demonstrate proof of concept for a novel, semi-synthetic PCV vaccine against SP3.

III. Materials and Methods

III. A. *In vitro / ex vivo*-Studies

III. A. i. Materials

III. A. i. 1. Bacteria

Highly encapsulated, alpha hemolytic serotype-3 *S. pneumonia* (PN36, NCTC7978, kindly provided by Prof. S. Hammerschmidt, Universität Greifswald, Germany), was employed in both OPA's and *in-vivo* experiments.

The preparation of bacteria for killing assays and challenge experiments was performed according to protocols previously established in our working group.

Bacterial stocks used in OPA's were established by cultivating bacteria from single colonies overnight in a medium of Todd-Hewitt broth supplemented with a 0.5% yeast extract to an optical density (OD) of 0.3-0.4 at a wavelength of 600 nm. This OD corresponds to a mid-logarithmic growth-phase for this strain.

The bacterial suspension was then diluted to obtain a concentration of ~10^6 CFU/mL and combined with glycerol to make a 15%-glycerol/suspension solution. This combination was frozen and stored at -80°C in 0.5 mL aliquots.

Bacteria-viability was verified by plating 50 μL (~5 x 10^4 CFU) of frozen aliquot taken at random, and diluted 1:1, 1:10 and 1:100 serially on Columbia Agar. The plates were incubated for 10 hours at 37°C and 5% CO_2 atmosphere, and the colonies counted manually.

III. A. i. 2. HL-60 Cells

Human, myeloproliferative leukemia HL-60 cells were implemented in the OPA's. Cryostocks of HL-60 cells were kindly provided by the working group of PD Dr. Andreas Nitsche at the Robert Koch Institute in Berlin, Germany.

To establish the growth pattern and optimal induction-conditions for these cells, multiple expansions were performed. We tracked both the viable cell density using 4% trypan blue exclusion, and the degree of differentiation using a combination of Giemsa-Pappenheim staining to resolve morphological features at pre-established time-points during the growth- and differentiation process.

In addition, nitro blue tetrazolium staining for superoxide anions, and therefore the presence of granules was used to identify more accurately mature granulocytic cells.

Expansion of HL-60 Stocks

A cryostock of 1×10^6 HL-60 cells was diluted to 1:20 in a solution of RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin-streptomycin. Cells were expanded to a concentration of $\leq 1 \times 106$ cells/mL in the upright position at 37°C and 5% CO^2 atmosphere. Cell-viability was verified using 0.4% trypan blue exclusion. The growth medium was changed every 24h by centrifuging the culture at room-temperature at 160 x g for 10 minutes, and decanting supernatant from the pellet. Following expansion, cells were cultured with a starting concentration of 2×10^5 cells/mL of the same medium, supplemented with 10% FBS instead of 20%. These growth cultures were incubated at 37°C and 5% atmosphere to a concentration of $\leq 5\text{-}7 \times 10^6$ cells/mL in the upright position. The medium was changed as described above every 48-72 hours. Counts of viable cells were performed at regular intervals using trypan blue, and showed logarithmic growth over time (Figure 9).
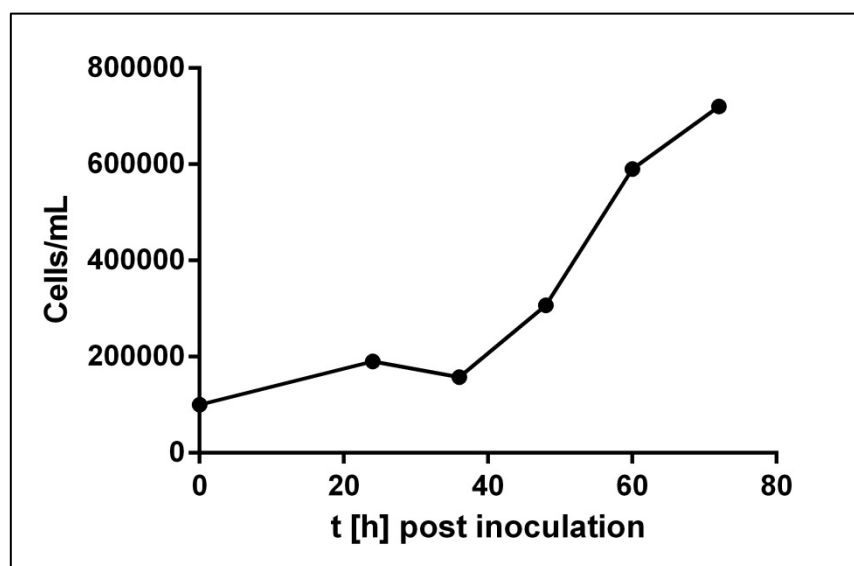
Figure 9. Average growth-rate of viable HL-60 cells over time started with an inoculation of 100,000 Cells/mL in a 200 mL solution. Shown are the average cell counts of 6 expansions.

HL-60 Cell Differentiation

The procedure for the differentiation of HL-60 cells to pseudogranulocytes was also modified from that described by Romero Steiner *et. al.*, 2007. We assumed that the concentration of DMF used to stimulate HL-60 would at once affect the morphology as well as the viability of the cells. The latter

characteristic we attribute to the toxic nature of this compound and the fact that terminal differentiation of myeloid cells generally involves a proliferative block[87,88]. To determine the optimal condition for inducing granulocytic differentiation with preservation of cellular viability, we therefore conducted differentiation of HL-60 cells in 3 different environments i.e. with different concentrations of DMF: to a standard medium 200 mL of RPMI 1640 supplemented with 20% FBS and 1% L-glutamine was added the required volume of DMF to produce either a 100 mM, 75 mM or 50 mM solution of this compound. (The protocol described by Romero-Steiner et. al. calls for a concentration of 100 mM DMF.)

Cells from the previous step were then added to this medium at a concentration of 2×10^5 cells/mL and incubated for 7 days at 37°C and 5% CO_2 atmosphere in the slanted position. The medium was not changed during this time. After 7 days, trypan blue exclusion was used to control for the number of viable cells. Giemsa staining combined with nitroblue tetrazolium for testing the presence of superoxide anions was used to verify differentiation to pseudogranulocytes. Absolute cell counts vs. non-viable cell counts were charted over time (Figure 10) to assess the optimal differentiation time for each of these conditions.
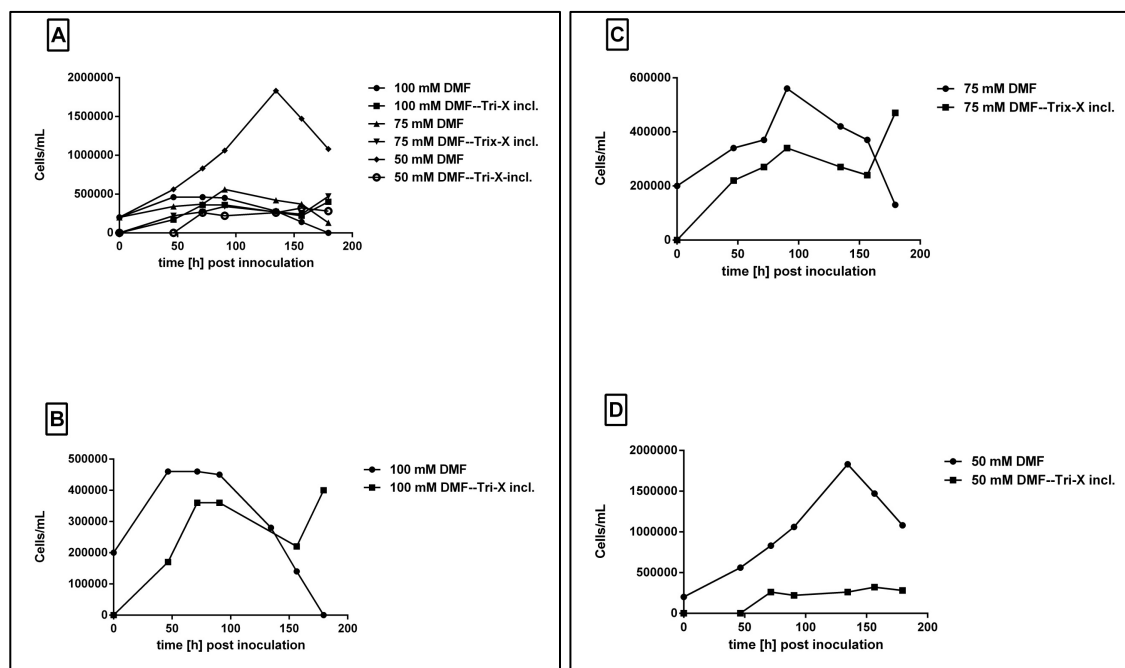
Figure 10. Relative growth ('X mM DMF) and mortality ('X mM DMF—Tri-X incl) of HL-60 cells in varied solutions of DMF inoculated with a starting concentration of 200,000 cells/mL. Cell mortality was quantified using a sample of cells from each time-point stained with Tri-X pan solution counted in a Neubauer cell-chamber. Cells showing inclusion of Tri-X pan were deemed unviable. All conditions showed an initial rise in viable cell concentration, with subsequent decline in viable cell concentration with a concomitant rise in cells showing Tripan Blue inclusion.

For each of these time-points, samples were plated and prepared using Giemsa-stain and nitroblue tetrazolium to establish the relative differentiation of cells in each of these respective conditions. (Micrographs of representative samples of cells from each set of conditions are shown in Figures 11-13). In order to select the optimal conditions for performing OPAs we compared samples from conditions with the optimal cell viability i.e. lowest cell-mortality, and highest degree of cell differentiation. The latter characteristic was determined by cell-morphology and superoxide anion content of granules. We assumed that an optimal culture would contain the least number of dead cells, which would presumably interfere with granulocyte-SP3 contact and thereby opsonization, and the greatest number of cells with the highest degree of differentiation.
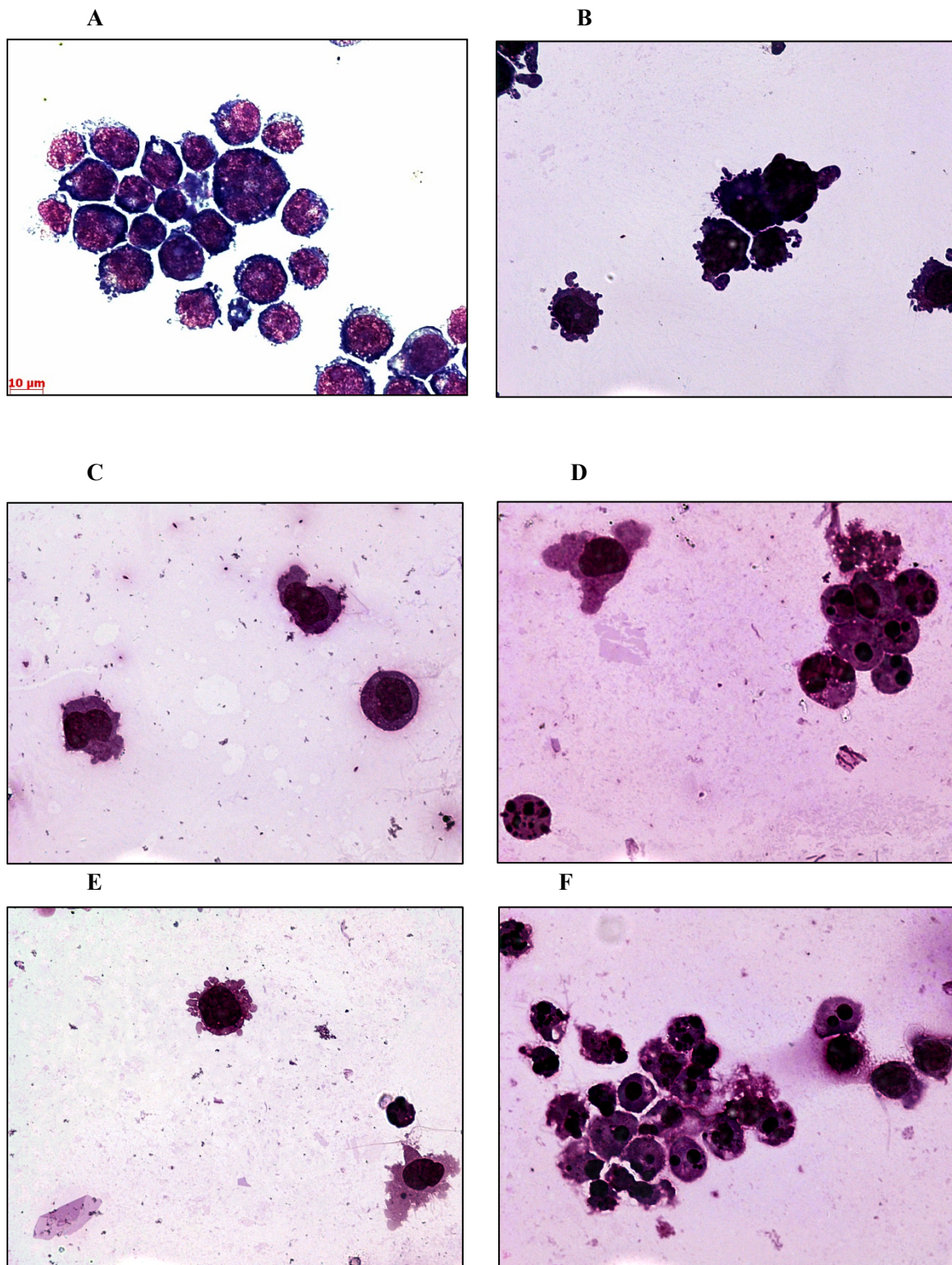
Figure 11 A-F showing author's micrographs of HL-60 cells prepared by the author with Giemsa stain and nitroblue tetrazolium in a native, unstimulated state (A), and in a 100 mM solution of DMF at 47(B), 72 (C), 91(D), 135(E) and 156(F) hours post-inoculation. Progressive differentiation can be observed from a morphology comparable to that of HL-60 cells in a native state, to one with increased segmentation of the nucleus and presence of granules containing superoxide anions.

A

B

C

D

E

F
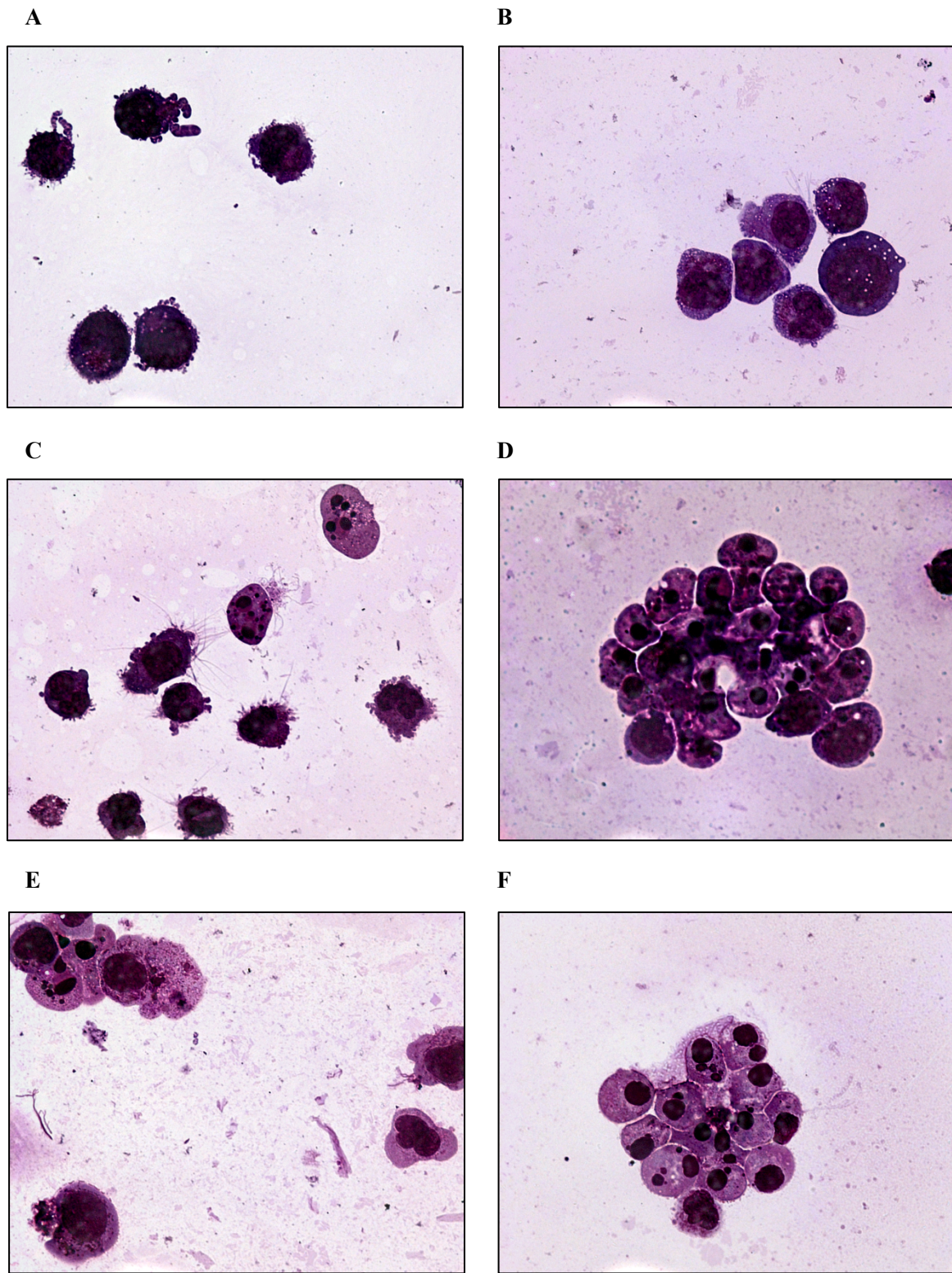
Figure 12 A-F. Author's micrographs of HL-60 cells, prepared by the author, stimulated with a 75 mM solution of DMF at 47, 72, 91, 135, 156 and 180 hours post-inoculation, respectively. The micrographs show progressive stages of differentiation, with a similar progression to a pseudogranulocytic state, and an increase in nuclear segmentation and the development of granules over time. Notably, there is an increased clustering of viable cells toward the end of the differentiation period and markedly less cell fragments, when compared to the differentiation-cultures containing a 100mM DMF solution.
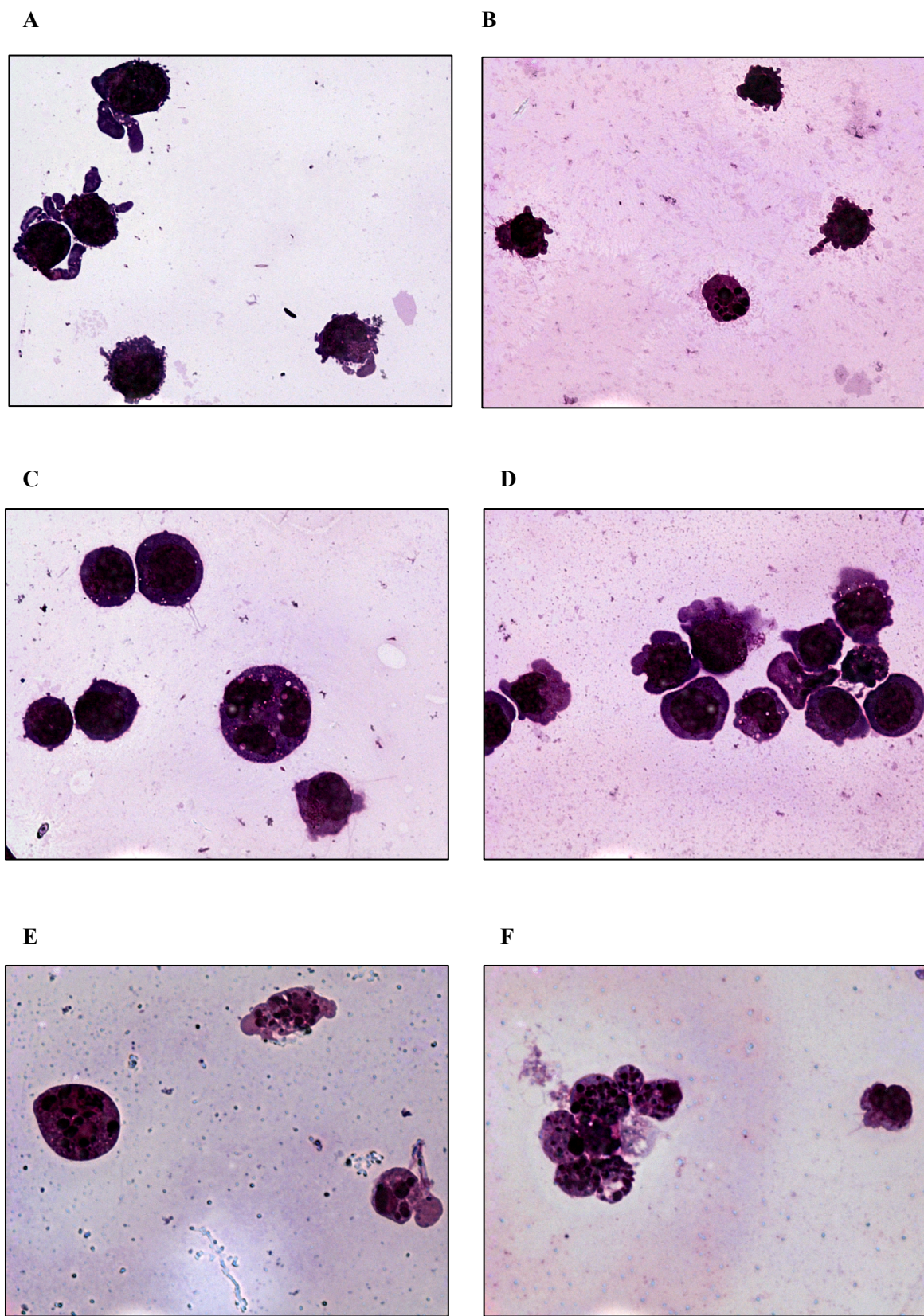
Figure 13 A-F. Author's micrographs of HL-60 cells, prepared by the author, stimulated with a 50 mM solution of DMF at 47, 72, 91, 135, 156 and 180 hours, respectively, post-inoculation. Progression to a pseudogranulocytic state where the characteristic, granulocytic appearance is also observed.

We concluded that cells differentiated in a 75 mM DMF Solution for 6 days showed, by comparison, the best ratio of differentiation to viability.

III. A. i. 3. Peripheral Human Neutrophils (PHN)

Peripheral human neutrophils (PHN) were extracted from human whole blood using combined Hisoplaque-1077 and -1119 gradients. Isolated neutrophils were substituted in the same quantity for the HL-60 cells in the OPA assays described above. To establish the gradient, 12 mL of Histoplaque-1077 solution was layered onto 12 mL of Histoplaque-1119 solution in a 50 mL centrifuge tube at room-temperature. 24 mL of whole blood, anticoagulated with 0.40 mL of a 250 mM EDTA solution, were then immediately layered onto this gradient and centrifuged at 700 x g for 30 minutes at 22°C with the brake off. The granulocyte layer was extracted and rinsed twice by suspension in PBS and centrifugation at 400 x g for 10 minutes. Following a third centrifugation, the cells were re-suspended with OPA-buffer and kept on ice until utilized in the OPA.

To verify the presence of granulocytes in the layer extracted, a sample was taken and was prepared with a Giemsa-Pappenheim stain to verify the cell-type, morphologically (Figure 14).
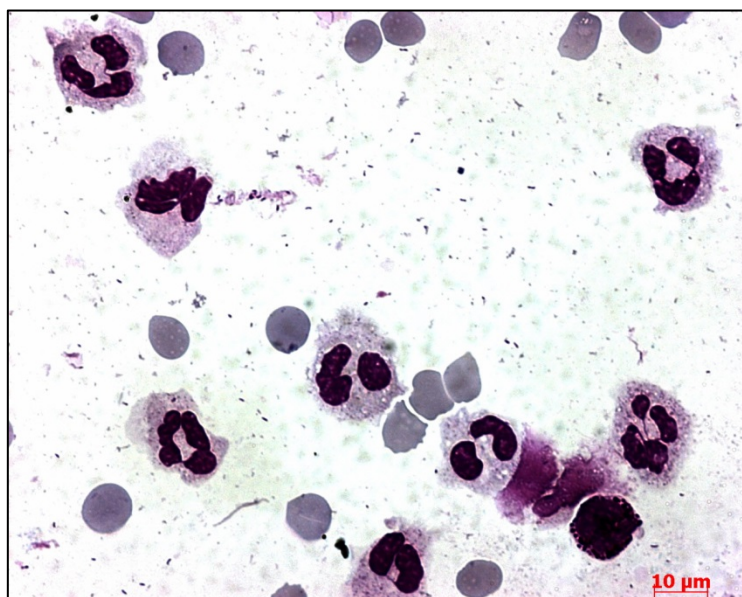
Figure 14. Author's micrographs, of Giemsa-Pappenheim stain of a representative sample, prepared by the author, of isolated PHN used in these experiments, with characteristic, multi-segmentation of the cell-nucleus.

III. A. i. 4. Serum

Serum used in OPAs stemmed from one of two groups: either from mice vaccinated in the experiments performed by Prof. Seeberger's group, or from mice vaccinated in our facilities. Mice from the former set of experiments had been vaccinated with SP3-Tetrasaccharide with a primary dose

of vaccine and either a Conjugate Freund's Adjuvant (CFA) or Aluminum Hydroxide adjuvant (Alum). Both groups received one boosting 28 days thereafter. Serum was extracted before vaccination, at 1, 2 and 4 weeks following primary vaccination, and at 1 and 2 weeks following boosting. Pooled serum samples from each of these conditions were then implemented in our OPAs.

A separate vaccination trial was carried out as above using 6 animals treated with SP3-Tetrasaccharide + CFA. Serum from individual animals drawn at 2 and then at 3 weeks post-boosting was used in OPAs.

In vaccination experiments conducted in our facilities (see description of *in-vivo* studies below) animals were treated with a primary dose of SP3-Tetrasaccharide + Alum, and with vaccine-boostings at 14 and 28 days thereafter. Serum was extracted at day 35 (i.e. 7 days following the second boosting) for use in OPAs.

Serum from naïve animals was used as a negative control in these experiments. WHO-standardized, human serum from humans vaccinated with the 23-valent polysaccharide vaccine was implemented as a positive control.

III. A. i. 5. Complement Factor

Complement factors, isolated from naïve baby rabbits, provided by two different manufacturers, Cedarlane™ and Biozol™, were compared for effectivity. OPAs showed that assays using Biozol™ produced significantly greater killing than those using complement obtained from Cedarlane™ (see Results section), and Biozol™ was therefore selected for use in comparative OPAs.

III. A. ii. Methods

III. A. ii. 1. Opsonophagocytosis Assay (OPA)

Differentiated cells obtained in the steps described above were harvested by centrifugation (160 x g for 10 minutes at room temperature). Trypan blue exclusion was used to first determine the viable cell count. The viable cell count was then used to calculate the volume of growth culture needed for the previous step. In order to obtain an effector-to-target cell ratio of 400:1, a volume of differentiated HL-60 cells or PHN was removed to provide 4×10^5 viable cells per well, multiplied by the number of wells used in the experimental conditions. On the day of the assay, the differentiated cells were centrifuged at 160 x g for 10 minutes at room temperature, the supernatant was discarded and the pellet then re-suspended in 15 mL of Hank's buffer without Ca^{++} or Mg^{++} (OPA-buffer). The suspension was then centrifuged a second time as above, and then re-suspended in the volume of the

same required for that specific assay. This suspension was kept in an ice-bath until it was implemented.

For the assay itself, 5 μL aliquots of serum from each condition (run in duplicate for each assay) were added to the first well of each row of a 96-well, round-bottom micro titer plate. 15 μL of OPA-buffer were then added to each of these wells, and 10 μL of OPA-buffer to all of the subsequent wells in that row. The serum was then serially diluted using a multi-channel pipette for a titer range of 1:32 to 1:4096. 10uL of OPA-buffer were then added to each row to bring the total volume of each condition to 90 μL. 20 μL of bacterial suspension (PN36, taken directly from thawed cryostocks prepared as described in the section above) were then added to each well. Control conditions, also run in duplicate, were set up as follows: 20 μL of PN36 + 70 μL of OPA-buffer, 20 μL of PN36 + 10 μL of complement + 60 μL of OPA-buffer, 20 μL of PN36 + 40 μL of differentiated Hl-60 cells + 30 μL of OPA-buffer, and 20 μL of PN36 + 10 μL of complement + 5 μL of WHO serum + 55 μL of OPA-buffer. The micro titer plate was then incubated for 15 minutes at 37°C and 5% CO_2 atmosphere. Phagocytosis was then initiated by adding 10 μL of complement suspension (Biozol™) to each well, followed immediately by 40 μL of differentiated HL-60 cells or PHN. The plate was then incubated for 45 minutes at 37° in a horizontal shaker operating at 220 rpm. The plate was then removed and kept on ice for the remainder of the protocol to halt the phagocytosis process. 5 μL of suspension from each condition was then plated onto 5% sheep blood agar plates and incubated for 10-12 hours at 37°C and 5% CO_2 atmosphere. The plates were then removed and the CFU's counted manually.

III. B. *In–Vivo* Studies

III. B. i. Materials

III. B. i. 1. Animals/Housing

All protocols involving animals were first approved by the University's board for the care and use of animals in research, as well as the local authorities (Landesamt für Gesundheit und Soziales Berlin, approval ID: A 305/12). Animals were housed and handled adherent to regulations on animals used for scientific purposes set forth by the European Commission for Environment and the Society for Laboratory Animal Science Association (FELASA). Housing for all mice used was kept pathogen-free.

III. B. i. 2. Bacteria

Bacteria for challenge-experiments were taken from frozen stocks and plated on Columbia Agar containing 5% sheep blood by volume and incubated at 37°C and 5% CO_2 atmosphere for 9 hours. Single colonies were used to inoculate a medium of Todd Hewitt Broth supplemented with 0.5% yeast extract (growth medium). Bacteria were then cultured at 37°C and 5% CO_2 atmosphere to an optical density of 0.3 – 0.4 at a wavelength of 600 nm, which corresponds to mid-logarithmic growth-phase. The suspension was then centrifuged and re-suspended with sterile, Phosphate-buffered Saline (PBS) to the appropriate concentration for direct implementation in infection (see below).

III. B. i. 3. Serum

Serum was obtained from mice in a pre-vaccinated state, and at 2 and 4 weeks following primary vaccination. Blood was drawn either from the tail- or facial-vein and centrifuged at 5000 x g for 10 minutes at room-temperature to separate serum from the cellular phase.

III. B. ii. Methods

III. B. ii. 1. Pneumococcal Challenge

8 week-old, Female C57BL/6N mice (Charles River, Sulzfeld, Germany) were injected subcutaneously with a 1:1 (v/v) suspension of SP3-Tetrasaccharide and Alum (Alhydrogel, Brenntag, Mühlheim, Germany) or CFA (Sigma-Aldrich, Steinheim am Albuch, Germany). This combination corresponds to a 100 µL solution containing 5 µg of conjugate. As controls, mice were injected with 100 µL of sterile PBS, or with a suspension containing SP3-Tetrasaccharide without an adjuvant.

S. pneumoniae challenge was performed using transnasal inoculation with bacteria at 35 days for short-term studies, or at 116 days for long-term studies. For this purpose, mice were anaesthetized by intraperitoneal (i.p.) administration of 80 mg/kg of Ketamine (Ketavet®, Pfizer, Berlin, Germany) and 25 mg/kg of xylazine (Rompun®, Bayer, Leverkusen, Germany). A 20 µL PBS/bacterial suspension containing 1×10^6 CFU, or 20uL PBS solution was administered to the anaesthetized mice. Mice were monitored at 12-hour intervals for signs of illness and distress, which was qualified according to behavior (activity, grooming and respirations), appearance, body weight and rectal temperature (BAT-12 Microprobe Thermometer, Physitemp Instruments, Clifton, NJ).

48 hours following pneumococcal challenge, mice were deeply anaesthetized with a combination of ketamine (160 mg/kg body weight (BW)) and xylazine (75 mg/kg BW), and subsequently prepared for tracheotomization and ventilation. This procedure was carried out by Dr. med. vet. Katrin Reppe

(Witzenrath laboratory). Mice were then heparinized and exsanguinated via the *Vena Cava Caudalis*. The lungs were then perfused by way of the pulmonary artery using sterile PBS. Broncheoalveolar lavage (BAL) was conducted in two steps with an administration of 800 µL of PBS plus protease inhibitors each (Roche™, Mannheim, Germany). Both lungs and the spleen were then subsequently removed for microbiological and histological examination.

Bacterial burden in BAL-fluid (BALF), lung-tissue and whole blood was measured. Lung-tissue was passed through a cell-strainer (100 µm, BD Bioscience), and the homogenate diluted serially and plated on Columbia agar with 5 % sheep blood and incubated at 37°C and 5% CO_2 overnight. BALF and whole blood samples were similarly diluted and then plated for incubation. CFU's were then counted manually.

To measure blood-lung barrier permeability, albumin concentration in BALF supernatant and plasma were determined using enzyme-linked immunosorbent assay (Bethyl Laboratories Inc., Montgomery, AL, USA) as per manufacturer's instructions. Albumin BALF/plasma ratio was calculated as an indicator and quantifier of alveolar edema.

Total BALF leukocytes were quantified by use of a Neubauer™ hemocytometer, and differentiated by FACS analysis (FACS Calibur; BD Biosciences, Heidelberg, Germany) using forward vs. side scatter characteristics and staining with CD45 PerCP (clone 30-F11) GR-1 PE (clone RB6-8C5, all from BD Bioscience™, Heidelberg, Germany) and F4-80 APC (Invitrogen™ by Life Technologies, Carlsbad, CA).

IV. Results

IV. Pooled reference serum from immunized humans potently opsonizes and facilitates killing of SP3 bacteria by HL-60 derived pseudogranulocytes or peripheral human neutrophils and complement.

Host-defense against *S. pneumoniae* is contingent upon effector cells' ability to phagocytize bacteria[29,30,89]. Phagocytosis of *S. pneumonia*, like most capsulated bacteria, is greatly enhanced by opsonization via specific antibodies, in particular IgG[28,90]. Enzyme-Linked Immunoabsorbant Assay (ELISA) is used to quantify the absolute serum-concentration of specific IgG[91,82], and opsono-phagocytosis assays (OPA) indirectly measures the presence and functionality of opsonizing and complement activating antibodies by measuring the ability of effector cells to clear bacteria in vitro[92].

In-vitro assessment of immune response: quantitative Enzyme-Linked Immunoabsorbant Assay (ELISA) and opsonophagocytosis assays (OPA).

IV. A. i. ELISA for verification of IgG concentration.

In-vitro experiments include the use of quantitative ELISA and OPA to establish specific serum IgG concentrations and the functionality of those immunoglobulins, respectively[82]. Similar to the glycan microarray studies described above, ELISA involves the immobilization of antigens—in this case the native SP3-CPS—in a solid matrix and application of serum from immunized mice. Secondary antibodies conjugated to substances that can be stimulated to produce chromographic reaction are subsequently applied. Quantification of this reaction verifies IgG concentrations[91,93].

IV. A. ii. OPAs as a tool for functional assessment of immunogenicity in response to vaccination.

OPAs combine immune serum (or non-immune serum as control) with complement (usually baby rabbit complement) with effector cells and a pre-determined number of bacteria. The finished product of an OPA is plated on sheep's agar. The functionality of the sera used for each condition is indicated by the degree of killing of bacteria. This procedure was first described and standardized by Romero-Steiner *et. al* in 1997 and has been adopted by the WHO in its guidelines for the evaluation of novel vaccines[82].
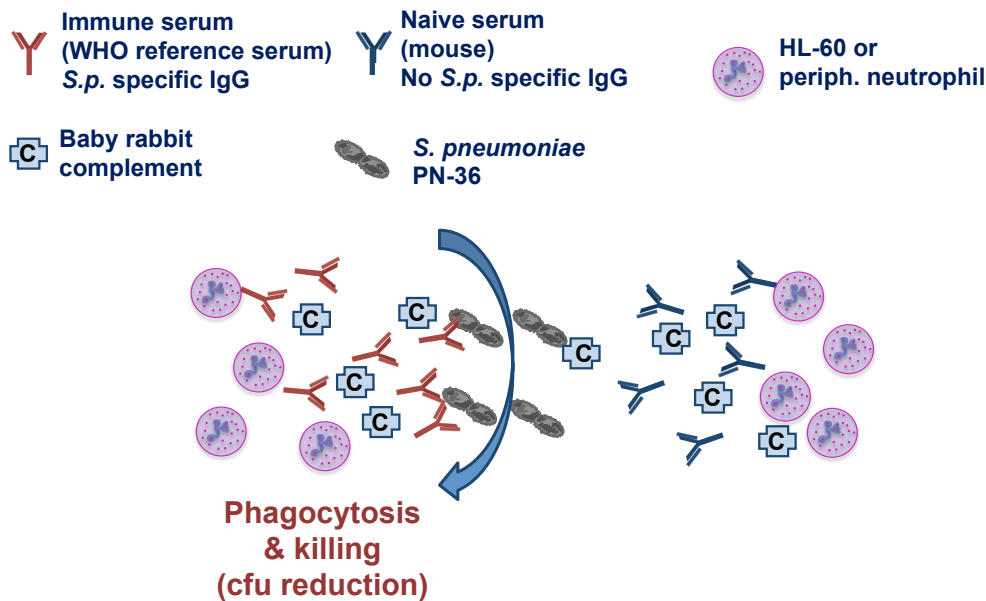
Figure 15. Author's rendering—using original artwork—of the process of opsonization of S. pneumoniae showing binding of specific IgG to pneumococci and subsequent phagocytosis with the help of complement.

A key component of OPAs is the effector cells: phagocytosis of *S. pneumoniae in-vivo* is executed largely by neutrophilic granulocytes[94–96]. The isolation of primary peripheral granulocytes represents a source of potential variability. The strategy employed to circumvent this problem has been to utilize differentiated, human myeloprolific HL-60 cells[92,97,98]. These cells were isolated from a patient in the late 1950's with acute myeloprolipic leukemia and have been utilized for a wide range of uses since[99]. These cells are pluripotent, and when exposed to certain stimuli will differentiate to express the phenotype of mature white blood cells[100]. It was found that exposure to the organic solvent, Dimethylformamide (DMF) would result in the differentiation of these cells to granulocytes or 'pseudogranulocytes' with the morphological and biochemical properties of peripheral human neutrophils[87] (Figure 15).

In order to establish the OPA testing of mouse immune sera in our laboratory, we acquired pooled serum standardized by the World Health Organization (WHO-S) from humans vaccinated with PSV23 (Pneumovax23®) to use as a positive control. Indeed, we found that WHO reference serum mediated significant killing of SP3 bacteria through opsonphagocytosis with HL60-derived pseudogranulocytes, achieving 50% killing at titers between 1:32 and 1:128 (Figure 16). By contrast, serum from naïve, nonimmune mice (NMS) failed to induce significant bacterial killing (Figure 16). Per WHO definition, an effective concentration of serum antibodies corresponds to one that achieves 50% or greater-reduction of CFU-count[98,101]. For further optimization, effectivity of two complement-factor sources

(Biozol™ and CedarLane™) was also compared, showing better killing in assays utilizing Biozol's™ baby rabbit complement (Figure 16).
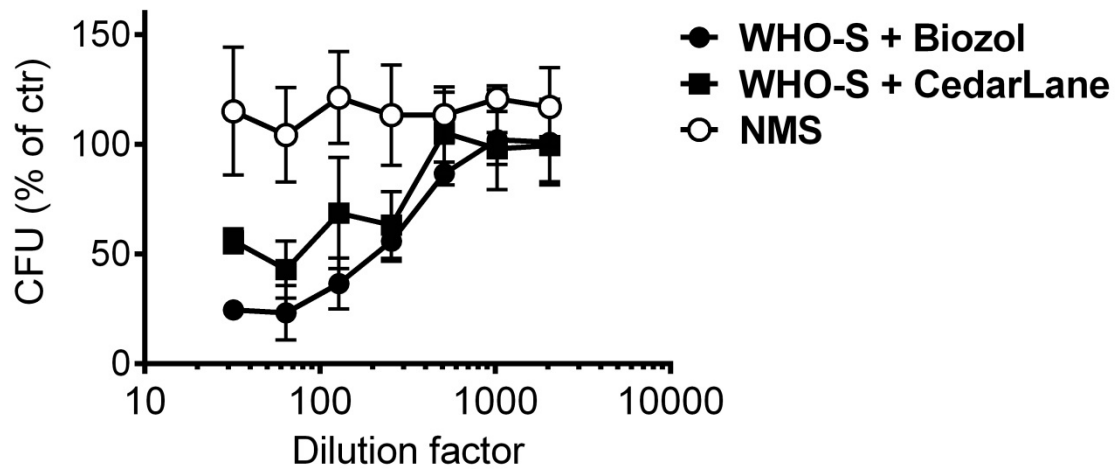
Figure 16. A comparison of bacterial killing curves in OPAs utilizing HL60-derived pseudogranulocytes with either Biozol™ or CedarLane™-sources of complement factor and nonimmune mouse serum (NMS) or WHO-S (Immunized, WHO-Serum). Shown is the mean ±SEM. n= 5.

To further confirm effectivity of OPAs with primary human cells, we isolated human neutrophils (PHN) from peripheral blood of healthy blood donors by density gradient centrifugation as stated in detail in the Methods section. PHN showed slightly better killing capacity in OPA compared to HL60 derived pseudogranulocytes (Figure 17).
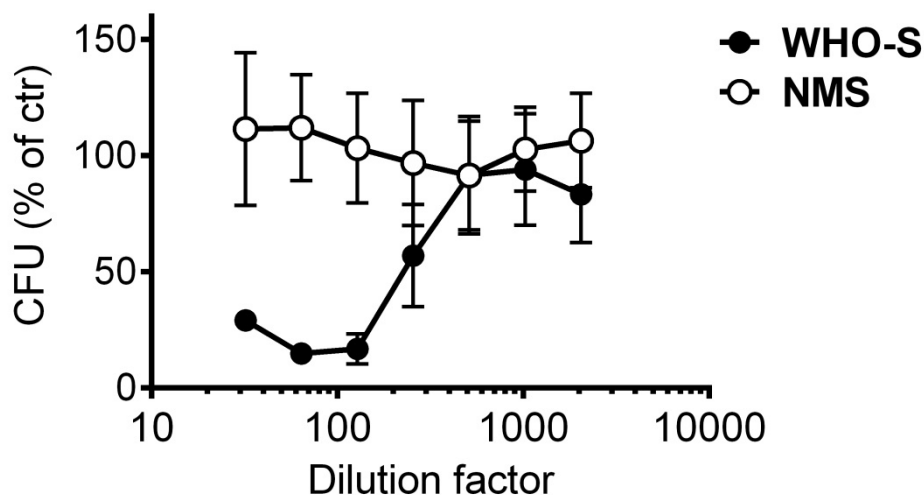
Figure 17. Bacterial killing in OPAs utilizing PHN as effector cells and Biozol™ complement and either WHO-S or NMS. Shown is the mean ±SEM. n=5

To sum up, we established a standardized *in vitro* assay (OPA) to assess the functionality of humoral immunity to *S. pneumoniae.*

IV. B. Results from Short Term Vaccination Studies.

IV. B. i. Preliminary Studies

IV. B. i. 1. OPAs

Immune sera from SP3-Tetrasaccharide vaccinated mice facilitate significant opsonophagocytosis.

Sera derived from experiments conducted by our collaborators at the MPIKG included mice immunized with SP3-Tetrasachharide + Alum following a prime-boost regimen (day 0, 14, 28), were analyzed by OPA (Figure 18).
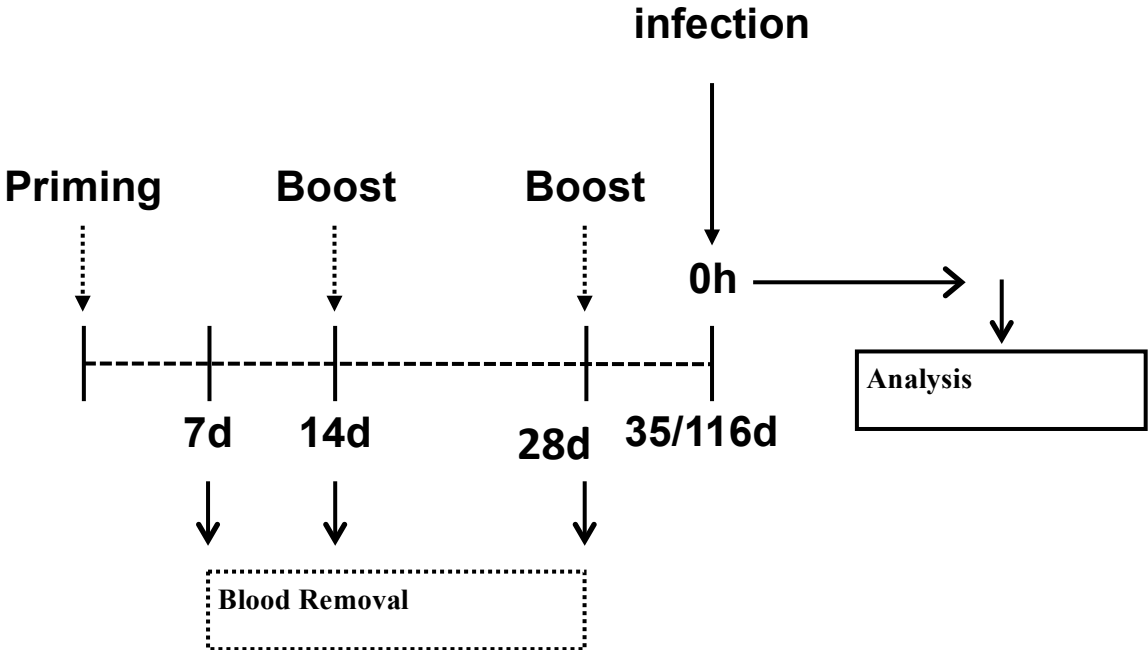
Figure 18. Time-line of the prime-boost SP3-Tetrasaccharide vaccinations, followed by infection and OPA.

Data from OPAs using sera from mice treated in this way, taken at 2 and 3 weeks following the second boosting, were pooled and compared to sera from naïve mice. The OPA analyses demonstrated efficient opsonophagocytic capacity of immune sera from mice at 2 weeks post-boosting, with 50%

killing or greater at titers up to 1:256. Sera taken from mice at 3 weeks post-booster effected killing at all concentrations (Figure 19).
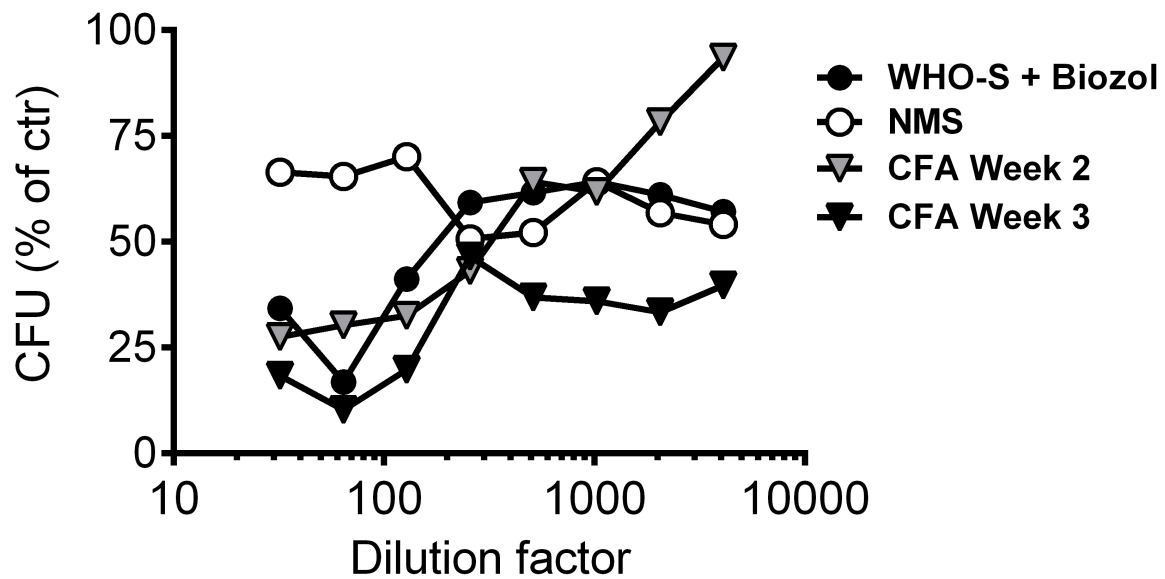
Figure 19. OPAs utilizing pooled sera from 6 mice treated with SP3-Tetrasaccharide (SP3-ts) + CFA at 2 and 3 weeks after second boosting. Shown is the mean killing achieved using pooled sera from 6 mice, with each experimental condition repeated a total of 6 times.

Pooled sera from mice treated with SP3-Tetrasaccharide + CFA taken at 4 weeks post primary-vaccination (i.e. before the second boosting) and at 1 and 2 weeks after the second boosting showed significant killing when compared to controls and earlier time-points (Figures 20 A-B & 21).
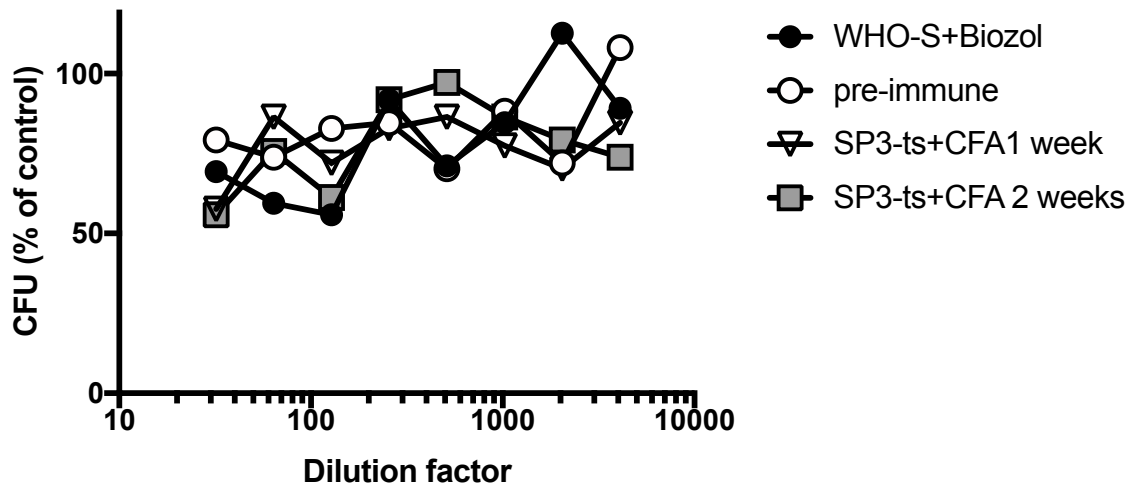
Figure 20-A. Killing achieved with Pooled sera from 6 Mice in a pre-immune state, at 1 and 2 weeks following vaccination.
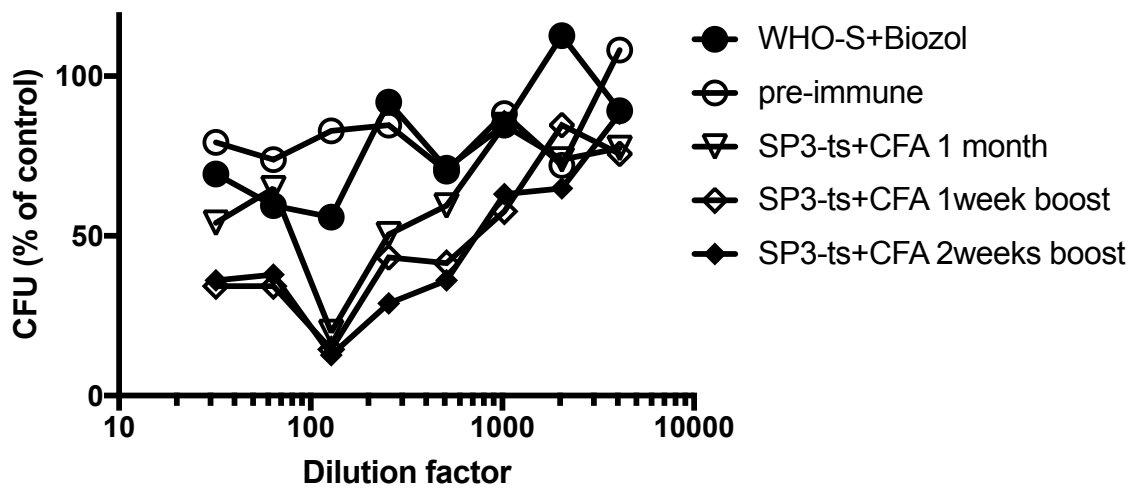
Figure 20-B. Killing achieved with Pooled sera from 6 Mice 4 weeks following primary vaccination (SP3-ts+CFA), and at 1 and 2 weeks following the second boosting (SP3-ts+CFA). Each experimental condition was carried out once, as there was insufficient serum to perform multiple trials.

A selection of data points from each curve from the data-sets described above corresponding to a dilution of 1:128 further illustrates effective bacterial killing a month after primary vaccination and at 1 and 2 weeks post boosting (Figure 21).
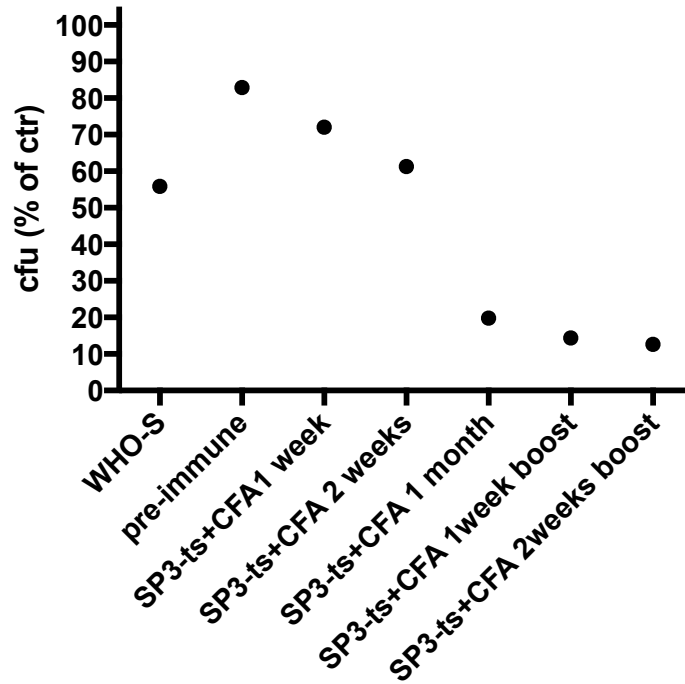
Figure 21. Selection of mean bacterial killing capacity in OPA of pooled sera (dilution 1:128) from animals vaccinated using SP3-Tetrasaccharide (SP3-ts) + CFA.

Using a standardized WHO OPA for preclinical testing, these results demonstrated a functional immunogenicity of the newly generated semi-synthetic Alum or CFA adjuvanted SP3-Tetrasaccharide vaccine.

IV. B. ii. Establishment and Execution of In Vivo Experiments.

Applying a mouse-model of pneumonia for *in-vivo* vaccine assessment.

While there is no standard animal-model of pneumonia, the effects of *S. pneumoniae* in mice have been well characterized in the literature[102,84]. A challenge-experiment in which animals are directly inoculated with a quantity of bacteria sufficient to cause fulminant pneumonia represents an effective means of testing vaccine protection[103]. Pneumococcal serotypes differ in terms of the effect they have on their host. SP3 often causes bacteremia and pneumogenic sepsis that often affects the liver and other organ-systems[102]. An effective vaccine for SP3 should therefore be able not only to protect against bacterial burden of the lungs and the effects of pneumonia, but also to reduce bacterial translocation to the bloodstream and involvement of other organ-systems.

The parameters used to evaluate the effects of SP3-Tetrasacharide were established to encompass the clinical, physiological, laboratory and histological measures of disease in mice.

SP3-Tetrasaccharide + Alum protects mice against challenge with SP3 35 days post immunization.

IV. B. ii. 1. Clinical Data: appearance, bodyweight and temperature.

Vaccinated animals showed clinical signs of protection from SP3 challenge, as well as maintenance of bodyweight and temperature as compared to unvaccinated animals.

Animals were monitored in 12-hour intervals for signs of disease. Clinically, mice with severe illness exhibit signs of inactivity, reduced feeding and grooming and excessive lacrimation[104]. Body weight declines over the course of progressive disease and body temperature drops in cases of fulminant bacterial invasion and sepsis[104]. Regular and frequent monitoring and documentation of these parameters can indicate the trajectory of the disease course in animals[104]. For the purposes of this study, these parameters were monitored at 12-hour intervals.

Clinical observation of the mice showed telltale signs of disease. Reduced activity, hunched posture, lacrimation and starry fur were seen in varying degrees in mice from the PBS-treated and SP3-Tetrasaccharide -only groups, whereas SP3-Tetrasaccharide + Alum mice were completely free from such signs.

The body weight of *S. pneumoniae* infected mice immunized with adjuvanted vaccine did not drop significantly from baseline (n=11; 92.48 ± 3.45%; p>0.05). In contrast, the control non-immunized group and the group immunized in the absence of Alum adjuvant showed significant bodyweight loss. SP3-Tetrasaccharide + Alum mice showed significantly better maintenance of body-weight at 48 hours after infection (n=11; 92.48 % ± 3.35 %) compared with infected mice treated with PBS (n=11; 84.14 % ± 7.04 %; p<0.001) and the group receiving SP3-Tetrasaccharide only (n=11; 84.6 % ± 5.67 %; p<0.01). Predictably, PBS-treated, uninfected mice showed the least drop in weight (n=9; 96.99 % ± 4.27 %: p>0.05) (Fig's. 22 & 23).
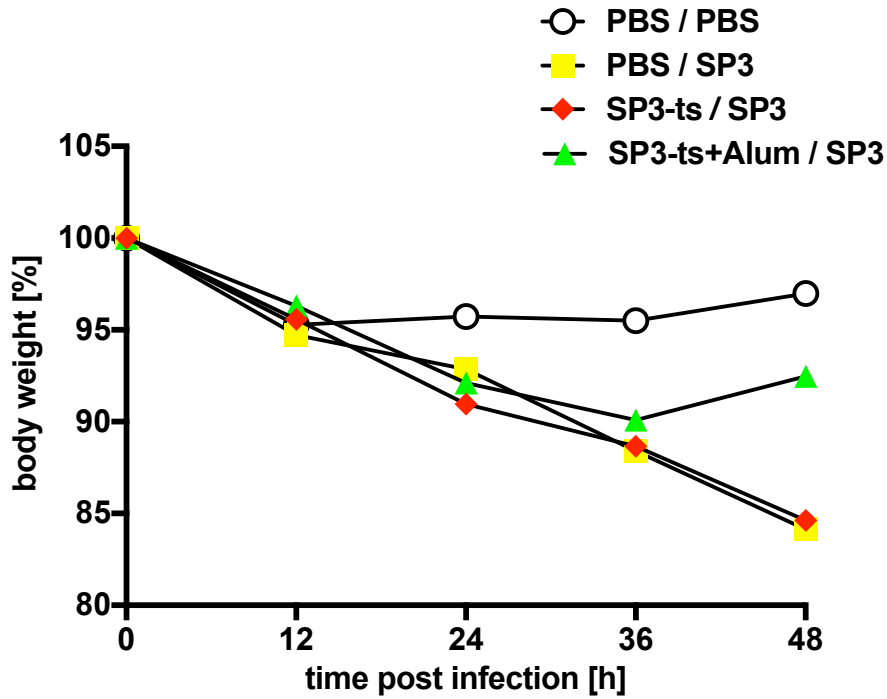
Figure 22. Average body weight over time in the 48 hours following pneumococcal challenge, shown as a percentage of body weight at time=0 (PBS / PBS = Phosphate-buffered saline - sham-infected, unvaccinated animals; PBS / SP3 = unvaccinated mice infected with SP3). Shown is the mean ±SEM. 1-Way ANOVA n=9 PBS/PBS; n=11 for PBS/SP3, SP3-Tetrasaccharide/SP3 and SP3-Tetrasaccharide+Alum/SP3
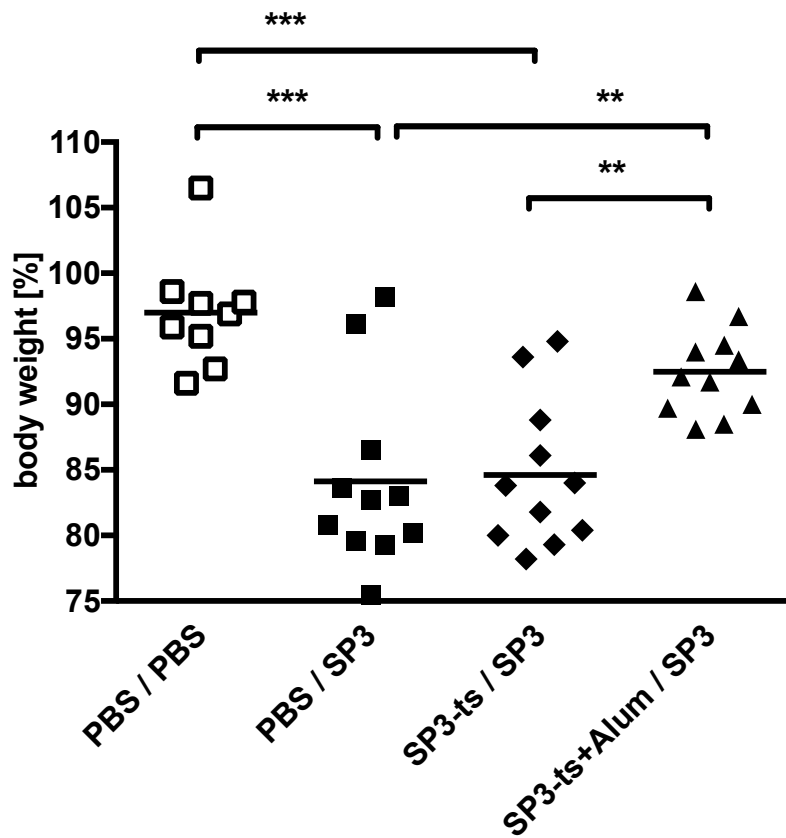
Figure 23. Average body weight of mice 48h post-challenge. [**p<0.01; ***p<0.001] 1 way ANOVA, Shown are the data for each animal and the mean (horizontal bar; PBS / PBS = Phosphate-buffered saline - sham-infected, unvaccinated animals; PBS / SP3 = unvaccinated mice infected with SP3). 1-Way ANOVA. n=9 PBS/PBS; n=11 for PBS/SP3, SP3-Tetrasaccharide/SP3 and SP3-Tetrasaccharide+Alum/SP3.

Fever in humans corresponds to a temperature drop in mice[104]. We found that the changes in body temperature in the experimental groups mirrored the changes in body-weight described above (Figures 24 & 25). Body-temperature 48 hours post pneumococcal challenge showed declines in the PBS-treated, infected group (n=11; 36.07 °C ± 0.88 °C; p<0.01) and the SP3-Tetrasaccharide-only control groups (n=11; 35.91°C ± 1.79 °C p<0.01); whereas the SP3-Tetrasaccharide + Alum group maintained body-temperature (n=11; 37.11°C ± 0.36 °C; p>0.5), reflecting the protective effect of the adjuvanted vaccine. Our negative-controls, which were unvaccinated and sham-infected, showed no significant decline in body temperature (n= 9; 37.68°C ± 0.28 °C p>0.5).
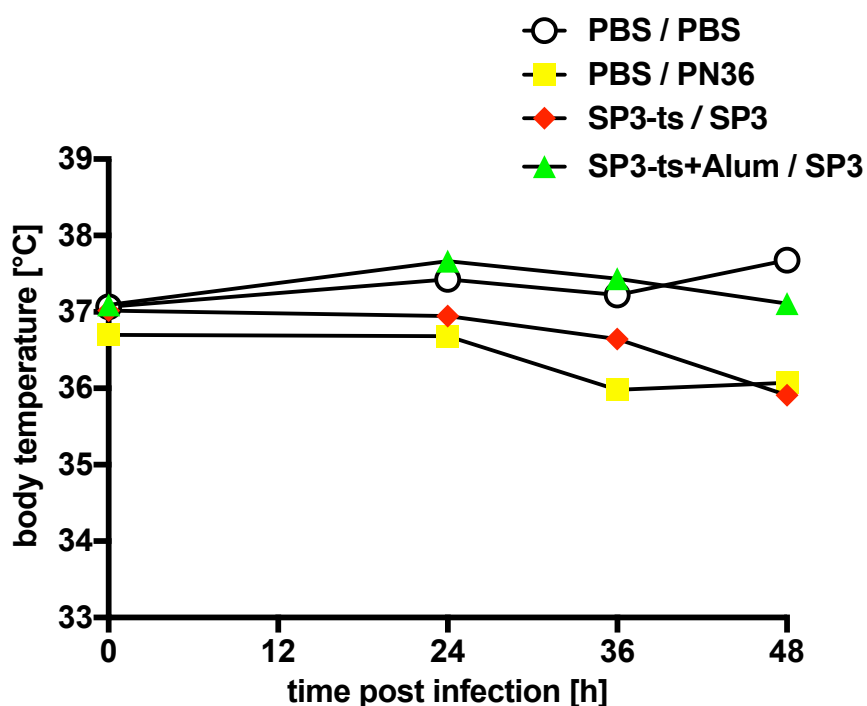
Figure 24. Average body-temperature in the experimental groups during the 48 hours following pneumococcal infection. Shown is the mean ±SEM, n=9 PBS/PBS; n=11 for PBS/SP3, SP3-Tetrasaccharide/SP3 and SP3-Tetrasaccharide+Alum/SP3.
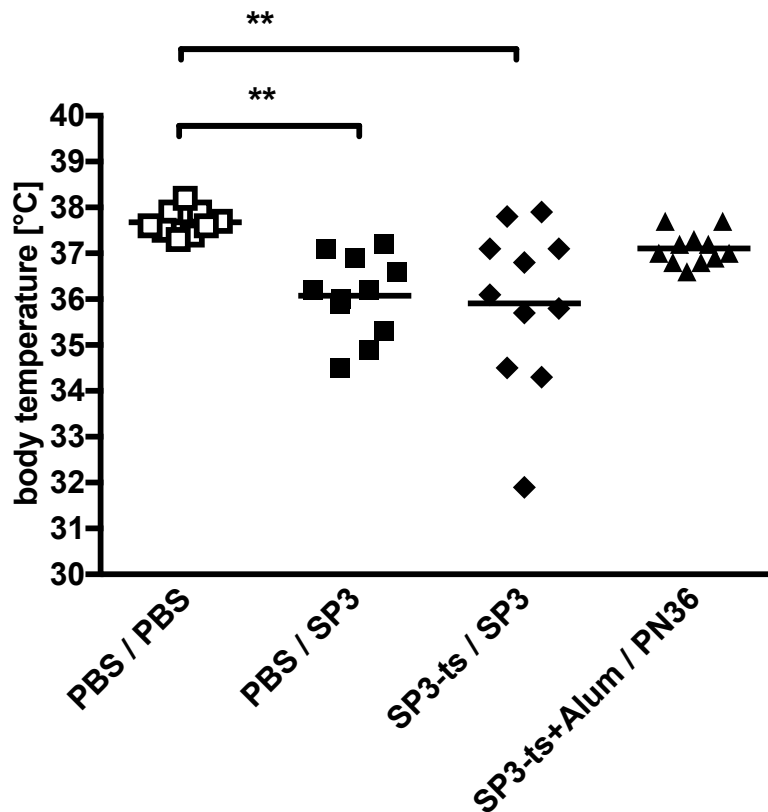
Figure 25. Body-temperature 48 hours post pneumococcal challenge. Shown are the values for each individual animal, the mean is represented by the horizontal bar. [**p<0.01] 1-Way ANOVA, n=9 PBS/PBS; n=11 for PBS/SP3, SP3-Tetrasaccharide/SP3 and SP3-Tetrasaccharide+Alum/SP3.

IV. B. ii. 2. Bacterial Burden in the Lung and in Blood.

Animals immunized with Alum-adjuvanted SP3-Tetrasaccharide showed overall lower bacterial burden of the lung, and lower levels of bacteremia compared to controls.

S. pneumoniae pneumonia in mice is associated with high bacterial burdens in pulmonary secretions and lung tissue[102,105]; bacteremia and sepsis are also frequent complications of pneumococcal pneumonia[106]. Quantification of colony forming units (CFU) in both bronchoalveolar lavage fluid (BALF) and homogenized lung tissue allows for the quantification of bacterial load in the lung; CFU counts in whole blood may be used to assess levels of bacteremia, and thereby sepsis[107,108]. Bacterial clearance was measured by CFU counts from plating BALF, lung-tissue homogenates, and whole blood samples on sheep-blood agar. We found that bacterial loads in the BALF of SP3-Tetrasaccharide + Alum animals (n=8; 3 CFU/μL ± 3/μL) were significantly lower than those in SP3-Tetrasaccharide-only controls (n=6; 5.18 x 10^2 CFU/μL ± 1.12 x 10^3/μL; p<0.05). Bacterial load in the vaccinated group was also lower than in PBS-treated, infected animals (n=8; 1.016 x 10^3 CFU/μL ±

1.844 x 10^3 CFU/µL), but this difference is not statistically significant at conventional levels (p>0.5) (see Figure 26).
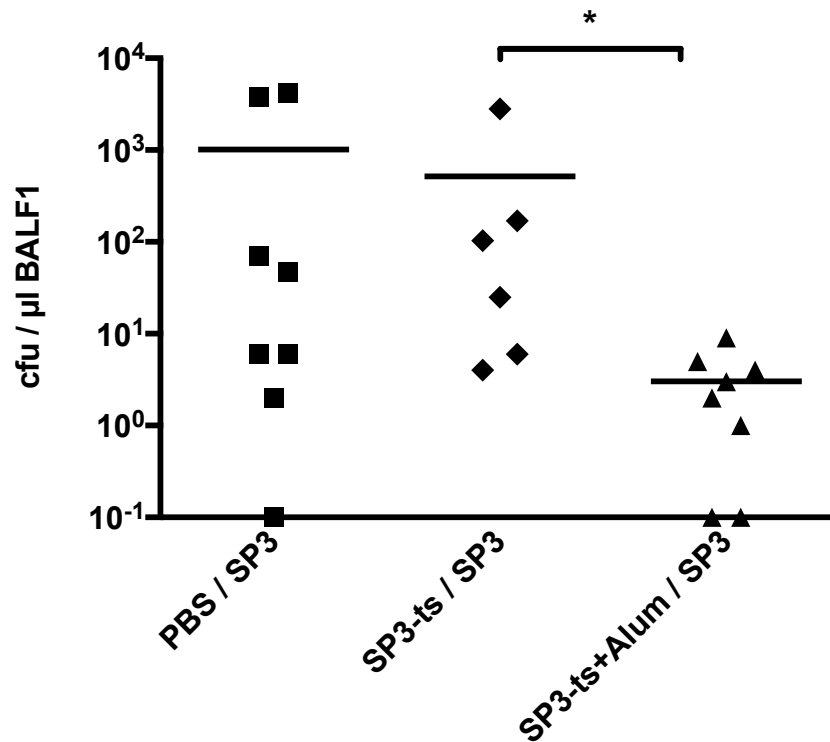
Figure 26. CFU counts obtained from BALF by plating samples on sheep-blood agar and overnight incubation. [*p<0.05]1-Way ANOVA. (n= 8 for PBS / SP3 and SP3-ts + Alum groups; n= 7 for SP3-ts / SP3 groups).

These results were reflected by bacterial burdens in homogenized lung tissue samples, in which significantly better bacterial clearance was seen in the vaccine-adjuvant group when compared to controls (Figure 27). Bacterial load in SP3-tetrasaccharide + Alum mice (n=8; 9.47 x 10^4 CFU/g ± 2.59 x 10^5 CFU/g) was significantly lower than in both the PBS-treated, infected mice (n=8; 1.75 x10^8 CFU/g ± 1.53 x 10^8 CFU/g; p<0.01) and the SP3-Tetrasaccharide only group (n=8; 2.15 x 10^8 CFU/g ± 2.99 x 10^8 CFU/g; p<0.05).
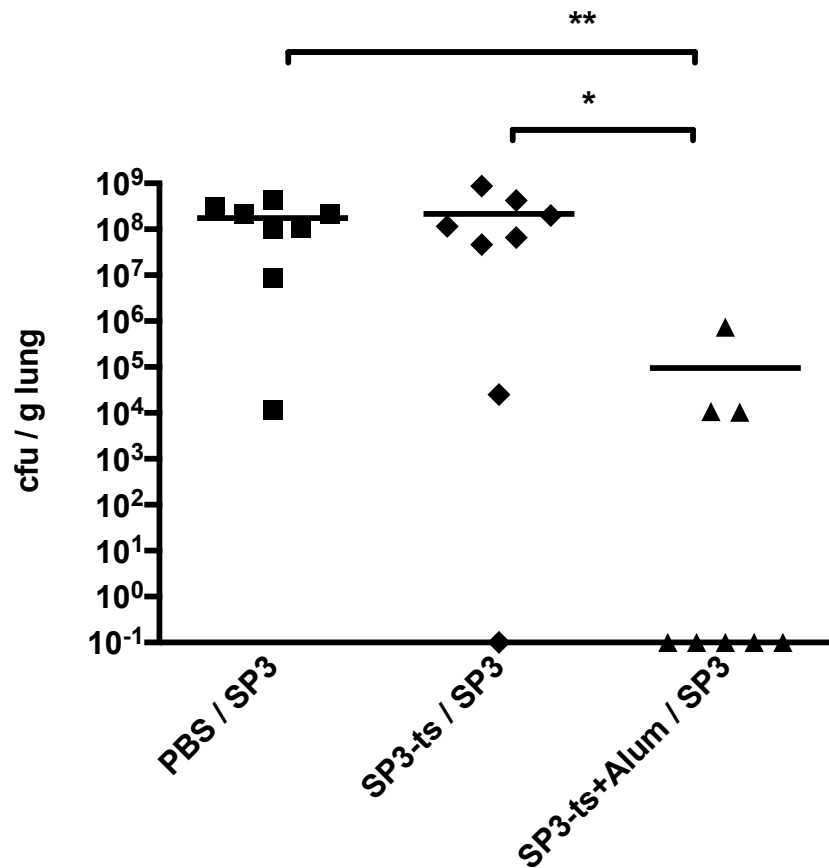
Figure. 27. Bacterial load in homogenized lung-tissue 48 hours post pneumococcal challenge. 1-Way ANOVA [*p<0.05; **p<0.01], n=8 for all groups.

Levels of bacteremia were pronounced in control animals, whereas the animals in the adjuvanated-vaccine group showed virtually no bacterial invasion into the blood stream (Figure 28). SP3-Tetrasaccharide + Alum treated mice (n=8; 0.5 CFU/μL $\pm$ 1.41 CFU/μL) were lower than those in both the SP3-Tetrasacharide only group (n=8; 5.10 x 10^2 CFU/μL $\pm$ 5.96 x 10^2 CFU/μL; p<0.05) and the PBS-treated group (n=8; 2.53 x $10^2\mu$L $\pm$ 2.36 x 10^2 CFU/μL; p<0.05).
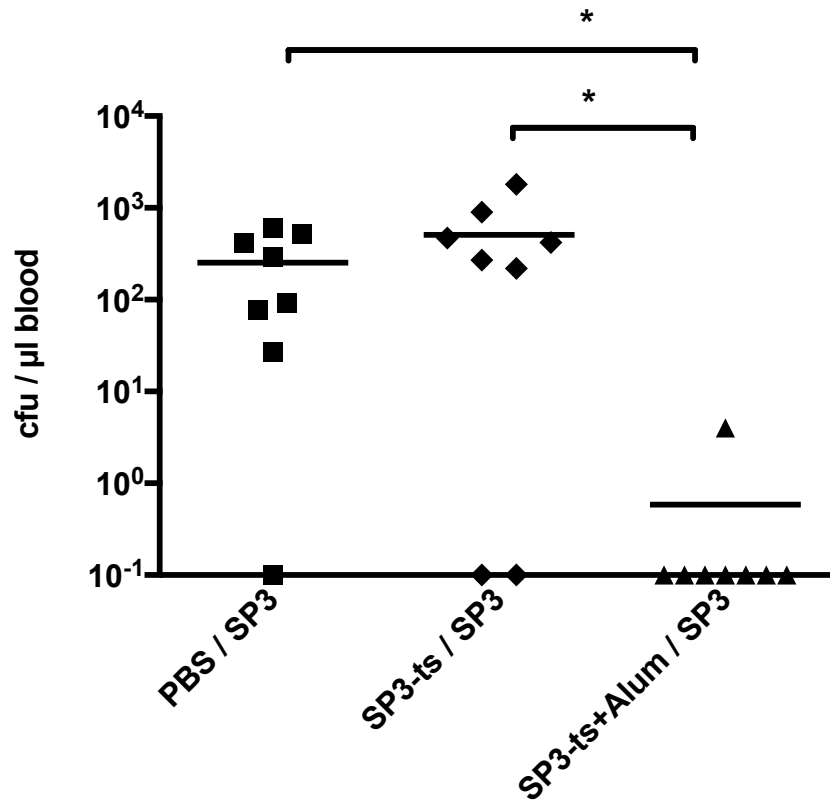
*Figure 28. CFU counts in whole blood 48 hours after pneumococcal challenge. 1-Way ANOVA. [*p<0.05] n=8 for all groups.*

IV. B. ii. 3. White Cell Counts in the Lung and in Blood.

Bacterial pneumonia is characterized by a massive influx of inflammatory leukocytes into the infected lung tissue, and extravasation of those cells and fluid into the air-spaces[109]. Leukocyte-, and especially Neutrophil infiltration in the case of *S. pneumoniae* plays a central role in the mediation of innate response to invasive organisms[110,111], involving activation and excretion of inflammatory factors such as pneumolysin, reactive-oxygen species and proteases, causing both bactericide and lung parenchyma edema, tissue damage and fluid-accumulation[112,113]. These inflammatory responses and the secondary effects of inflammation are the hallmarks of pneumonia, and are typically reflected as "pulmonary infiltrate" in the chest X-ray of pneumonia patients[114,115]. Here, as a marker of disease-severity, we quantified leukocyte infiltration of the lung and the alveolar space using flow cytometry. Leukocyte counts in BALF were performed 48 hours after infection, and revealed a significantly greater number of leukocytes in the PBS-treated, infected mice (n=8; 2.02×10^4 cells/mL $\pm$ 1.60×10^5/mL) and the SP3-Tetrasaccharide only group (n=7; 2.53×10^5 cells/mL $\pm$ 1.50×10^5 cells/mL) than in the PBS-treated, uninfected mice (n=7 2.02×10^4 cells/mL $\pm$ 9.38×10^3 cells/mL) (p<0.05 & p<0.01, respectively). A higher leukocyte count in the SP3-Tetrasaccharide + Alum group was also detected,

but is not statistically significant at conventional levels (n=8; $1.39 \times 10^5 \pm 6.74 \times 10^4$ cells/mL; $p>0.05$) (Figure 29).
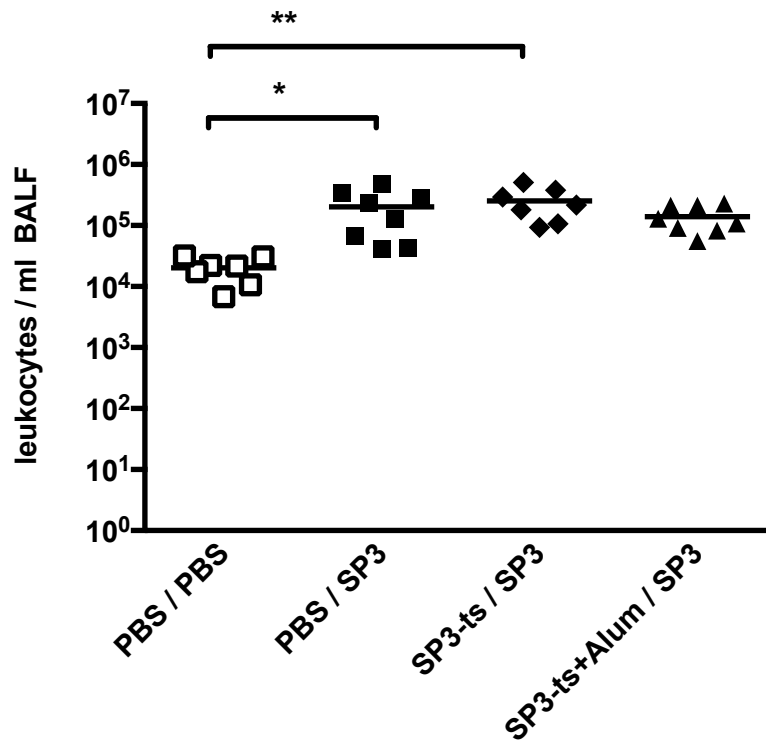
Figure 29. Leukocyte counts in BALF 48 hours post pneumococcal infection. 1-Way ANOVA. [*p<0.05; **p<0.01] , n=8 for all groups.

The relative proportions of Leukocytes may shed light on specific- vs. non-specific immune response to bacterial challenge. The nonspecific response to bacterial invasion is characterized primarily by granulocytes, which play a role in phagocytocis[116] and secretion of bacteriotoxic substances[96,98,117]. The specific or adaptive immune response, by contrast, is characterized by a mobilization of B-Lymphocytes, and specifically Plasma-Cells, which produce opsonizing antibodies important for assisting phagocytosis and cell-clearance[118,119]. Performing differential cell-counts of BALF and peripheral blood allows for an assessment of which of the two immune-responses predominate in vaccinated or non-vaccinated animals[84,120,121].

To further differentiate and quantify the infiltrating leukocyte populations, we performed multi-color flow cytometry. Absolute neutrophilia was observed in both the PBS-treated, infected mice (n=7; 1.53×10^5 cells/mL $\pm 1.23 \times 10^5$ cells/mL) as well as the SP3-Tetrasaccharide-only mice (n=6; 1.67×10^5 cells/mL $\pm 1.32 \times 10^2$ cells/mL) compared to the PBS-treated uninfected mice (n=6; 8.85×10^2 cells/mL $\pm 4.82 \times 10^2$ cells/mL) where $p<0.05$ in both cases (Figure 30-A).

In comparison to PBS-treated, uninfected mice, the percentage of Neutrophils (%Neu) in BALF was dramatically higher in the infected control mice (n=7; 56.29 %Neu ± 28.06 %Neu; $p<0.01$), in mice treated with SP3-Tetrasaccharide only (n=6; 63.92 %Neu ± 8.07 %Neu; $p<0.05$) and in SP3-Tetrasaccharide + Alum treated mice (n=7; 48.54 %Neu ± 17.34 %Neu) (Figure 30-B).

The absolute Macrophage count in BALF did not differ significantly between groups. Unvaccinated, uninfected controls showed an absolute number of cells in BALF (6.48 x 10^4 cells/mL ± 4.53 x 10^4 cells/mL) that did not differ significantly from either the SP3-tetrasaccharide-only mice (n=6; 6.98 x 10^4 ± 10^4 cells/mL; $p>0.05$) SP3-tetrasaccharide + Alum (n=7; 6.48 x 10^4 cells/mL ± 1.82 x 10^4 cells/mL; $p>0.05$) or the PBS-treated, infected animals (n=6; 2.07 ± 7.83 x 10^3 cells/mL; $p>0.05$). In contrast, the relative number of macrophages in PBS-treated, infected mice (n=7; 38.32 %Ma ± 23.28 %Ma; $p<0.001$), the SP3-tetrasaccharide-only mice (n=6; 37.9 %Ma ± 7.9 %Ma; $p<0.001$) and SP3-Tetrasaccharide + Alum (n=7; 31.86 %Ma ± 7.77 %Ma; $p<0.001$) were all significantly lower than those observed in PBS-treated, infected mice (n=6; 91.52 ± 3.54 %Ma)(Figure. 30-C/D).

Greater absolute numbers of Lymphocytes in SP3-tetrasaccharide+Alum animals (n=7; 1.69 x 10^4 cells/mL ± 1.24 x 10^4) were observed when compared to both PBS-treated, uninfected animals (n=6; 7.71 x 10^2 ± 3.74 x 10^2 cells/mL; $p<0.01$) and PBS-treated, infected animals (n=7; 4.35 x 10^3 ± 2.29 x 10^3 cells/mL; $p<0.05$), but not when compared to the SP3-tetrasaccharide-only animals (n=7; 7.71 x 10^3 cells/mL ± 6.15 x 10^3; $p>0.05$). The latter group did not show absolute numbers of Lymphocytes that differed from those of the other groups.

The percentage of Lymphocytes (%Ly) were observed in SP3-Tetrasaccharide + Alum mice (n=7; 12.57 %Ly ± 9.76 %Ly) were also shown to be significantly higher than in PBS-treated, uninfected mice (n=6; 3.44 %Ly ± 1.58 %Ly; $p<0.05$), PBS-treated, infected mice (n=7; 3.16 %Ly ± 2.83 %Ly; $p<0.05$), and SP3-tetrasacharide-only mice (n=6; 3.31 %Ly ± 1.89 %Ly; $p<0.05$) (Figure. 30-E/F). These findings strongly suggest an adaptive immune response in adjuvanted, vaccinated animals. When taken together with the findings on bacterial burden described above, they further suggest that the (highly protective) effect of vaccination in these animals is facilitated by antibody-producing lymphocytes.
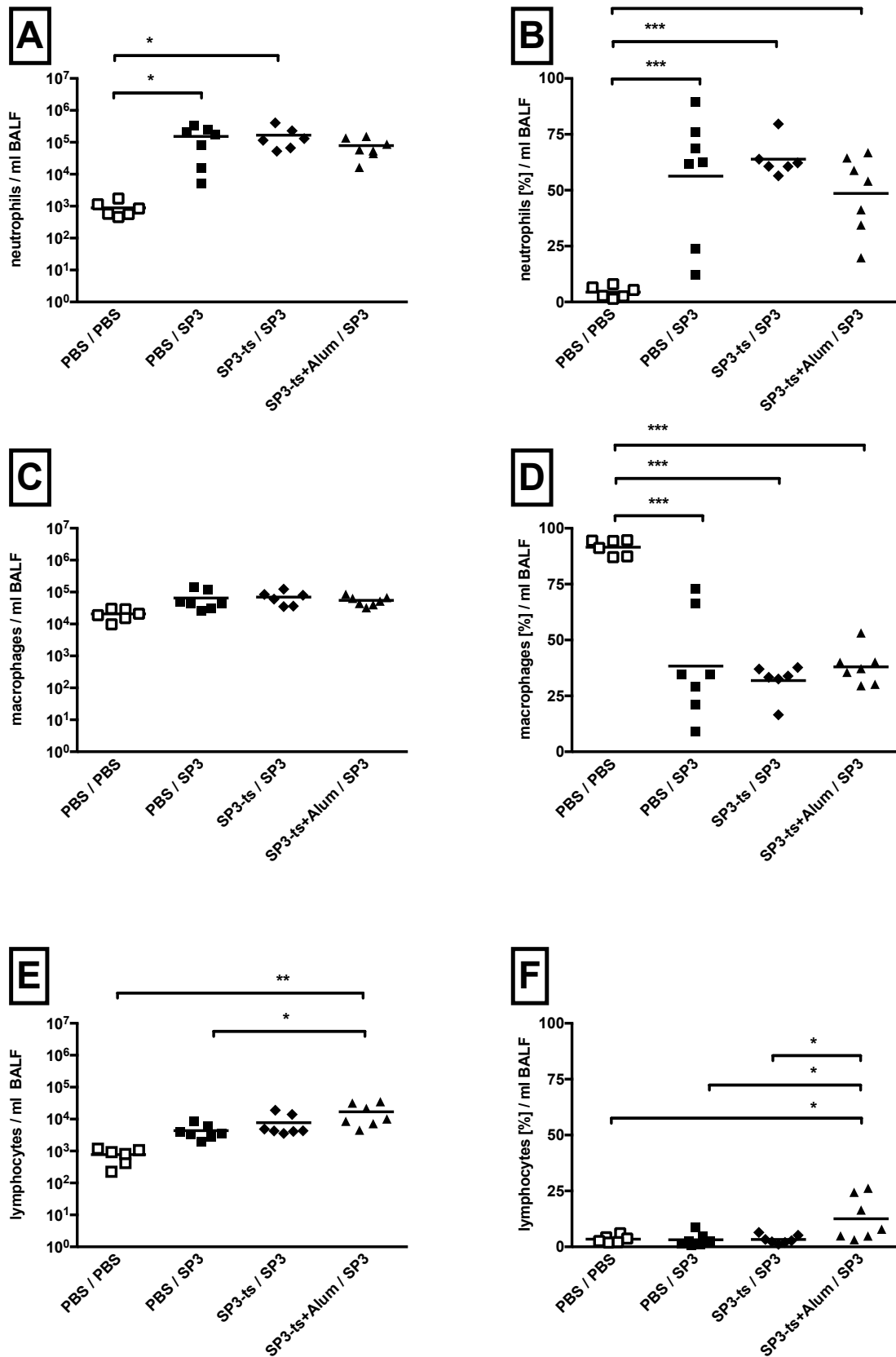
Figure 30 A-F. Figure 13-A: Differential cell-counts of BALF obtained using FACS. 1-Way ANOVA (*p<0.05 **p<0.01 ***p<0.001) n=7 for PBS / PBS; n=8 for PBS / SP3, SP3-ts / SP3 and SP3-ts + Alum / SP3.

Differential cell counts performed in whole blood: leukocytosis of peripheral blood as a marker for disease severity and immune-response.

The mobilization of leukocytes in pneumonia is a hallmark sign of disease severity[96,122,123]. We measured absolute leukocyte counts in peripheral blood samples. In comparison to SP3-tetrasaccharide+Alum mice (n=8; 2.68 ± 7.64 x 10^2 cells/µL), absolute Leukocyte counts were significantly lower in PBS-treated, infected animals (n=8; 1.27 ± 7.03 x10^2 cells/µL; $p<0.01$) and in SP3-tetrasaccharide-only mice (n=8; 1.36 ± 5.98 x 10^2 cells/µL). Leukocyte counts in PBS-treated, uninfected mice (n=7; 2.04 x 10^3 cells/µL ± 7.22 x10^2 cells/µL; $p<0.05$) did not differ significantly from any of the other groups, probably representing an unstimulated state with regard to Lymphocyte proliferation (Fig 31).
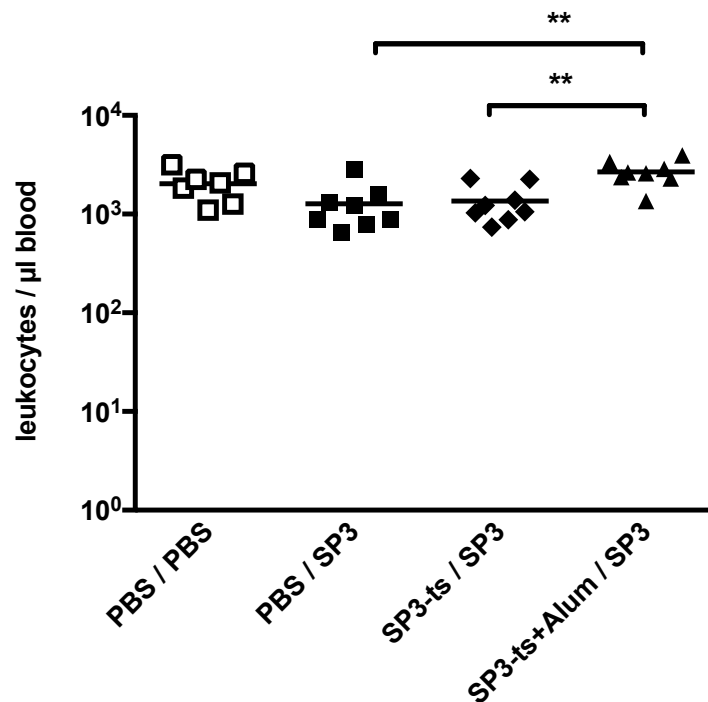
*Figure. 31. Absolute cell counts in whole-blood performed 48 hours after pneumococcal challenge. TruCOUNT™ of whole-blood. 1-Way ANOVA . [** p<0.01] n=7 for PBS / PBS; n=8 for PBS / SP3, SP3-ts / SP3 and SP3-ts + Alum / SP3.*

We then further differentiated the blood leukocytes by flow cytometry (Figure 32). Absolute Neutrophil-counts differed significantly only between SP3-tetrasaccharide+Alum animals (n=8; 6.04 x $10^2 \pm 4.13$ cells/ µL) and PBS-treated, uninfected animals (n=7; 1.83 x $10^2 \pm 79.3$ cells/µL; $p<0.05$). Counts performed in whole blood from either PBS-treated infected animals (n=8; 3.19 x$10^2 \pm 2.12$ x10^2 cells/ µL) and SP3-tetrasaccharide only animals (n=8; 3.02 x $10^2 \pm 1.62$ x 10^2 cells/ µL; $p>0.05$) revealed no significant differences between groups.

The relative proportion of Neutrophils showed no significant variance (p>0.05) at all between groups. (PBS/PBS: n=7; 8.88 ± 2.24 %Neu); (PBS/SP3: n=8; 24.80 ± 13.33%Neu); (SP3-tetrasaccharide+Alum/SP3: n=8; 22.21 ± 14.03 %Neu); (SP3-tetrasaccharide/SP3: n=8; 23.14 ± 9.81 %Neu).

Some significant differences were observed in the absolute count of blood Monocytes between PBS-treated, uninfected mice (n=7; $1.73 \times 10^2 \pm 6.73 \times 10^2$ cells/ μL) and SP3-tetrasaccharide-only animals (n=8; $3.52 \times 10^2 \pm 1.02 \times 10^2$ cells/μL), where the latter showed higher numbers than the former. Unvaccinated, infected mice (n=8; $3.02 \times 10^2 \pm 84.78 \times 10$ cells/μL) and SP3-tetrasaccharide +Alum mice (n=8; $2.89 \times 10^2 \pm 1.08 \times 10^2$ cells/μL) also showed slightly higher monocyte counts than the unvaccinated, uninfected mice, but these were not significant at the conventional level (p>0.05).

Measures of the relative number of blood monocytes (%Mono), revealed that PBS-treated, infected animals (n=8; 27.63 %Mono ± 9.5 %Mono) and SP3-tetrasaccharide –only mice (n=8; 28.48 %Mono ± 9.16 %Mono) had significantly higher proportions (p<0.001 in both cases) of circulating monocytes than in both SP3-Tetrasaccharide + Alum, infected (n=8; 10.60 %Mono ± 2.07 %Mono) and PBS-treated, uninfected groups (n=7; 8.82 %Mono ± 2.39 %Mono) (Figure 32-D).

By contrast, both the absolute and relative lymphocyte counts for the PBS-treated, uninfected mice and, more importantly, for the SP3-tetrasaccharide+Alum group were significantly higher than those of the unvaccinated, infected group, and the mice treated with the conjugate without adjuvant.

Absolute counts of lymphocytes in the SP3-tetrasaccharide+Alum group (n=8; $1.76 \times 10^3 \pm 5.80 \times 10^2$ cells/μL) were significantly higher than those in both the PBS-treated, infected mice (n=8; $6.33 \times 10^2 \pm 5.73 \times 10^2$ cells/μL; p<0.01) and in mice treated only with SP3-tetrasaccharide (n=8; $6.85 \times 10^2 \pm 4.73 \times 10^2$ cells/μL; p<0.01). Unvaccinated, infected animals (n=7; $1.66 \times 10^3 \pm 6.12 \times 10^2$ cells/μL) showed higher levels of absolute lymphocyte counts than both the unvaccinated, infected animals (p<0.05) and the mice treated with only SP3-tetrasaccharide (p<0.01). (Figure 32-E).

Significantly lower rates of relative lymphocyte counts compared to the SP3-tetrasaccharide+Alum mice (n=8; 66.31 %Ly ± 14.51 %Ly) were seen in both the PBS-treated, infected controls (n=8; 46.23 Ly% ± 12.26 %Ly; p<0.05) and the SP3-tetrasaccharide-only, infected group (n=8; 47.17 %Ly ± 11.39 %Ly; p<0.05). The PBS-treated, uninfected controls (n=7; 81.15%Ly ± 4.30 %Ly) showed significantly higher relative numbers of circulating lymphocytes than both the PBS-treated, infected animals and the SP3-tetrasaccharide-only animals (p<0.001 for both cases). (Figure 32-F).
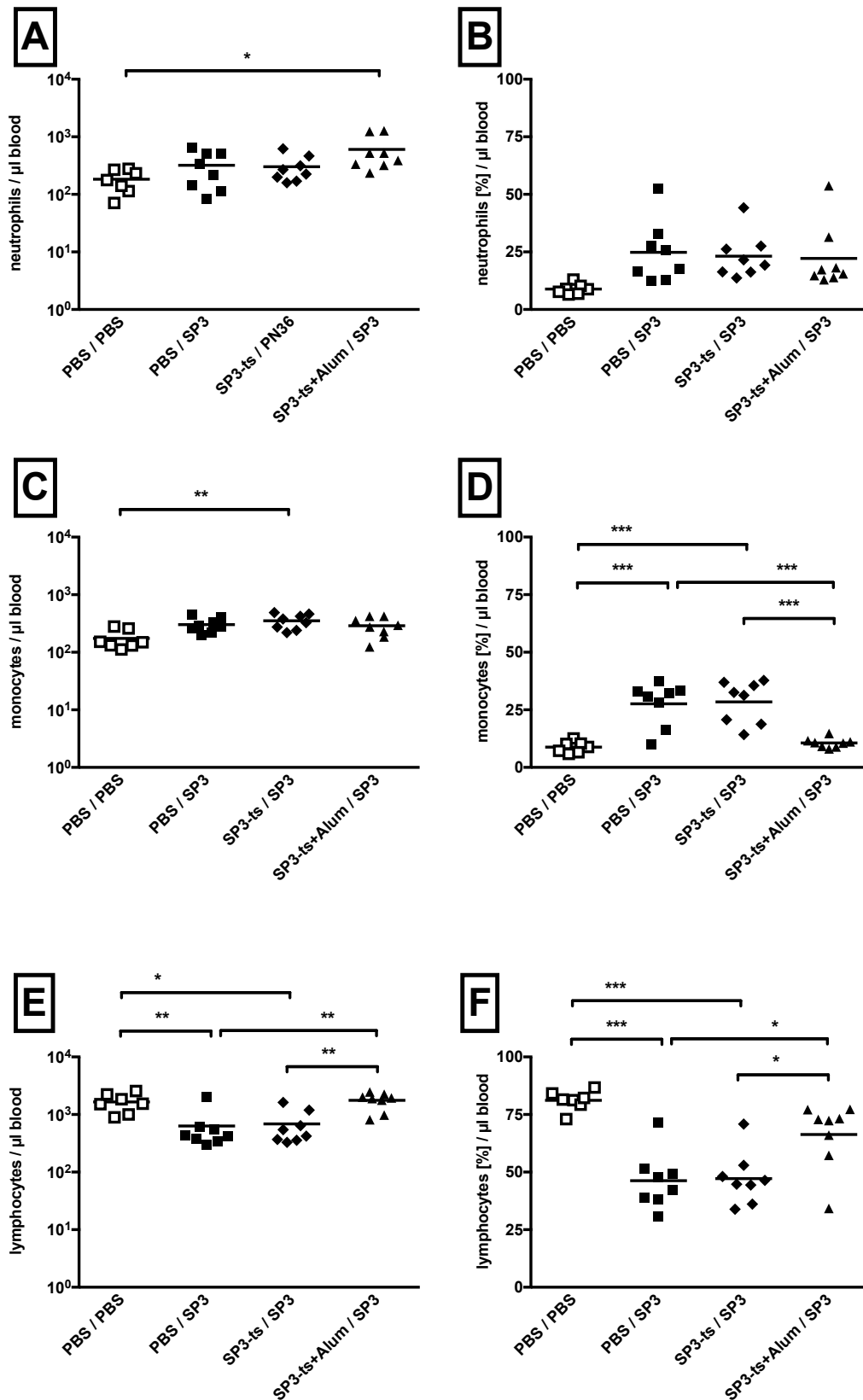
Figure 32 A-F. FACS analysis of leukocyte populations in whole blood. 1-Way ANOVA*p<0.05 **p<0.01 ***p<0.001) n=7 for PBS / PBS; n=8 for PBS / SP3, SP3-ts / Sp3 and SP3-ts + Alum / SP3.

IV. B. ii. 4. Pulmonary Function Tests

SP3-Tetrasaccharide + Alum vaccination protects against lung damage and decreased lung function following *S. pneumoniae* infection.

We next assessed lung function parameters to determine the effects of SP3-Tetrasaccharide vaccination on pneumonia induced organ dysfunction. These measurements were performed by Dr. vet. med. Katrin Reppe and kindly provided for inclusion in this thesis for completeness.

As discussed above, bacterial pneumonia caused by *S. pneumoniae* is hallmarked by leukocyte extravasation and edema[124]. The functional consequences are reduced pulmonary compliance and increased airway-resistance[125,126,85].

Pulmonary compliance refers to the distensability of lung parenchyma in response to changes in (pleural) pressure[127]; pulmonary edema reduces pulmonary compliance by increasing the rigidity of parenchyma[124], thereby reducing the lung's ability to expand in a response to reduced pleural pressure, as for example during inspiration[128,129].

Airway resistance refers to airflow through the upper and lower pulmonary airways, and is determined by two significant factors:

1) airway radius, as shown by Poiseuille's law[130]:

$$Airway\ Resistance = \frac{8}{\pi} \cdot \frac{fluid\ viscosity \cdot tube\ length}{radius^4}$$

and

2) the degree of turbulent airflow vs. laminar airflow[127,130].

In order to maintain a given airflow with increasing resistance, an increase in respiratory work to change airway pressure is required, as described by the equation[130]:

$$Airflow\ in\ \frac{L}{s} = \frac{\Delta\ Airway\ Pressure}{Airway\ Resistance}$$

Inflammation of the mucosa in pneumonia surrounding the inner lumen of the airways, and the accumulation of mucus and pus in airways cause an decrease in airway diameter, an increase in physical irregularities in airway lumen, and thereby increase of airway resistance and turbulent airflow, respectively [131,132,130].

The resulting effect is increased respiratory work to produce to maintain an airflow that will ventilate the lungs and to maintain gas-exchange[130]. As respiratory work decompensates, so too does airflow and gas exchange; this leads to a state of respiratory insufficiency, which is characterized by hypoxemia and hypercapnia[133].

Pulmonary functions tests showed that average lung compliance was lower in sham-vaccinated, infected mice (n=8; 15.78 μm/cm H_2O ± 4.17 μm/cm H2O; p<0.01) and SP3-tetrasaccharide-only, infected mice (n=8; 16.06 μm/cm H2O ± 2.45 μm/cm H_2O; p<0.01) than in the sham-vaccinated, uninfected mice (n=7; 22.6 μm/cm H2O ± 1.0 μm/cm H_2O). Compliance in SP3-tetrasaccharide+Alum mice (n=8; 16.06 μm/cm H_2O ± 2.45 μm/cm H_2O) was better than in the PBS-treated, infected animals and the animals treated only with SP3-tetrasaccharide, but not significantly so (p>0.05). At the same time, adjuvanted, vaccinated animals did not have significantly lower compliance than uninfected controls (p>0.05), indicating a partial restoration (Figure 33).
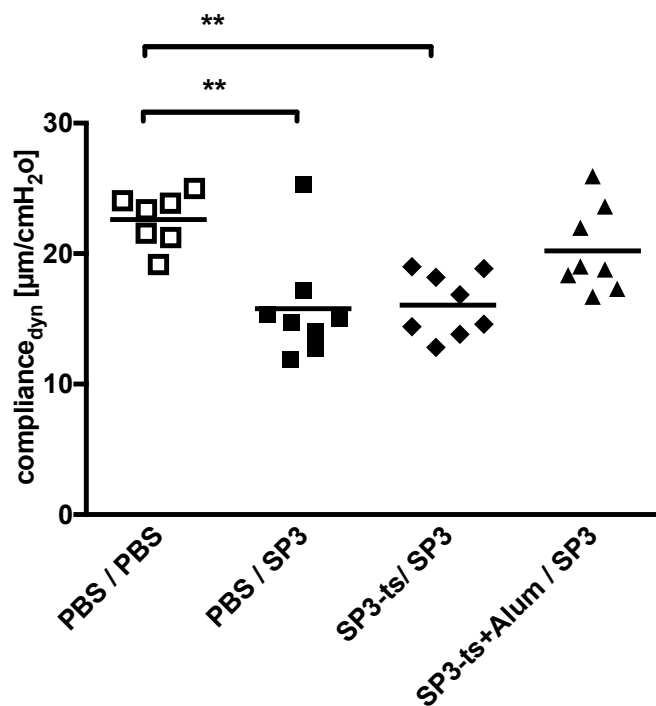
*Figure 33. Lung function test measuring lung compliance in the experimental groups depicted. 1-Way ANOVA. [**p<0.01]. n=7 for PBS / PBS; n=8 for PBS / SP3, SP3-ts / SP3 and SP3-ts + Alum / SP3.*

Lung resistance was significantly higher only in sham-vaccinated, infected mice (n=8; 2.5 cmH_2O/mL/s ± 0.5 μm/cm H_2O; p<0.01) when compared to PBS-treated, uninfected mice (n=7; 1.9 μm/cm H_2O ± 0.22 μm/cm H_2O). The former group and the group treated only with SP3-tetrasaccaride (n=8; 2.53 ± 0.38 μm/cm H_2O) showed a resistance that was higher than the SP3-tetrasaccaride+Alum animals (n=8; 2.04 ± 0.24 μm/cm H_2O), but not significantly so (p>0.05 in both cases).
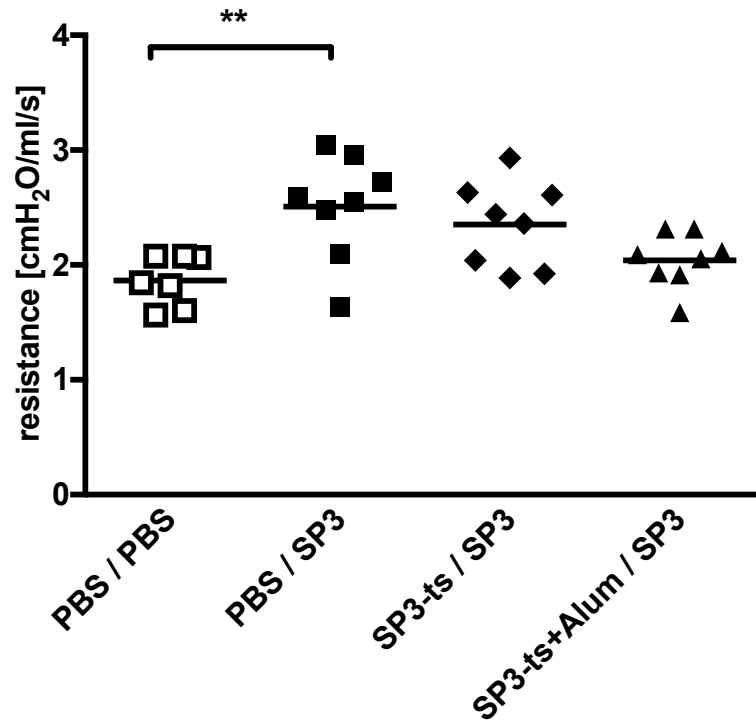
Figure 34. Lung function test measuring lung resistance in the experimental groups depicted. 1-Way ANOVA. [**p<0.01]. n=7 for PBS / PBS; n=8 for PBS / SP3, SP3-ts / SP3 and SP3-ts + Alum / SP3.

IV. B. iii. *In vitro/in vivo* Data from Challenge Experiments

IV. B. iii. 1. OPAs

OPAs confirm production of protective antibodies in SP3-Tetrasaccharide + Alum-vaccinated animals.

We next assessed the OPA activity of sera in the experimental groups used for the challenge experiments described above. Sera from blood draws performed at day 35 (1 week following the second boosting) were implemented in OPAs and showed effective killing in the vaccinated groups at titers up to 1:32 (Figure 35), essentially confirming the protective effects observed in the vaccination studies.
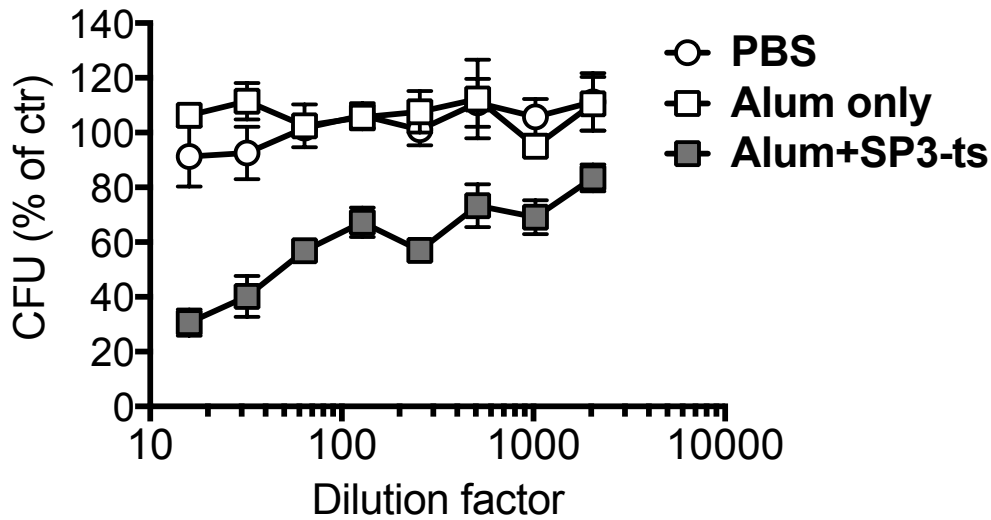
Figure 35. OPAs were performed with pooled serum from mice taken at 1 week following the second boosting. Sera from 10 mice were pooled for Alum only and Alum + SP3-Tetrasaccharide (SP3-ts)groups, and 11 mice for the PBS groups . Each experimental condition was repeated a total of 4 times. PBS= sham-vaccinated mice, Alum=adjuvanted only, Alum + SP3-ts= mice treated with adjuvanted vaccine.

These results are comparable with those obtained from the preliminary experiments described in the above section on short-term immunity. For the purposes of comparing the effectiveness of the two adjuvants used, data-plots from the experiments implementing CFA instead of Alum were added to the graph in Figure 35. A comparison of the results indicate that vaccines adjuvanted with CFA seem to elicit a more robust antibody OPA-response than the Alum adjuvanted vaccine, producing a reduction of 50% of CFU at a titer up to 1:256 (Figure 36).
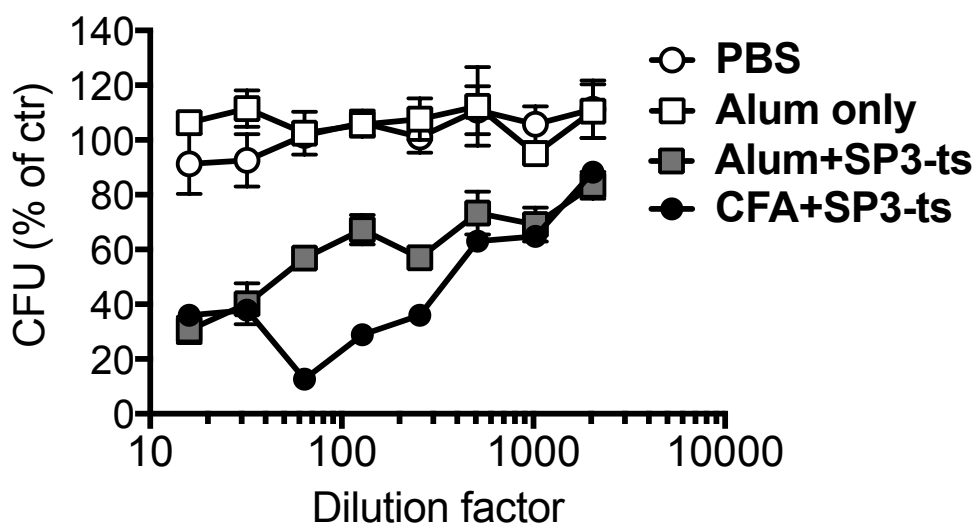
Figure 36. Comparison of bacterial killing with sera obtained from mice treated either with an Alum- or CFA-conjugate. Sera from 10 mice were pooled for Alum only and Alum + SP3-ts groups, 11 mice for the PBS groups and 6 for the CFA groups. Experimental conditions were repeated a total of 8 times for conditions using mice treated with PBS, Alum, Alum + SP3-Tetrasaccharide (SP3-ts), and 4 times for mice treated with CFA + SP3-Tetrasaccharide.

IV. C. Results from the Long-Term Vaccination Study.

SP3-Tetrasaccharide + Alum vaccination does not provide clinically effective long-term immunity.

One of the big advantages of vaccines is their capacity to afford long-lasting protective immunity against infections. It was therefore critical to assess the long-term efficacy of the newly generated semi-synthetic conjugate vaccine in our model. Given the relatively short life span of laboratory mice, we chose a 4-month time point as a proxy to determine long-term immune protection.

A challenge at 35 days, i.e. 7 days following the most recent boosting, corresponds to the end of the window in which a primary immune response to a new antigen can still be in effect[72,134]. Long-term memory must take effect for protection to be observed greater than 21-day after boosting[134,72]. Performing a challenge at 116 days following the second boosting creates experimental conditions in which the primary response to vaccination will have phased out completely, and the secondary immune response to vaccination can be specifically examined for effectivity[135].

Disappointingly, mice were not protected against pneumonia 4 months after vaccination with SP3-Tetrasaccharide + Alum, as evidenced by high levels of all indices of infection.

IV. C. i. Clinical Data: appearance, bodyweight and temperature.

Bodyweight and temperature as indicators of disease-severity dropped in vaccinated animals by a margin comparable to non-vaccinated animals.

Body weight (Figures 37 & 38) in all animals decreased from baseline over the 48 hours following challenge, without an observable significant difference between groups. SP3-Tetrasaccharide + Alum animals (n=10; 85.1% ± 3.4%) showed a drop in weight similar to that seen in control groups (p>0.05 for both cases) PBS/SP3-Tetrasaccharide (n=11; 83.4% ± 3.7%) and Alum/SP3 (n=9; 84.1% ± 2.8%), thus indicating fulminant disease in all groups, with no protective response to challenge observed in vaccinated animals.
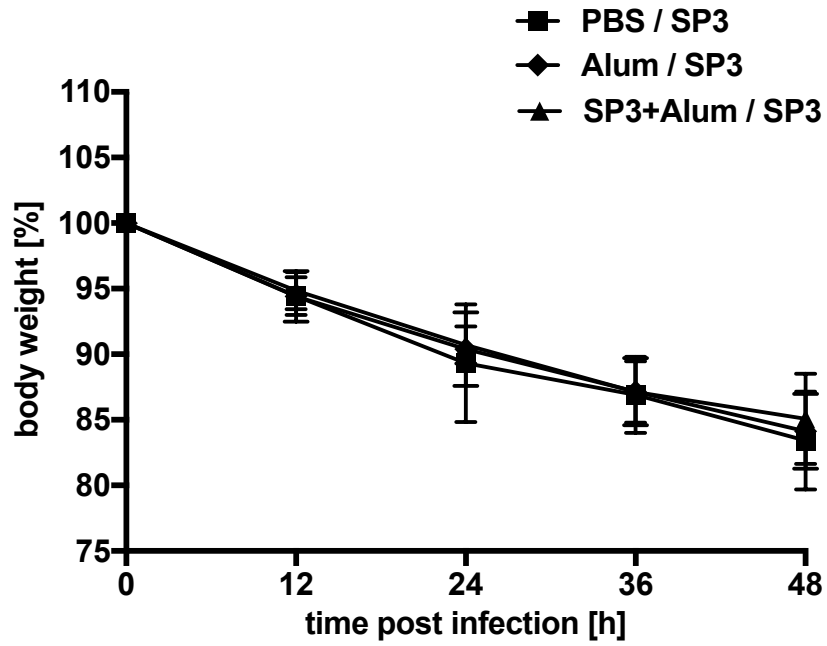
Figure 37. Average body-weight across groups showing a uniform decline across groups in the 48 hours post- pneumococcal challenge, with no statistically significant difference between groups. 1-Way ANOVA. n=10 for SP3-Tetrasaccharide (SP3-ts) + Alum, n=11 for PBS/SP3-Tetrasachharide, n=9 for Alum/SP3
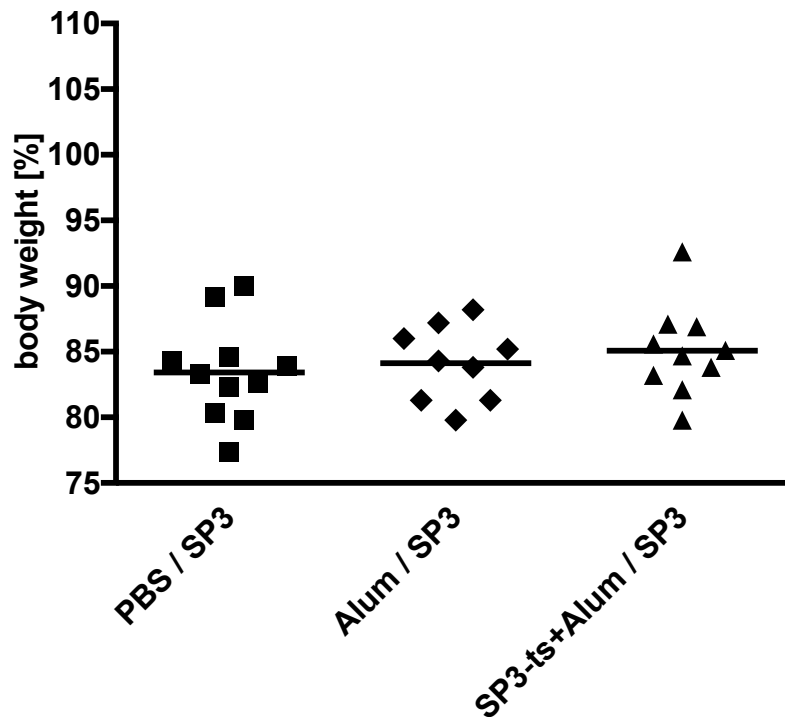
Figure 38. Distribution of body-weight percent of base-line 48 hours following pneumococcal challenge. 1-Way ANOVA n=10 for SP3-tetrasacchride (SP3-ts) + Alum, n=11 for PBS/SP3-ts, n=9 for Alum/SP3.

Similarly, all groups showed a comparable decline in body-temperature in the 48h following pneumococcal challenge. The average value of this parameter did not vary significantly between groups (PBS/SP3 (n=11; 35.0 °C ± 1.7 °C); Alum/SP3(n=9; 35.0 °C ± 2.1); SP3-Tetrasaccharide + Alum (n=9; 34.8 °C ± 3.1 °C), with p>0.05 in all cases), indicating that vaccinated animals were not more protected from disease progression than non-vaccinated animals (Figures 39 & 40).
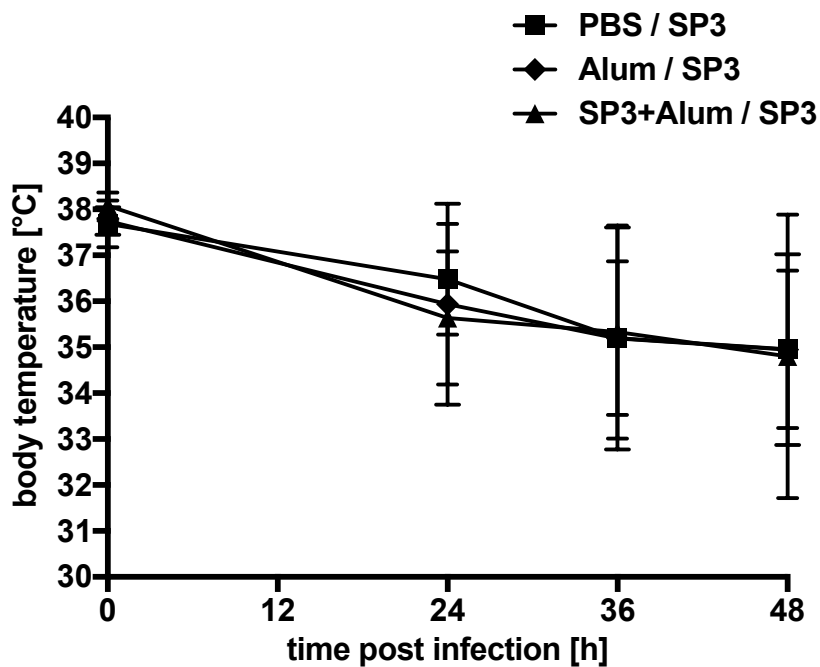
Figure 39. Average body-temperature across groups during the 46 hours following pneumococcal challenge, with no statistically significant differences between groups. n=10 for SP3-Tetrasacchraide (SP3-ts) + Alum, n=11 for PBS/SP3-ts, n=9 for Alum/SP3.
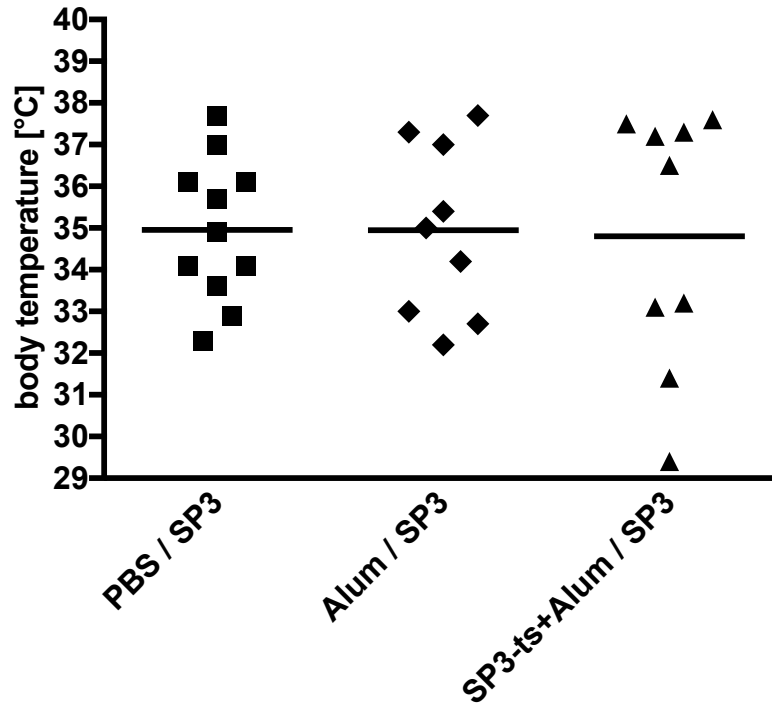
Figure 40. Distribution of temperatures across groups 48 hours following pneumococcal challenge. 1-Way ANOVA. n= 9 for PS3-ts + Alum; n= 11 for PBS/SP3 (n=11); n=9 for Alum/SP3

IV. C. ii. Bacterial Burden in the Lung and in Blood.

CFU-counts in BALF, lung-tissue and whole-blood, performed 116 days following challenge show no significant differences across groups.

CFU-counts in BALF

These general, clinical signs of disease progression and severity in the animals were reflected in the more specific indices of disease. CFU counts from BAL fluid, lung-tissue and blood were similar across all groups, including high bacterial burden in both SP3-Tetrasaccharide + Alum and control groups, reflecting ineffective clearing of bacteria from the lungs. The CFU counts conducted using BAL-fluid were as follows: SP3-Tetrasaccharide+Alum treated mice showed bacterial burden (n=7; $5.77 \times 10^3 \pm 1.30 \times 10^4$ CFU/μL BALF) similar to those in sham-vaccinated animals (PBS/PBS: n=8; $6.87 \times 10^3 \pm 1.67\ 7 \times 10^4$ CFU/μL BALF) and mice treated only with Alum (n=6; $1.53 \times 10^4 \pm 3.76 \times 10^4$ CFU/μL BALF), p>0.05 in all cases. (Figure 41).

CFU-counts in Lung-Tissue

Similarly high CFU-counts were seen in the lung-tissue of SP3-tetrasaccharide+Alum treated mice (n=7: $8.20 \times 10^7 \pm 1.49 \times 10^8$ CFU per 1/2 Lung and $3.89 \times 10^8 \pm 6.92 \times 10^8$ CFU/g lung-tissue), sham-vaccinated mice (PBS/SP3) (n=8: $1.50 \times 10^8 \pm 2.59 \times 10^8$ CFU per ½ lung and $7.98 \times 10^8 \pm 1.30 \times 10^9$ CFU/g lung-tissue), as well as only Alum adjuvanted mice (n=6: $4.21 \times 10^7 \pm 6.10 \times 10^7$ CFU per ½ lung and $2.23 \times 10^8 \pm 3.09 \times 10^8$ CFU/g lung-tissue) (Figure 42).
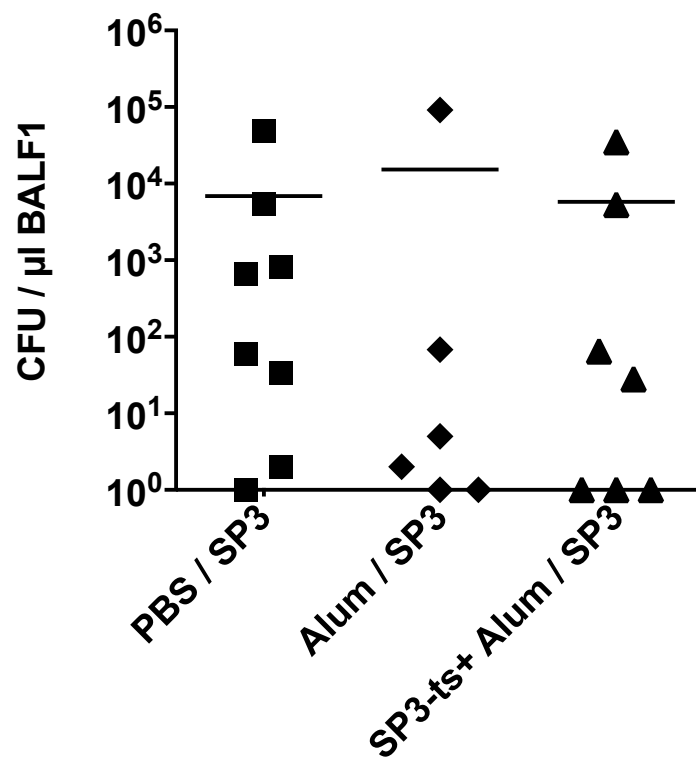
Figure 41. Distribution of CFU counts obtained from BALF. 1-Way ANOVA. n=8 for PBS / SP3; n=6 for Alum/SP3; n=7 for SP3-Tetrasaccharide (SP3-ts) + Alum.
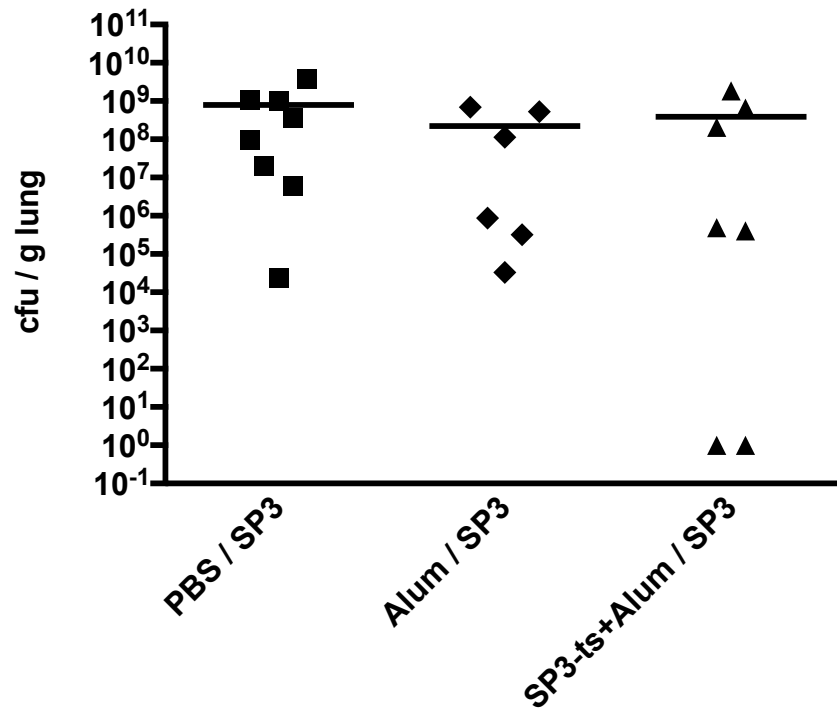
Figure 42. Distribution of CFU counts obtained from homogenized lung tissue Distribution of CFU counts obtained from BALF. 1-Way ANOVA. n=8 for PBS / SP3; n=6 for Alum/SP3; n=7 for SP3-ts + Alum.

CFU-counts in Whole-Blood

Bacteremia was similarly high in all groups, with no significant difference in average CFU counts. Animals treated with Alum-aduvanted SP3-Tetrasaccharide showed a tendency towards lower bacterial loads in the blood (n=7. $1.77 \times 10^3 \pm 2.21 \times 10^3$ CFU/µL), but this did not differ significantly from those in animals sham-treated with PBS (n=8; $3.05 \times 10^4 \pm 6.29 \times 10^4$ CFU/µL; $p>0.05$), and importantly, animals treated only with Alum showed a similar trend (n=6; $6.27 \times 10^3 \pm 1.51 \times 10^4$ CFU), indicating that there was no protective immunity in SP3-Tetrasaccharide vaccinated animals 4 months after vaccination (Figure 43).
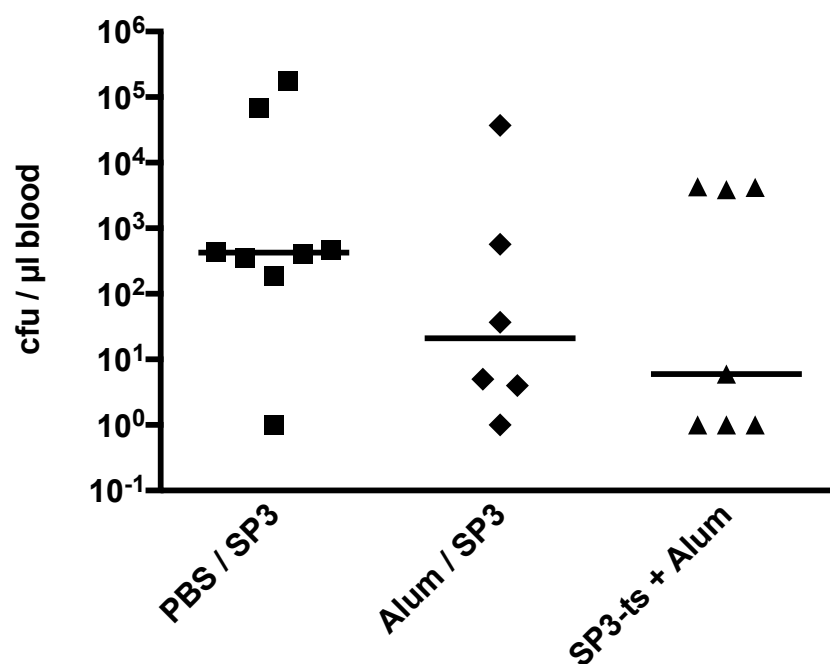
Figure 43. Distribution of CFU counts from whole blood across groups. 1-Way ANOVA. n=8 for PBS / SP3; n=6 for Alum/SP3; n=7 for SP3-ts + Alum.

IV. C. iii. Leukocyte Counts in the Lung and in Blood.

Cell recruitment determined by FACS in vaccinated, adjuvanted animals after 116 day shows no difference when compared to control animals.

Absolut and differential cell-counts, as measured by FACS, can indicate both the scale and quantity of disease and immune response. An increase in certain cell-lineages can be a gross indicator of disease; the relative proportion of those cell-lineages can indicate the predominance of certain elements of the immune response[135]. To this end, absolute and differential cell counts in whole-blood and BALF were performed.

Absolute Leukocyte Counts in BALF and Whole-Blood

Absolute cell counts (as determined by TruCOUNT™ and FACS) showed an immune-response that was similar across all groups, with increases in total leukocytes in both BALF and whole-blood. Results for counts performed in BALF: (PBS/SP3 (n=8; 4.32×10^5 cells/mL $\pm 3.55 \times 10^5$ cells/mL; p>0.05); Alum/SP3 (n=6; 5.49×10^5 cells/mL $\pm 7.14 \times 10^5$ cells/mL); SP3-Tetrasaccharide+Alum/SP3 (n=7; 5.15×10^5 cells/mL $\pm 5.35 \times 10^5$ cells/mL) (Figure 44). Results for counts performed with whole blood: PBS/PBS (n=8; 1.49×10^3 cells/μL $\pm 1.11 \times 10^3$ cells/μL); Alum/SP3 (n=5; 1.81×10^3 cells/μL

$\pm$ 1.14 x 10^3 cells/μL); SP3-Tetrasaccharide+Alum/SP3 (n=6; 1.80 x 10^3 cells/μL $\pm$ 6.79 x 10^2 cells/μL) (Figure 45). No significant differences could be found between groups (p>0.05 in all cases).
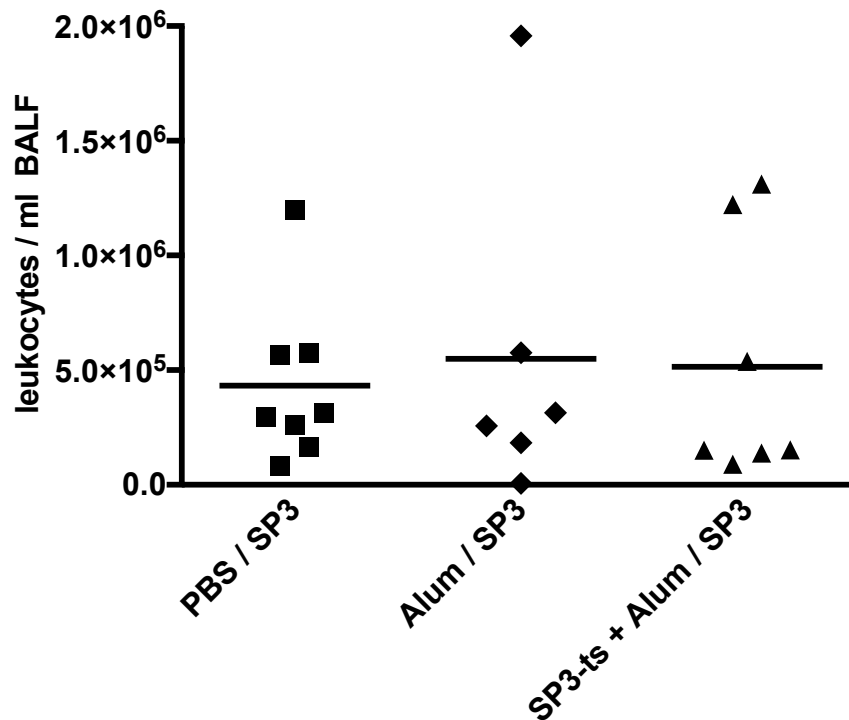
Figure 44. Distribution of absolute leukocyte-counts obtained from BALF with TruCOUNT. n=8 for PBS / SP3; n=6 for Alum/SP3; n=7 for SP3-Tetrasaccharide (SP3-ts) + Alum.

IV. C. iv. Pulmonary Endothelial Permeability

Pulmonary endothelial permeability increased in all experimental groups following bacterial challenge.

As discussed above, *S. pneumoniae* disrupts the integrity of the lung parenchyma via several virulence factors, including pneumolysin and autolysin, resulting in a concomitant reduction in pulmonary compliance and an increase in resistance. Destruction of the lung epithelia by the same mechanism compromises the integrity of the blood-lung barrier, resulting in the accumulation of exudate and consequently pulmonary edema from the accumulation of albumin[105,107,136].

Barrier integrity can be measured by administering human serum albumin (HSA) to mice, and then quantifying the level of HSA in the blood and BALF by ELISA. The resulting quotient of the

concentration of HSA in the BALF and blood provides an indirect measure of barrier integrity; a higher ratio indicates greater compromise of the alveolar-capillary barrier[137,138].

Albumin BALF/plasma ratios showed slightly lower ratios in animals treated with Alum-only when compared to the two other groups, but this difference was not statistically significant. Albumin BALF/plasma ratio in PBS-treated animals (n=8, 36.72 ± 43.71 albumin BALF/Plasma) in adjuvant-only animals (n=6, 17.83 ± 20.13 albumin BALF/Plasma) and in adjuvanted, vaccinated animals (n=7, 28.38 ± 39.97 albumin BALF/Plasma) showed no significant differences (Figure 48), reflecting comparable histopathological changes in the examined lungs following pneumococcal challenge, and thereby no significant protectivity of the adjuvanted vaccine.
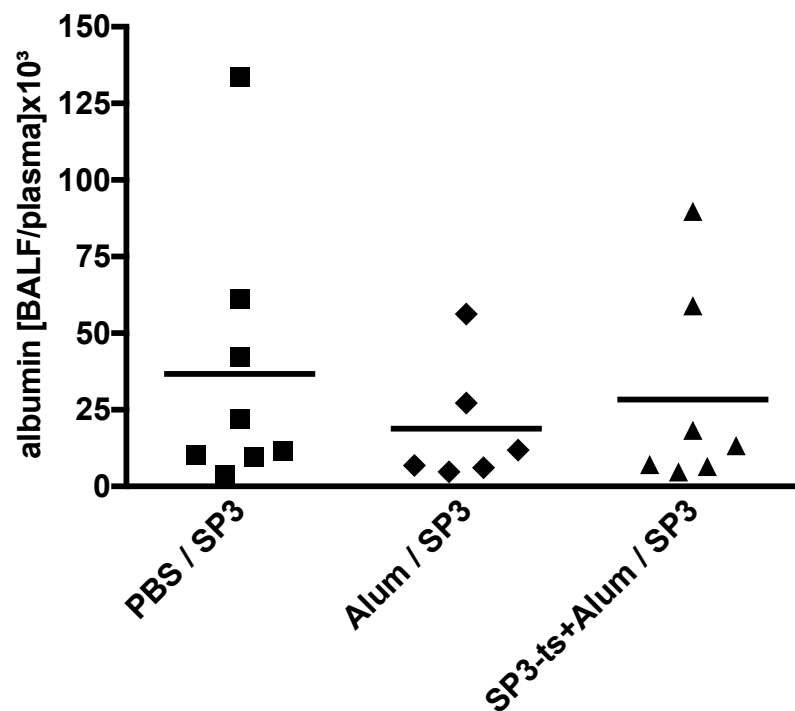
Figure 48. Pulmonary endothelial permeability as determined by HSA ELISA and expressed as albumin BALF/plasma-ratio. 1-Way ANOVA . n=8 for PBS / SP3; n=6 for Alum/SP3; n=7 for SP3-Tetrasaccharide (SP3-ts) + Alum.

V. Discussion

In this study, a semi-synthetic glycoconjugate vaccine candidate for *S. pneumoniae* type 3 (SP3-Tetrasaccharide) was evaluated for efficiency both in *in-vivo* and *in-vitro* trials. Experiments were carried out to determine both the short-term and the long-term efficiency of the vaccine, and to characterize the responses *in-vitro/ex vivo* and *in-vivo*.

Our principal finding is that this novel vaccine candidate is generally immunogenic. It provides robust protection against SP3 in vaccinated mice challenged at 5 weeks, but the protective effect rapidly wanes, and is not detectable at 16 weeks post immunization. These results indicate that additional immune activators, potentially targeted adjuvants may need to be added to increase long term immunogenicity of the vaccine.

V. A. Short-Term Immunity

Applying WHO-criteria for vaccine efficacy to results from the current study.

As discussed in the Introduction, the WHO-guidelines for determining conjugate vaccine protection include parameters from both *in-vitro/ex vivo* and *in-vivo* studies. These guidelines outline measures for both the conference of protection from morbidity and mortality to vaccinated subjects, as well as the quantity of specific IgG and the functionality of those immunoglobulins, as measured by ELISA and OPAs, respectively[82,139,81]. The guidelines also quantify efficacy for both parameters as follows:

Vaccine efficacy =

1 - [% of vaccine subjects with [Ab]<Ab(protective)/% of control subjects with [Ab]<Ab(protective)]

= 1 – [probability of disease in vaccinated group/probability of disease in the control group].

(The established, specific antibody concentration associated with clinical efficacy lies between 0.20 and 0.35 µg IgG/mL. [81,139])

In-vitro studies confirm vaccine efficacy when SP3-Tetrasaccharide is combined with CFA.

OPAs represent a test for the functionality of the antibodies identified with the ELISA by giving a direct indication of whether bacterial killing can be affected by these antibodies. Titer-response curves yield further, more precise information about the *relative* efficacy of the antibody response. A CFU-counts equal to, or below 50% of the control-CFU count is considered to be indicative of effective killing [82,139,140,101], and to correspond to a minimum IgG concentration of 0.20 µg/mL[141].

The preliminary studies conducted by Prof. Seeberger's group in MPIKG described in the previous section using quantitative ELISA conducted with sera from mice treated with SP3-Tetrasaccharide + CFA showed a high concentration of specific IgG (unpublished data).

OPAs performed with these sera show significant killing of bacteria at one and two-weeks after administration of booster-vaccine, with titers as high as 1:256 achieving 50% killing or greater at both intervals.

Notable, also, is the significant increase in killing achieved with sera taken from mice at 2 weeks post-boosting compared to that taken from 1 week post-boosting. These findings could suggest antibody maturation and avidity after booster-vaccination [142].

In contrast, this killing efficacy was not seen in animals treated with SP3-Tetrasaccharide + Alum. This is probably a reflection of the influence of confounding variables on that particular day of testing: poor killing was achieved not only with serum from vaccinated mice, but also with the WHO-serum. The factors influencing efficacy in killing can range from degree of differentiation of HL-60 derived psudogranulocytes to improper execution of the experiment. Unfortunately, there was insufficient serum available to perform repeat-trials.

Scarcity of material represents one barrier in normalizing the OPA-data. From our experience in establishing this procedure, the variability of results (as reflected in the date displayed in Figures 16, 17 and 20) can be applied to a multitude of factors. The differentiation of HL-60 cells probably represents the process which introduces the greatest variability into this procedure. Reasons for this may include: differentiation of HL-60 cells to pseudogranulocytes-stock will likely vary between trials; and survivability of differentiated cells may shift as well, often leaving significant detritus which may interfere with opsonization and even phagocytosis. The former of the two potential problems might be addressed by adding further criteria for better assessing the degree of differentiation: using flow cytometry for accurately identifying cell size may be used to better categorize cells in terms of their morphology thereby adding an additional reference-point for adjusting conditions in which HL-60 are differentiated.[1] The latter of the two issues might be addressed by filtering viable cells from deceased ones, possibly with a density gradient as described above for sorting granulocytes.

Another likely factor that might introduce variability is the bacterial stock itself: freezing stabs of bacterial culture, and their subsequent expansion may both represent steps in which significant fluctuation of viable cells between trials might arise. And, while controlling for optical density as a marker for the relative reproducibility of the bacteria, it makes no provision for viability. It is conceivable that bacterial components from lysed or otherwise deceased cells may also interfere with

opsonization by adsorbing antibodies, complement or both. Establishing viability using cytology or plating samples of bacterial culture might both represent methods for further reducing this factor's contribution to inter-trial variability.

Note that CFA is not licensed for human use because of its tendency to produce severe local and systemic side-effects[78]. For this reason, CFA was not implemented as an adjuvant for the *in-vivo* experiments conducted in mice in our facilities. Instead, an adjuvant containing aluminum was implemented, which has been accepted widely for use in humans, including the licensed PCV13 vaccine Prevenar®[31].

The clinical data gathered, as well as the laboratory-parameters measured, in the short-term, *in-vivo* experiments conducted in our facilities indicate that vaccinated mice are protected when compared to controls. Mice from the SP3-Tetrasaccharide + Alum group showed stable body-weights and body-temperatures, in contrast to unvaccinated controls. Clinically, vaccinated mice also showed far fewer signs of illness than control mice; the latter progressed into states of inactivity, showed a tendency towards progressive illness in mice[104].

Similarly, laboratory analyses showed pronounced signs of illness in control groups, but none in vaccinated animals. Effective bacterial clearance, as measured by CFU-counts, was observed in the blood, BALF and lung-tissue of vaccinated animals. Unvaccinated animals showed heavy bacterial burden in BALF and lung-tissue, as well as severe bacteremia. These findings are in line with previous studies showing that elevated CFU counts from BALF, whole blood and lung tissue from mice correlate positively with disease severity in mice, and negatively with a protected, vaccinated state[107,108].

The immune response to infection in the different groups was measured using FACS, and encompassed total leukocyte, neutrophil, lymphocyte and monocyte levels in BALF and whole blood. FACS studies using BALF showed, most notably, pronounced increases in % neutrophils and reductions in % macrophages across all groups when compared to healthy controls. Lung neutrophilia is well-documented as an important innate response to bacterial invasion[96,95,120]. The role of macrophages seems to be less well understood, but some evidence does suggest that these cells play less of a role in bacterial clearance than they do in regulating the inflammatory response of, and phagocytosis by, lung-neutrophils[120]. It is conceivable, therefore, that this inverse relationship in the two populations is a reflection of alveolar macrophage-consumption in response to massive neutrophilia.

In whole blood, the most prominent finding was the higher level of monocytes and a concomitant lower level of lymphocytes in both infected control groups compared to the vaccinated group. The

latter finding is consistent with previous studies showing changes in the proportion of lymphocytes in peripheral blood[105].

Pulmonary function tests revealed both a reduction in compliance and an increase in resistance in control-mice. As discussed in the Results-section, these findings likely reflect the effects of key virulence factors such as autolysin and pneumolysin, both of which play a central role in the destruction of lung-paranchyma observed in pneumococcal pneumonia[107,105,136]. Vaccinated mice, in contrast, showed no change relative to healthy controls. These contrasting findings show further support for the protective effects of the vaccine within this time-frame.

In summary, the data stemming from the *in vivo* evaluation of short-term efficacy of the vaccine present a convincing picture of the efficacy of the SP3-Tetrasaccharide vaccine when adjuvanted with Alum.

OPAs conducted with sera obtained at day 35 in the vaccination procedure (i.e. 1 week following the second boosting at day 28) showed significant killing of bacteria in vaccinated animals compared to controls, indicating an effective immune response to the vaccine. OPAs using serum from mice vaccinated using an Alum adjuvant only were not conducted, as other experiments using these sera were prioritized. Ideally, confirmation of adequate IgG production and functionality should have been obtained.

Thus, applying equation (1), we obtain:

Vaccine efficacy = 1 - (0 morbidity in vaccinated group)/(22 mice with morbidity/22 mice in control group) = 1.

We conclude that SP3-Tetrasaccharide, when adjuvanted with Alum, shows highly effective protection from SP3.

The role of Alum in facilitating the immune-response to vaccination.

Results from the afore-mentioned *in vivo* experiments underscore the importance of adjuvants in supporting an effective response to vaccines; protectivity, or lack thereof, in those mice treated only with SP3-Tetrasaccharide was essentially the same as that observed in PBS-treated animals, demonstrating that an adjuvant is necessary for the elicitation of immune-response to the conjugate vaccine.

Many substances have been employed as adjuvants, ranging from Alum, to oil-emulsions, to heat-inactivated bacteria, as in the case of CFA. The precise mechanism through which these substances have their effect is still not completely understood[75], but it is generally accepted that all induce a local inflammatory reaction that results in recruitment of phagocytotic cells and subsequent antigen presentation by dendritic cells and B-cells to T-helper cells[143,71,80,144]. Adjuvants therefore represent an

essential component of non-live vaccines, as they are required for bridging the innate and adaptive arm of the immune system, both of which are needed to elicit protective immunity.

This effect is not the same for all adjuvants: different adjuvants produce different qualitative effects upon the immune system[70,143,71,80]; the magnitude of the immune response also differs across substances[144,145]. The latter point, at least, is reflected in our own findings demonstrating higher killing in OPAs using sera from mice treated with SP3-Tetrasaccharide + CFA compared to mice treated with SP3-Tetrasaccharide + Alum (see Results). The importance of adjuvants in vaccinations will be revisited in a later section addressing strategies for improving a long-term response to SP3-Tetrasaccharide.

V. B. Long-Term Immunity

The same measures of morbidity and mortality implemented in the short-term *in vivo* experiments described above were applied to animals challenged 16 weeks following the same vaccination regime. We also conducted pulmonary endothelial/capillary barrier studies on these mice.

The protective effects of Alum-adjuvanted SP3-Tetrasaccharide at 5 weeks were not observed at 16 weeks. Indeed, no significant differences in morbidity and mortality could be established between vaccinated animals and controls, nor were there significant differences in any of the gross clinical indicators of disease between groups. All infected groups exhibited the telltale signs of clinical illnesses described in the section above. Body-weight and temperature both decreased dramatically from their baseline values in infected mice, with no significant differences between vaccinated and non-vaccinated animals.

Bacterial burden in both blood and in the lungs (both BALF and tissue-samples) was elevated in all groups, indicating no improvement in bacterial clearance in vaccinated animals.

In all groups, FACS-studies showed marked, non-specific leukocytosis in BALF and whole-blood samples, without the differentiated expression of cell-types between cell-groups observed in the FACS studies from the short-term *in-vivo* experiments.

We included lung-endothelial/capillary barrier integrity as an additional parameter to establish disease in these animals. Inflammation of endothelium causes an increase in permeability in the microvasculature[146,147]. In pneumococcal pneumonia, inflammation leads to a weakening of the pulmonary endothelial/capillary barrier and extravasation of bacteria into the blood-stream[148,85]. To estimate the integrity of the lung- endothelial/capillary barrier, the albumin BALF/plasma ratio was measured by comparing serum and BALF- human serum albumin (HAS) concentrations with ELISA. Higher quotients were observed in all infected animals, with no significant differences between

groups, indicating a compromised barrier function as a result of pneumococcal invasion in all groups examined[105,137]. These studies further underline the inefficacy of the vaccine in protecting animals from pneumococcal challenge in the long term.

Results from the OPAs conducted with sera obtained 16 weeks after vaccination showed no significant killing of bacteria, and no efficacy according to the WHO-criteria outlined above.

As the vaccinated animals in this series of studies examining the long-term protectivity of SP3-Tetrasaccharide showed pronounced morbidity in all cases, with no significant differences in the described parameters from those of non-vaccinated, infected mice, we conclude that the vaccine's efficacy in conveying long-term protection is 0.

Inducing a long-term immune-response and implications for the current study.

The immune-response to a threat can be described as arising in roughly two stages: the primary response to a new, invasive pathogen, and the secondary response to re-introduction of that pathogen into the host.[36] The current model of the secondary immune response suggests that the generation of memory B-cells and long-lived plasma cells, supported by memory-T cells, is required for a sustained, long-term immune response[36,149,150,151]. Plasma cells represent the population of B-cells that produce specific levels of antibodies to a pathogen; their production and maintenance in the long-term appear to be contingent upon the presence of so-called long-lived plasma cells (LL-PC) and the rapid re-invigoration of a protective immune response is contingent on the presence of memory B-cells[152,153]. One can surmise that the vaccine's lack of efficacy in conferring long-term protection was due to a failure in the systems producing a long-lived memory response.

Establishing whether memory B-cells and/or long-lived plasma cells were produced would be a guiding step in characterizing the response that took place. Following the primary immune response, plasma cell-levels in peripheral blood are reduced, and a few are sequestered by the bone marrow, where they survive for longer periods and are replenished by memory B-cells located either in the spleen or lymph nodes[149,150,151,152]. An examination of bone marrow, lymph nodes and spleen for antigen-specific plasma cells and memory-B cells could help to shed light on whether a memory-response was induced at all.

In an effort to expand these data on long-term protection by SP3-Tetrasaccharide, it might be advisable to include another correlates of vaccine-protection by conducting survival studies in mice. This would give an absolute measure of clinical protectivity by establishing whether improved survivability following challenge can be observed.

Strategies for improving long-term immune-response to vaccination.

Adjuvants

Alum is a commonly employed adjuvant, because it has a long record of safely producing a robust immune response and consequently immunity[154,74–76]. The relative safety of this adjuvant comes at a cost, however, when compared to the efficacy of other adjuvants, such as Incomplete Freund's Adjuvant (IFA) and CFA. The former has been shown to produce significantly stronger immune responses to vaccination than aluminum[144], but is not approved for use in humans because of a relatively high frequency of potentially severe local and systemic reactions[144]. Even in our own experiments we were able to show that SP3-Tetrasaccharide administered with CFA produced significantly greater killing than SP3-Tetrasaccharide administered with Alum, demonstrating that also in this case, adjuvants significantly influence immune response to vaccines.

The identification and development of novel adjuvants is a topic of growing interest in research[73], as they play a critical role not only in eliciting a significant immune response to vaccines, but also in influencing the course of the innate, and subsequently the adaptive, immune-response[155]. Identifying a suitable adjuvant for this particular pathogen may play the decisive role in promoting a sustained immune-response to the vaccine in the current study.

Carrier Proteins

As discussed in the Introduction, carrier proteins are necessary for inducing a thymus-dependent response to the vaccine to which it is conjugated[39,40]. Both the carrier protein itself and the linking bond to the carbohydrate-antigen play a role in the response of the immune system to a vaccine[36,39,40]. The type of carrier protein and its respective linkage have been shown to correlate with a varying degree of robustness of long-term immune-response when coupled with the same carbohydrate antigen[35,156,157]. As mentioned above, it is the magnitude of the immune response, not antigen specificity, that appears to govern the production of long-term immunity in the case of this antigen. However, the exact mechanisms that underly the generation of long-lived immune memory are still a matter of debate. An alternative carrier-protein may more strongly amplify the immune response and promote immune-memory longevity. In addition, alternative carriers, e.g. conserved pneumococcal surface proteins or virulence factors may provide useful additional vaccine targets and increase the overall efficacy of semi-synthetic and conventional PCVs.

VI. Conclusions

The apparent, clear specificity of the immune response elicited by the novel semi-synthetic conjugate vaccine candidate turned out not to be long-lived under the conditions described here. Yet the achievement of that response strongly supports the conjecture that the vaccine's design and synthesis hold out considerable promise as a way of developing new, efficacious vaccines of this kind.

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VIII. Statutory Declaration

"I, Roland Bell, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic A semi-synthetic glycoconjugate vaccine for Streptococcus pneumoniae serotype 3 confers short-term protection against pneumonia in mice, independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I am aware of the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice and that I commit to comply with these regulations.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date   Signature

IX. Declaration of contribution to publications

Roland Bell contributed the following to the below listed publication:

Publication 1: Parameswarappa SG, Reppe K, Geissner A, Ménová P, Govindan S, Calow ADJ, Wahlbrink A, Weishaupt MW, Monnanda BP, Bell RL, Pirofski LA, Suttorp N, Sander LE, Witzenrath M, Pereira CL, Anish C, Seeberger PH. A Semi-synthetic Oligosaccharide Conjugate Vaccine Candidate Confers Protection against Streptococcus pneumoniae Serotype 3 Infection Cell Chemistry and Biology. Volume 23, Issue 11, p1407–1416, 17 November 2016.

Specific Contributions : Figure 5- Page 1412, Figure 6-Page 1413, Figure 7-Seite 1414, and Figures S5 & S6 in ‚Supplemental Figures'

_____

Signature, date and stamp of supervising university professor / lecturer

_____

Signature of doctoral candidate

X. Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

XI. Publikationen

Abstrakte

R.L. Bell, N. Omelchenko, S.R. Sesack. Lateral habenula projections to the ventral tegmental area in the rat synapse onto dopamine and GABA neurons. *Society for Neuroscience Annual Meeting*. Nov. 3-7 San Diego, CA., 2007.*

J. Balcita Pedicino, N. Omelchenko, R. Bell, S. R. Sesack. The rostromedial mesopontine tegmentum as a relay between the lateral habenula and dopamine neurons in the ventral tegmental area: Ultrastructural evidence in the rat. *Society for Neuroscience Annual Meeting.* Oct. 17-21, Chicago, IL., 2009.*

Reppe K, Anish C, Bell R, Parameswarappa SG, Govindan S, Pereira CL, Dietert K4, Sander L, Gruber AD, Suttorp N, Seeberger PH, Witzenrath M. The synthetic Streptococcus pneumoniae oligosaccharide conjugate protected mice from severe pneumonia. *European Society of Clinical Microbiology and Infectious Diseases*, Annual Meeting. 27-30 April, 2013. Berlin, Germany.

Reppe K, Chakkumkal A, Bell R, Pereira CL, Dietert K, Sander L, Gruber AD, Suttorp N, Seeberger PH, Witzenrath M. Eine synthetische S. pneumoniae Oligosaccharid-Konjugatvakzine schützt Mäuse vor Pneumonie. *Herbsttagung der Sektion Zellbiologie der Deutschen Gesellschaft für Pneumologie*. 25-16 October, 2013. Marburg, Germany.

Artikel

Natalia Omelchenko, Roland Bell, and Susan R. Sesack. Lateral Habenula Projections to the Rat Ventral Tegmental Area: Sparse Synapses Observed Onto Dopamine and GABA Neurons. *Eur. J. Neurosci.,* 30(7): 1239–1250.*

Judith Joyce Balcita-Pedicino, Natalia Omelchenko, Roland Bell, Susan R. Sesack. The inhibitory influence of the lateral habenula on midbrain dopamine cells: Ultrastructural evidence for indirect mediation via the rostromedial mesopontine tegmental nucleus. *The Journal of Comparative Neuroanatomy. 2011 Apr 15; 519(6): 1143-1164.**

* Die Daten dieser Publikationen im Bereich Neuroanatomie habe ich im Jahr 2007 auch auf einem Jahrestreffen der Society For Neuroscience vorgestellt.

Sharavathi Guddehalli Parameswarappa, Katrin Reppe, Andreas Geissner, Petra Ménová, Subramanian Govindan, Adam D.J. Calow, Annette Wahlbrink, Markus W. Weishaupt, Bopanna Ponnappa Monnanda, Roland Lawrence Bell, Liise-Anne Pirofski, Norbert Suttorp,

Leif Erik Sander, Martin Witzenrath, Claney Lebev Pereira, Chakkumkal Anish, Peter H. Seeberger. A Semi-synthetic Oligosaccharide Conjugate Vaccine Candidate Confers Protection against *Streptococcus pneumoniae* Serotype 3 Infection. *Cell Chemical Biology.* Published online: November 3, 2016

Symposien

***Habenula: Crossroad between the Basal Ganglia and the Limbic System.* Die präsentierenden Autoren waren: Okihide Hikosaka, Susan R. Sesack, Lucas Lecourtier, and Paul D. Shepard.** Zitat: The Journal of Neuroscience, November 12, 2008, 28(46):11825-11829; doi:10.1523/JNEUROSCI.3463-08.2008

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