Improving the Immunoprotective Effect of Carbohydrate Vaccine Against Bacterial Pneumonia

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Declaration

This is to certify that the entire work in this thesis has been carried out by Paulina Kaplonek. The assistance and help received during the course of investigation have been fully acknowledged.

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(Date, Place) (Signature)

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Scientific Publications and Reviews

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2. Broecker F, Wegner E, Kaplonek P, Ensser A, Pfister F, Daniel C, Martin CE, Mattner J, Seeberger PH. *Synthetic oligosaccharide-based vaccines protect mice from Clostridium difficile infections*, under revision.
3. Downey, A. M, Kaplonek P, Seeberger P. H. *MAIT Cells As Attractive Vaccine Targets*, FEBS Letter 2019, 593(13):1627- 1640.
4. Kaplonek P, Khan N, Reppe K, Schumann B, Emmadi M, Lisboa M, Xu F, Calow A, Parameswarappa SG, Witzenrath M, Pereira CL, Seeberger PH. Improving vaccines against Streptococcus pneumoniae using *synthetic glycans*, Proceedings of the National Academy of Sciences 2018: 201811862
5. Schumann B, Reppe K, Kaplonek P, Wahlbrink A, Anish C, Witzenrath M, Pereira CL, Seeberger PH. *Development of an Efficacious, Semisynthetic Glycoconjugate Vaccine Candidate against Streptococcus pneumoniae Serotype 1*. ASC Central Science 2018; 4(3):357-361
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7. Zhang Q, Savagatrup S, Kaplonek P, Seeberger PH, Swager T, *Janus Emulsions for the Detection of Bacteria*, ACS Central Science 2017, 3(4): 309-313

Scientific Conferences and Symposia (* oral presentation)

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3. * 24th International Symposium On Glycoconjugates; Korea, 2017
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6. 2nd International Conference “Innate immunity of the lung”; Germany, 2016
7. SFB-TR84 retreat Kloster Drübeck; Germany, 2016

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List of Abbreviations

APC	Antigen-Presenting Cell
a.u.	Absorbance Units
BCR	B Cell Receptor
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CDR	Complementarity Determining Region
CFA	Complete Freund's Adjuvant
CFU	Colony-Forming Units
CPS	Capsular Polysaccharide
CRM197	Corynebacterium diphtheria Mutant CRM197
CWPS	Cell-Wall Polysaccharide
DC	Dendritic Cell
DLS	Dynamic Light Scattering
Da	Dalton
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DTT	1,4-Dithiothreitol
EAP	External Aqueous Phase
EDC	*N*-ethyl-*N*'-(diethylaminopropyl)-carbodiimide
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
FACS	Fluorescent-Activated Cell Sorting
FCS	Fetal Calf Serum
FELASA	Federation of European Laboratory Animal Science Associations
FITC	Fluorescein Isothiocyanate
HPLC	High Performance Liquid Chromatography
HRP	Horse Radish Peroxidase
IgM/IgG	Immunoglobulin M/G
IAP	Internal Aqueous Phase
IC_{50}	The half maximal inhibitory concentration
IL	Interleukin

IFN	Interferon
iNKT	Invariant Natural Killer T Cells
i.p.	Intraperitoneally
IPD	Invasive Pneumococcal Disease
i.v.	intravenous
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization With Time-Of-Flight Detection
MS	Mass Spectrometry
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MW	Molecular Weight
NHC	N-Hydroxysuccinimide
NK	Natural Killer Cell
OD	Optical Density
OP	Organic Phase
OPKA	Opsonophagocytic Killing Assay
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate-Buffered Saline
PBST	Phosphate Buffered Saline with 0.1% Tween-20
PCV	Pneumococcal Conjugate Vaccine
Ply	Pneumolysin
PLGA	Polylactic acid nanoparticles
PspA	Pneumococcal surface antigen A
PRR	Pattern-Recognition Receptor
Prev13	Prevnar13®
PVA	Polyvinylalcohol
RBC	Red Blood Cells
RPM	Revolutions per minute
RT	Room Temperature
RU	Response Units
s.c.	Subcutaneous

SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
SEM	Scanning electron microscopy
SPR	Surface Plasmon Resonance
ST1/3/8…	*Streptococcus pneumoniae* serotype 1/3/8…
TCR	T Cell Receptor
T_D	T cell-dependent
Th	T Helper Cell
TI	T cell-independent
TLR	Toll-Like Receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor Necrosis Factor
TrBS-T	Tris Buffered Saline with 0.1% Tween-20

Summary

High mortality rates of bacterial pneumonia and increased antibiotic resistance are major reasons to develop novel vaccine strategies against *Streptococcus pneumoniae. S. pneumoniae* serotype 3 (ST3) is one of the most frequent serotypes isolated from patients with invasive pneumococcal diseases, even though it is included in the routine immunization schedule. To improve immunogenic properties of ST3, synthetic antigenic tetrasaccharide based on capsular polysaccharide repeating units have been conjugated to a carrier protein and used as a vaccine candidate. The study illustrates the principle of proper optimization of variable aspects of vaccine formulation, such as dosage, adjuvant, and carrier protein.

Use of highly pure and well-characterized synthetic oligosaccharide allowed to significantly decreased the dosage of antigen while maintaining sufficient protection shown by *in-vitro* opsonophagocytic killing assay. In defiance of the general notion “the more, the better”, the higher dose did not improve the protective effect of immunization but could even diminish the final success of the vaccination.

In order to improve the immunogenicity of semi-synthetic ST3-glycoconjugates, several commercially available adjuvants were used and incorporated into biodegradable poly-lactic-acid (PLA) microparticles. Screening experiments in mice yielded promising results for agonists of TLR7/8, namely resimiquimod (R848) and bacterial RNA, as well as MPLA, a TRIF-biased TLR4 agonist used in several commercial vaccines.

The carrier protein derived from *S.pneumoanie* can serve as “double-action bullet”, being both a carrier essential for glycan presentation and an additional vaccine antigen providing broader protection, so-called "additional valency". Hence, ST3-tetrasaccharide was conjugated to pneumolysin and PspA protein and vaccine was evaluated *in-vivo* in the mouse and swine model. The mouse study showed that ST3-tetrasaccharide pneumolysin conjugates decrease the bacteria load in blood and lungs as well as reduce the disease severity in mice challenged with *S.pneumoniae* serotype 3. Additionally, the synthetic oligosaccharide conjugated to pneumolysin and PspA inhibited colonization of the nasopharynx after infection with bacteria. The immunization of piglets provides the first evidence for the immunogenicity of the synthetic glycoconjugate vaccine in a swine model. The generated antibodies were able to kill

pneumococci and neutralize the toxic effect of pneumolysin *in-vitro*. However, the protective activity of the glycoconjugate vaccines in the swine *in-vivo* infection model has to be further investigated.

The study presented in the thesis combined a series of innovations, which enhance the efficacy and applicability of glycoconjugate vaccines and help to further clarify the principles of anti-carbohydrate- and anti-bacterial immunity.

Zusammenfassung

Hohe Sterblichkeitsraten bei bakteriellen Lungenentzündungen und eine erhöhte Antibiotikaresistenz sind wichtige Gründe für die Entwicklung neuer Impfstrategien gegen Streptococcus pneumoniae. S. pneumoniae Serotyp 3 (ST3) ist einer der häufigsten Serotypen, die bei Patienten mit invasiven Pneumokokkenerkrankungen isoliert wurden, obwohl er in den routinemäßigen Impfplan aufgenommen wurde. Um die immunogenen Eigenschaften von ST3 zu verbessern, wurden synthetische antigene Tetrasaccharide, die auf sich wiederholenden Polysaccharid-Kapseleinheiten basieren, an ein Trägerprotein konjugiert und als Impfstoffkandidat verwendet. Die Studie veranschaulicht das Prinzip der richtigen Optimierung verschiedener Aspekte der Impfstoffformulierung, wie Dosierung, Adjuvans und Trägerprotein.

Die Verwendung von einem hochreinen und gut charakterisierten synthetischen Oligosaccharid ermöglichte eine signifikante Verringerung der Antigendosis unter Aufrechterhaltung eines ausreichenden Schutzes, belegt durch einen *in-vitro*-Opsonophagozytentötungstest. Entgegen dem Sprichtwort „Viel hilft viel“ verbesserte die höhere Dosis die Schutzwirkung der Immunisierung nicht, sondern konnte den endgültigen Erfolg der Impfung sogar beeinträchtigen.

Um die Immunogenität von halbsynthetischen ST3-Glykokonjugaten zu verbessern, wurden mehrere im Handel erhältliche Adjuvantien verwendet und in biologisch abbaubare Polymilchsäure (PLA) -Mikropartikel eingearbeitet. Screening-Experimente an Mäusen ergaben vielversprechende Ergebnisse für TLR7/8-Agonisten, nämlich Resimiquimod (R848) und bakterielle RNA, sowie MPLA, einen TRIF-voreingenommenen TLR4-Agonisten, der in mehreren kommerziellen Impfstoffen verwendet wird.

Das von S.pneumoanie abgeleitete Trägerprotein kann als "Mehrzweckwaffe" dienen, da es ein für die Glykanpräsentation wesentlicher Träger, und gleichzeitig auch ein zusätzliches Impfstoffantigen ist, das einen breiteren Schutz, auch "zusätzliche Wertigkeit" genannt, bietet. Folglich wurde ST3-Tetrasaccharid an Pneumolysin und PspA-Protein konjugiert und der Impfstoff *in-vivo* im Maus- und Schweine-Modell bewertet. Die Mausstudie zeigte, dass ST3-Tetrasaccharid-Pneumolysin-Konjugate die Bakterienbelastung in Blut und Lunge reduzieren sowie die Schwere der Erkrankung bei Mäusen, die mit *S. pneumoniae* Serotyp 3 in Kontakt

gebracht wurden, verringern. Zusätzlich inhibierte das synthetische Oligosaccharid, das an Pneumolysin und PspA konjugiert war, die Kolonisierung des Nasopharynx nach Infektion mit Bakterien. Die Immunisierung von Ferkeln liefert den ersten Nachweis für die Immunogenität des synthetischen Glykokonjugat-Impfstoffs in einem Schweinemodell. Die erzeugten Antikörper konnten Pneumokokken abtöten und die toxische Wirkung von Pneumolysin *in-vitro* neutralisieren. Die Schutzwirkung der Glykokonjugat-Impfstoffe im *In-vivo*-Infektionsmodell für Schweine muss jedoch weiter untersucht werden.

Die in der Dissertation vorgestellte Studie kombinierte eine Reihe von Innovationen, die die Wirksamkeit und Anwendbarkeit von Glykokonjugat-Impfstoffen verbessern und zur weiteren Klärung der Prinzipien der antikohlenhydrat- und antibakteriellen Immunität beitragen.

1 Introduction

1.1 *Streptococcus pneumoniae*

S. pneumoniae is the leading source of life-threatening diseases like pneumonia, septicemia, and meningitis (1), as well as a major cause of death in children under five years old in developing countries (2-4). According to a UNICEF report from 2017, pneumonia globally kills 5.6 million children every year (16%). More than 95% of cases occur in emerging nations (5). In the US, pneumonia is less often fatal for children, but it is still the most common reason for them to be hospitalized. According to the American Thoracic Society debriefing, among American adults, pneumonia is the most common cause of hospital admissions other than women giving birth. About 1 million adults in the US seek care in a hospital due to pneumonia every year, and 50,000 die from this disease (6). Pneumococcus is also the leading cause of bacterial meningitis in older adults, causing substantial morbidity and mortality (7).

Although the clinical significance of community-acquired pneumonia (CAP) is very high, a clinical disease with the pneumococcus is rare compared to bacteria colonization. Up to 10% of the adolescent population, and more than 40% young infants, mainly those exposed in daycare settings, are colonized (8). Thus, in order to protect most individuals against clinical syndromes such as pneumonia and invasive pneumococcal disease, an effective immune response must already have been developed in them (9).

S.pneumoniae enter the nasal cavity and attach to the nasopharyngeal epithelial cells. Bacteria may either stay as a colonizer or spread to other organs, such as the ears, sinuses, or via bronchi down to the lungs. The initiation of pneumonia requires bacteria to escape from mucous defenses by the interaction of bacterial components with the alveolar epithelium (inhibiting the mucociliary beat of respiratory cells and separating epithelial cell tight junctions). From the lung, it can penetrate the mucosal barrier to enter the bloodstream and cross the blood-brain barrier to cause meningitis. Lack of innate defenses in cerebrospinal fluid (CSF) allows bacteria to multiply easily. Pneumolysin and hydrogen peroxide, produced by bacteria cause the toxin-mediated neuronal death. Pneumococcal infections spread from person to person via droplets/aerosols (Figure 1) (8, 10).

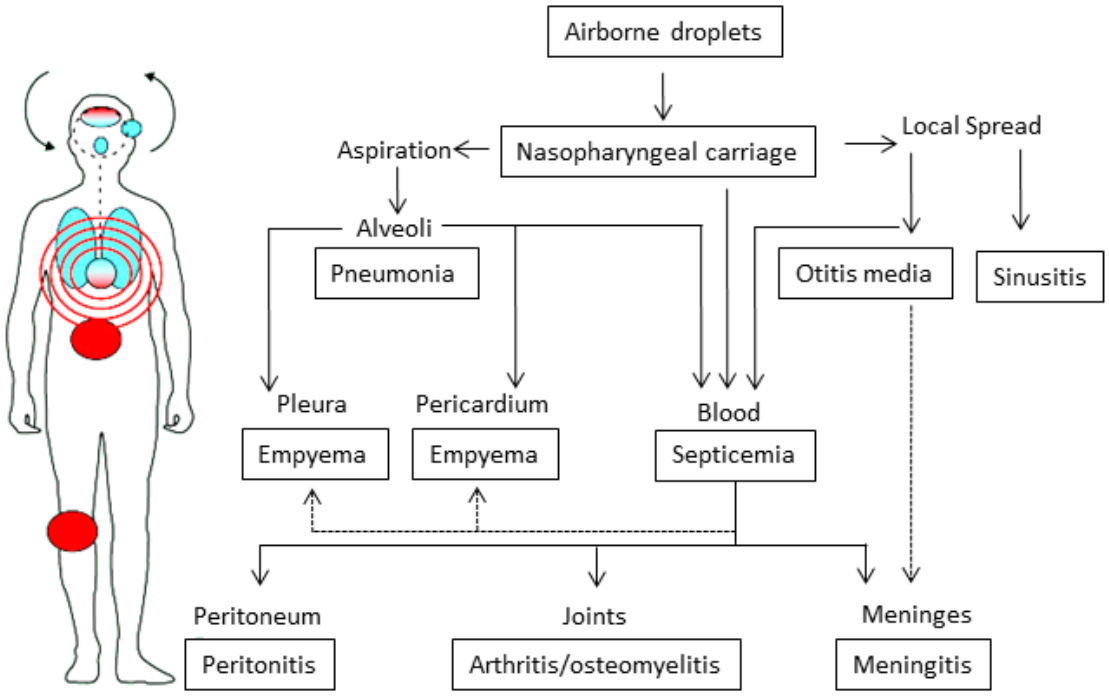

Figure 1. Pathogenic route for *S. pneumoniae* infection.
Adapted from *Bogaert et al.*, 2004 (8).

At least 98 *S. pneumoniae* serotypes can be distinguished based on their CPS (11, 12). Currently available CPS-based pneumococcal vaccines contain the serotypes most frequently associated with invasive pneumococcal diseases (IPDs). Although the licensed 23-valent polysaccharide vaccine (Pneumovax 23®) is not effective in younger children (3, 13), the conjugate vaccines Prevnar13® and Synflorix®cover thirteen and ten serotypes respectively and are highly successful in all age groups (14). Nevertheless, the plasticity of the pneumococcal genome means that the pathogen has the potential to adapt to the selective pressure of vaccines (9). The serotype replacement due to vaccination and regional differences in dominant serotypes necessitate the expansion of existing vaccines to include additional serotypes. New strains, e.g., serotype 19A and 22F, arise and replace previous colonizing strains under this selective pressure (15).

1.1.1 Biology of *S. pneumoniae* and virulence factors

Streptococcus pneumoniae is a gram-positive facultatively anaerobic organism. Its growth is enhanced in 5% carbon dioxide or anaerobic conditions. On blood agar, colonies are α hemolytic and can be identified as lancet-shaped short chains diplococci. The specific pneumococcal types based on polysaccharide capsule can be identified using typing serum, microscopic examination, and molecular techniques (16).

1.1.1.1 The capsular polysaccharide of *S. pneumoniae*

S.pneumoniae has a capacity to produce a capsule, which is structurally distinct for each of the 98 recognize serotypes and is the dominant surface structure of the organism. In 1881, Louis Pasteur observed that colony variants of a pneumococcal strain react with the serotype-specific protective sera, which was described as an "aureole (halo)". The studies of pneumococcal culture supernatants containing materials reacting with serotyping sera revealed a polysaccharide (PS) nature of the capsule (17). Capsular polysaccharide (CPS) plays a critical role in virulence of *S. pneumoniae* and all fresh clinical isolates are encapsulated. CPS appears to act as a safeguard, preventing activation of the complement pathway and also an interaction between the pathogen and receptors on phagocytic cells. The spontaneous nonencapsulated (rough) derivatives of the bacteria are almost avirulent. Most pneumococcal capsules are anionic, except 7A, 7F, 14, 33F, 33A, and 37 that are not charged. The negative charge helps to inhibit clearance by mucus and prevent phagocytes through electrostatic repulsion (16, 18). However, antibodies to CPS are highly protective and result in opsonization as well as rapid removal of the invading pneumococci by the host opsonophagocytic clearance mechanisms.

The genes necessary for capsular synthesis are present in a cassette-like arrangement. Genes, named *wzg*, *wzh*, *wzd*, and *wze* (more commonly known as *cpsA*, *cpsB*, *cpsC*, and *cpsD*, respectively) are widely conserved. The *cps* loci are transcribed as a single operon. CPSs are generally synthesized by the Wzx/Wzy-dependent pathway. The locus begins with conserved, or "common," genes whose products are involved in the regulation of capsule: *cpsA*, *cpsB*, *cpsC*, and *cpsD (*Figure 2*a).* The *cps* locus encodes the enzymes to build the repeat units, including

glycosyl phosphate transferase, and additional transferases responsible for the formation of the linkages and addition of sugars, as well as a repeat-unit flippase and polymerase *(19)*.

CPSs are synthesized by transfer of an initial monosaccharide phosphate from a nucleotide diphosphate sugar to a membrane-associated lipid carrier and the sequential transfer of further monosaccharides to produce the lipid-linked repeating unit. The lipid-repeating unit is transferred to the outer face of the cytoplasmic membrane by the repeat-unit transporter or flippase, polymerized to form the mature CPS, and then attached to the peptidoglycan (Figure 2b) (19).

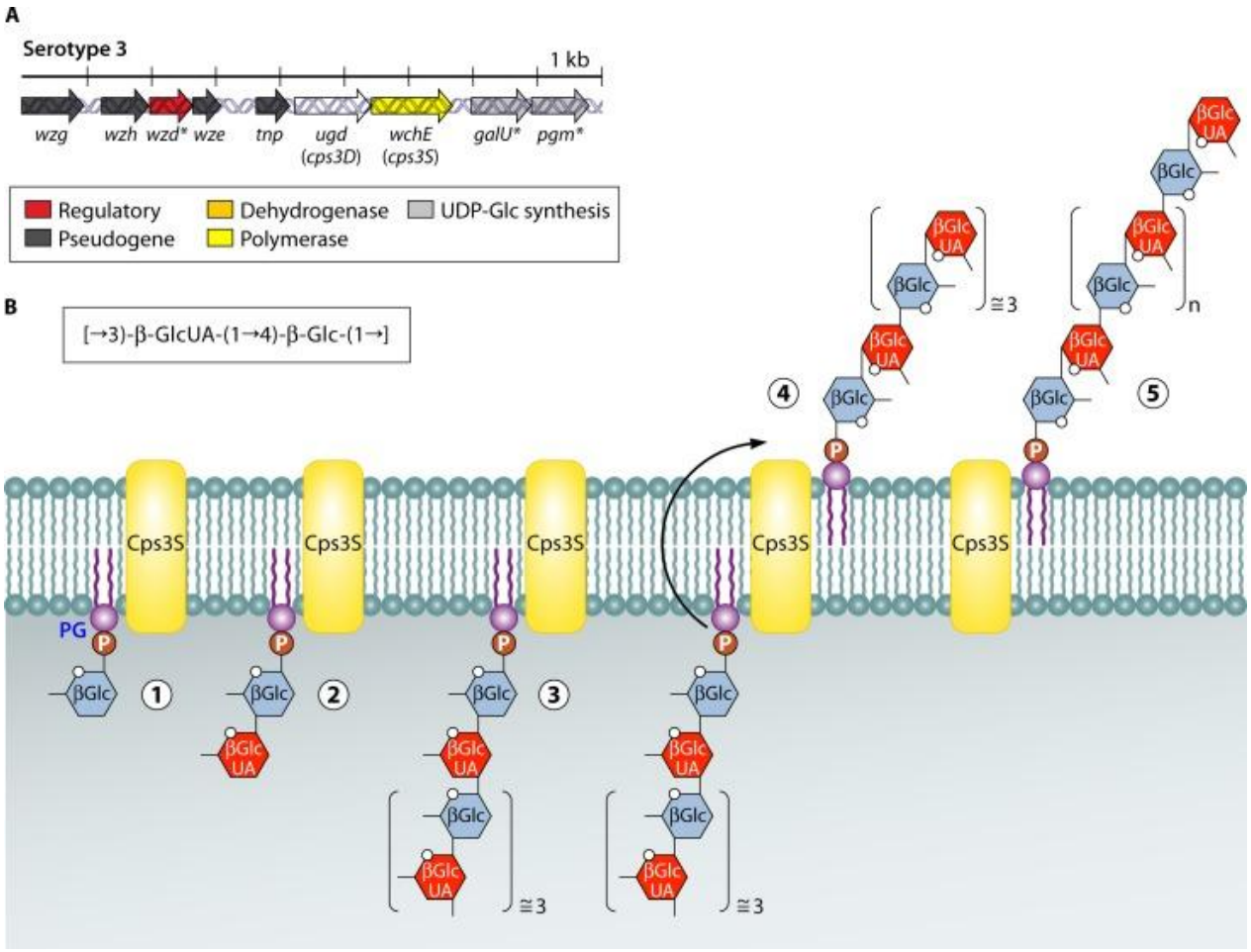

Figure 2. Genetic and biochemical bases of *S.pneumoniae* serotype 3 capsular synthesis.
a) The serotype 3 cps locus b) Synthesis of serotype 3 PS by Cps3S synthase, which initiates a transfer of glucose (Glc) from UDP-glucose to a phosphatidylglycerol (PG) acceptor (1). Glucuronic acid (GlcUA) is transferred from UDP-GlcUA to the PG-linked Glc (2), and the capsule is extended to approximately an octa-saccharide (3). Cps3S translocates the PS chain to the outer face of the membrane (4) and increases chain length by a processive capsular synthesis mechanism (5). (Adapted from *Geno K.A. et al*., 2015)

The PSs of different serotypes are built of structurally varying repeating units, which include recognized saccharide residues, their order, and linkages. The repeating units contain from two to eight saccharides and are very often substituted with O-acetyl, phosphoglycerol, and pyruvyl acetal, and other groups. The substitutions are located at various sites with various degrees of substitution. (18). Monosaccharide composition can be determined with a gas-liquid chromatography, a mass spectrometry method, the size of repeating units can be solved. Modern nuclear magnetic resonance approaches (including ^{1}H, ^{13}C, ^{31}P, and two-dimensional (2D) NMR) can reveal structural details of intact capsular PS with minimal degradation (20).

1.1.2 Virulence factors *of S. pneumoniae*

S.pnuemoniae virulence factors are classified based on different functions during *in vivo* infection: (a) surface adhesins (b) enzymes involved in the invasion of host tissues (invasins) and (c) enzymes destabilizing and suppressing the host defense. Most of the pneumococcal virulence factors are cell surface located proteins. They can either be the classical cell wall proteins or house-keeping cytosolic enzymes that are secreted and attached to the bacterial cell wall (21). Multiple virulence factors have been identified and considered for use as vaccine candidates, such as pneumolysin, neuraminidases, hyaluronidase, choline-binding proteins (e.g. autolysin, pneumococcal surface protein A (PspA) and choline-binding protein A (CbpA)), lipoprotein (pneumococcal surface antigen A (PsaA)) and the immune subverting factor Immunoglobulin A1 (IgA1) protease.

1.1.2.1.1 Pneumolysin

Pneumolysin (Ply) is a 53 kDa protein that belongs to the family of cholesterol-dependent cytolysin (CDC). Ply is not actively secreted from the bacterium as it lacks a typical signal secretion leader sequence but escapes from the cell by either autolysis or the action of lytic antibiotics. However, there have been some reports of active secretion of the protein. Ply forms pores in the cell membrane by oligomerization and conformational change in the structure. The pores formed can be up to 350 Å in diameter with each pore up to 50 Ply monomers. The formation of pores results in a cell membrane disintegration which helps the bacteria to spread in

a body as well as increase the host cells death and disease manifestation. Pneumolysin is a relatively conserved protein across all serotypes of *S. pneumoniae*. However, at least 16 different naturally-occurring variants of Ply are expressed in specific strains of serotypes 1, and 8 pneumococci have been identified (22, 23).

Pneumolysin is a multifunctional toxin with distinct activities such as:

- Complement activation - Ply plays a central role in protecting the pneumococcus from complement attack and helps the spread of the bacteria to other tissues/organs. The secreted protein can activate the classical complement pathway, even in the absence of Ply specific antibody. This mechanism results in depletion of complement in the host and decreases inflammation (24). Patients with an active pneumococcal infection have reduced serum complement levels, while Ply-treated serum has reduced opsonic activity (25).
- Lysis of red blood cells (hemolysis) – it was proved that Ply forms pores in the membrane of red blood cells and cause the hemoglobin release. The toxin has lectin activity and binds glycans, including the Lewis histo-blood group antigens (26)
- Production of immune regulatory molecules - Ply has highly pro-inflammatory properties and stimulates the production of cytokines including TNFα, IL-1, and IL-6. The production of these cytokines may play a role in pro-inflammatory disease as well as in the regulation of the immune response to pneumococcus (27).
- Impact neutrophil activity and neutrophil extracellular traps formation (NETs) - it has been proven that Ply reduces the killing of pneumococci by neutrophils in vitro (28). Other studies showed the toxin induces vital NETosis in human neutrophils, which depends on the intensity of the inflammatory response during pneumococcal infection and may either contribute to host defense or worsen disease severity (29).
- Helps to spread bacteria – Ply breaks the tight epithelial junctions (by reducing stable and dynamic microtubule content and by modulating VE-cadherin expression), increases alveolar permeability and inflammation, thus allows bacteria spread in the blood (bacteremia). The toxin also damages the blood-brain barrier allowing for the bacteria to reach the brain and cause meningitis (30).
- Influences cell-signaling, cytoskeletal rearrangement and induces DNA damage (31).

- Colonization of the host - studies using *S.pneumoniae* PLY-deficient mutants revealed decreased colonization of the nasopharynx, increased bacterial clearance from the lung and prolonged survival of animals following the infection (32).
- Play a role in pneumonia pathogenesis by, causing the endothelial hyper-permeability (pulmonary permeability edema), a major complication of pneumonia (33).

The evidence from animal infection studies points clearly to an integral role of pneumolysin in invasive pneumococcal diseases. Neutralization of the toxin seems to be a potentially valuable approach to treat pneumococcal diseases as well as an exciting vaccine candidate.

1.1.2.1.2 Pneumococcal surface antigen A – PspA

PsaA is a very well conserved 37-kDa lipoprotein composed of 309 amino acid residues directly attached to the lipid of the cytoplasmic membrane. The flexible nature of the PsaA structure enables its dual functions of metal ion transport and adhesion to the epithelial cells (34). PspA is composed of four different distinct regions (1) the C-terminal anchoring the protein to the pneumococcal surface, (2) a stretch of ten highly conserved 20-amino-acid repeats, (3) a proline-rich region which acts as a tether and allows greater flexibility and movement of the amino-terminus, and (4) a highly charged amino-terminus (23, 35). The amino-terminal end extends from the cell wall and sticks outside the capsule (36). Based on amino acid sequence ahead of the proline-rich region, PspA is classified into three families and six clades (Family 1, clades 1 and 2; Family 2, clades 3, 4 and 5; and the rarely isolated Family 3, clade 6) (21). The interaction of PspA through helix 3 and helix 4 of the N-terminal domain with the iron-saturated lactoferrin helps bacteria to adhere on the surface of the host (35). A highly polar electrostatic charge of PspA increase the capsule charge stabilization and the predominant part of protein prevents C3-mediated binding of the host complement to pneumococci by competing with the C-reactive protein (23, 37).

The multifunctional immune evasive properties of PspA are essential for pneumococcal nasopharyngeal colonization and invasion of *S.pneumoniae (38).* Many studies show that $PsaA^{-}$ mutants of *S. pneumoniae* were significantly less virulent than the parent strains. This may be due to their impaired growth in an Mn^{2+} deficient environment, reduced capacity to adhere to

lung cells, or hypersensitivity to oxidative stress (34). Active immunization with PspA in animal models protects against the nasopharyngeal carriage and invasive disease. Intranasal immunization of mice with the cholera toxin B subunit-PsaA was shown to protect them against pneumococcal colonization without changing their healthy flora (39).

PsaA is immunogenic and induces both the humoral (antibody production) and cellular (activation of phagocytes, cytotoxic T-lymphocytes, and the release of various cytokine) immune response. Asymptomatic or symptomatic pneumococcus carriage results in robust production of antibodies to PsaA and inhibits colonization and disease. The protein has a high potential to be used both in the diagnosis and as a component of a protein vaccine (34).

1.2 Vaccines

Vaccines are one of the most successful medical advances in modern times and one of the greatest success stories within the health sector. The eradication of several life-threatening diseases, such as smallpox, whooping cough, mumps, and polio, has put vaccines on one of the highest pedestals in disease prevention (40, 41). Unfortunately, political instability, conflict and not a transparent strategy of pharma industries has posed a challenge for public trust in vaccines. (42). Vaccine hesitancy has been stated as one of the ten main issues that demand attention from WHO and health partners in 2019 (43). Another problem facing world health is antimicrobial resistance (AMR). Overuse or inappropriate consumption of antimicrobials in people and animals has led to the rapid development of AMR. It is estimated that AMR currently causes 700,000 deaths annually. Use of vaccines can prevent antibiotic-resistant infections directly by reducing or eliminating the risk of infection. The secondary effect of vaccines decrease viral infections such as influenza and measles, concomitantly reduces the risk of secondary infections that are often a result of the use of antibiotics (44).

1.2.1 History of vaccines

Recognition of the pathogens (mostly viruses or bacteria) as a cause of the infectious disease was already made in 400 b.c by Hippocrates. He was the first to recognize infections such as mumps and diphtheria in neonates and children and combine the knowledge with the occurrence of deadly diseases. At that point, the disease could not be prevented but only treated by herbal medicine with a very high rate of mortality. Many decades later, around the 12^{th} century, the first vaccine-like related procedures were described in China and India. Healthy people were inoculated with ground smallpox scabs by blowing them into the nostril or applying onto the scratched skin (45, 46). However, the title of “the father of vaccination and immunology” belongs to Edward Jenner, who in 1796 successfully introduced the Cowpox immunization in humans. He transferred a cowpox sore from an infected milkmaid to a healthy boy and then challenged him with a human virus. With the experiment, Jenner proved that when a cowpox sample is transferred from one person to the other, it has a protective effect.

The new era of vaccinology began in the 19th century, when scientists, such as Louis Pasteur, Robert Koch, and Paul Ehrlich discovered and developed essential knowledge related to pathogens and immunization. Their achievements led to the expansion of research in vaccinology and paved the way for the production of new vaccines around the world. The milestones in the field of vaccinology are summarized in Table 1.

Table 1. Milestones in vaccinology research.
Table prepared based on (45, 46).

	Date	Achievement
XII century	1100	Early Chinese inoculation with Smallpox
XVII century	1759	Heberden described how parents could inoculate their children against smallpox
	5/14/1796	Jenner's breakthrough
XIX century	1885	Rabies attenuated vaccine used in human (Pasteur)
	1890	Serological treatment by Emil Von Behring and Paul Ehrlich; tetanus toxoid was introduced
	1896	Typhoid Fever (*Salmonella typhi*) vaccine Cholera (*Vibrio cholerae*) vaccinee
	1897	Plague (*Yersinia pestis*) vaccine
XX century	1915	Pertussis - whooping cough (*Bordetella pertussis)* vaccine
	1927	BCG (*Bacillus Calmette–Guérin*) vaccine against tuberculosis
	1936	Yellow Fever (*Yellow fever virus*) vaccine
	1945	The first influenza vaccine approved (*Influenza virus* A and B)
	1948	Pertussis, Tetanus, Diphtheria (DTP) combined vaccine
	1960	Polio (*Enterovirus*) vaccine
	1963	Measles (*Paramyxovirus*) vaccine
	1964	Use of adjuvant was recommended

	1967	Mumps (*Rubulavirus*) vaccine licensed (Mumpsvax®)
	1969	First Rubella (*Rubivirus*) vaccine licensed
	1971	Combined MMR vaccine (measles, mumps, and rubella)
	1974	WHO Expanded Program of Immunization for BCG, Polio, DTP, measles, yellow fever, and hepatitis B. First meningococcal B polysaccharide vaccine
	1977	First pneumococcal vaccine (14 different strains)
	1980	Rabies: HDCV (human diploid cell) vaccine
	1981	Hepatitis B first subunit viral vaccine
	1983	Expanded pneumococcal vaccine PCV23 (Pneumovax23®)
	1987	Conjugate Hib (*Hemophilus influenza* type B) vaccine
XXI century	2000	Pneumococcal conjugated 7-valent vaccine (Prevnar7®)
	2008	Rotavirus vaccine licensed (Rotarix™)
	2010	Cconjugate pneuomococcal 13-valent vaccine (Prevnar13®)
	2014	Group B Meningococcal vaccine approved (Trumenba®)

1.2.2 Vaccine types

Vaccines, one of the best defenses against infectious diseases, can exert a protective effect against various invading pathogens. With time, significant effort has been made to improve and control vaccine-safety as well as efficacy. Vaccines are classified into different categories, based on specific characteristics and the spectrum of protection (46). The first most traditional generation of vaccines includes live attenuated (e.g., MMR vaccine, Yellow Fever, Influenza) and inactivated (e.g., Rabies, Pertussis, Hepatitis B) vaccines, which can be produced easily. Both types contain whole pathogens, respectively weaken through several cells passages or inactivated by heat, radiation, or chemical methods. Nevertheless, the pathogens are able to return to an infective form. The second generation of vaccines offers a safer solution and are made by utilizing the specific microbial elements of the microbes or recombinant antigens that

can elicit a protective response. The subunit vaccines might contain one on more immunogenic compounds; either protein/peptide or a polysaccharide; for example tetanus, diphtheria, and pertussis toxoid vaccines are non-toxic forms of a main pathogenic factor of the respective bacteria. Another example of the second generation of vaccines is the use of the polysaccharide capsule of the bacteria as an antigen. It can be conjugated to the carrier protein to increase the more robust immune response or used alone as in the case of Pneumoniae, Meningococcus or *H.influenza*. The recombinant antigens of hepatitis B virus, herpes simplex virus, or rotavirus respectively also belong to this family. Since there are no genetic components involved with this method, these highly immunogenic particles are not able to cause the disease anymore (46-48). The third generation of vaccines utilizes the genetic material of pathogens as antigens. Either liner DNA and RNA or plasmid can be employed. Many ongoing clinical trials are based on the use of the most modern type of vaccines for cancer, HIV, influenza or Ebola prevention as well as for immunotherapy. So far, DNA vaccines have been licensed only for animal use (49).

1.2.3 Immunology of antigen recognition

The main goal of vaccination is to induce the long-lasting protective immune memory, so that upon exposure to the pathogen, a quick and forceful response will be generated. Vaccination induces both the evolutionarily ancient and immediate, innate response and the highly specific, but temporally delayed, adaptive immune response. However, the quality, the intensity, and the duration of the final adaptive response are greatly influenced by the innate arm (50, 51).

The immunological mechanism of immunization has been studied for many years, and the understanding of the processes occurring after the injection of antigen is very well established. The main impact into the field was made by Charles Janeway, who described the mechanism of T cells recognition of cognate antigen, the interactions between the T cell receptor and antigen presented by Major Histocompatibility Complex (MHC) molecule, the role of “co-receptor” for activation of T cells, mechanism of pattern recognition by the mammalian Toll-like receptor family, as well as connection between innate signals and adaptive immunity (52).

The innate immune system can recognize invariant features, characteristic for different classes of microbes, so-called pathogen-associated molecular patterns (PAMPs), by using germ-line

encoded receptors (pattern recognition receptors - PRRs). The mechanism is highly specific and efficient in distinguishing self and non-self antigens, classes of pathogens, and most importantly, determining the need for either immediate or future defense (51). Numerous classes of PRRs, such as Toll-like Receptors (TLRs), Nucleotide-binding Oligomerization Domain (NOD)-like Receptor (NLRs), C-type Lectin Receptors (CLRs**)** and Retinoic Acid-Inducible Gene (RIG)-I-like Receptors (RLRs) have been identified. Physical association between antigen and PRR on antigen-presenting cells, such as dendritic cells and macrophages, initiates a cascade of various signaling pathways. The most critical molecules included in the cascades are Myeloid Differentiation primary response gene 88 (MyD88), Toll-interleukin-1 Receptor Adaptor Protein (TIRAP) inducing interferon β, and TRIF-related adaptor molecule (TRAM). Their activation results in the initiation of the mitogen-activated protein kinases (MAPKs), followed by the transcription of a nuclear factor (NF)-κB and interferon regulatory factor (IRF)-responsive genes which finally lead to the regulation of the phagocytosis of the pathogen as well as the cytokines production by antigen-presenting cells (APCs) (53). The uptake of intact microbes into the endocytic pathway may occur by receptor-mediated endocytosis, phagocytosis, or nonspecific fluid-phase endocytosis. It has been hypothesized that TLR signaling may regulate the phagocytic pathway to improve antigen presentation and host defense (53). TLRs are also able to recognize PAMPs in the extracellular space and endosomes (54).

The signals from TLRs via the signaling adaptor protein MyD88 and the MAPK p38) activate fusion of nascent phagosomes with endosomes and lysosomes. The low pH of the phagolysosome, created after fusion of the phagosome with the lysosome, causes the fragmentation of the bacteria into small pieces (proteolysis). The phagolysosome containing pathogen-derived proteins fuse with the MHC bearing endosome allowing bacterial peptides to bind to the groove of the MHC class II molecule. MHC-II-peptide loaded endocytic compartments change morphology from vacuolar to tubular, and is transported to the area of the plasma membrane where contact with the TCR on $CD4^+$ T cells occurs. Only phagosomes containing TLR ligand can mediate the presentation of antigens. Apoptotic cells which do not involve TLR signaling (their phagosomes mature into different compartments) are not receptive to MHC-II presentation. This mechanism allows APCs to distinguish between phagosomes with self (non-stimulating) antigens and non-self (stimulating) antigens (53, 55).

APCs activated by microbial components via pattern-recognition receptors such as TLRs secrete a precise set of cytokines that attract T cells and induce a specific T cell response (T helper type 1 (Th_1), T helper type 2 (Th_2), T follicular helper (T_{FH})). Thus, APCs are a bridge between innate and adaptive immunity (56). The antigen is taken up by dendritic cells at the site of immunization and, within a few days, is transported to the peripheral lymphoid organs such as spleen or lymph nodes. The activation of the B cell receptor (BCR) by a T cell-dependent antigen provides further activation signals triggering the formation of the germinal centers (GC) within lymph nodes and the spleen. The maturation, proliferation, and differentiation of B cells occurs within GCs (57).

T cells develop in the thymus by undergoing positive and negative selection which results in naïve CD4 and CD8 T cells population displaying distinctive T-cell surface receptor (TCR). The TCR is composed of two membrane-anchored polypeptides (α and β), and each contains one constant (C) and one variable (V) domain. The variable domain-containing complementarity determining regions (CDRs) recognizes the peptide:MHC complex and delivers activation signals to the B cell. However, first, T cells by themselves require antigen-induced three-step activation (Figure 3). The initial binding between a TCR and the antigen-MHC is called the first signal. The second signal comes from co-stimulatory molecules, e.g., in a case of T helper, CD28 costimulatory ligand which binds to one of two receptors on the APC – B7.1 (CD80) or B7.2 (CD86. The third signal comes from released cytokines, which determine the functional subclasses of T cells, like T_{FH} or Th_1**,** Th_2**,** or Th_{17} with different cytokine profiles and functions. Activated T cells synthesize both cell-bound and secreted effector molecules that synergize in the B cell activation (58).

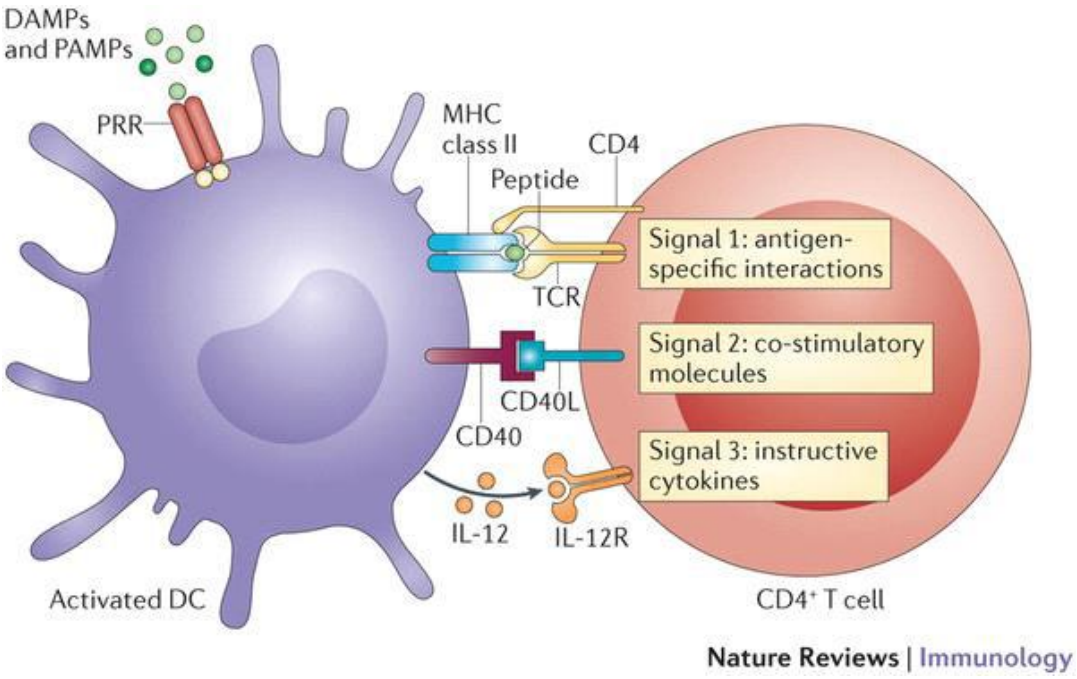

Figure 3. Naïve T cell activation.
The activation of a naive T cell requires communication with a professional APC which provides multiple signals. CD40L, CD40 ligand; DAMP, damage-associated molecular pattern; IL-12, interleukin-12; IL-12R, IL-12 receptor; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor. Adopted from *Kambayashi et.al*., 2104 (59)

B cell development and rearrangement of B cell immunoglobulin genes occurs in the bone marrow. The immature B cells with the surface-bound B cell receptor (BCR) of the IgM isotype first go through negative selection. The self-antigen recognizing B cells are removed from the cell's repertoire by apoptosis. The immature B cells continue to differentiate into transitional and mature B cells before and after they travel to the spleen (60). In a light zone of the spleen positive selection takes place. B cells that are able to interact with an antigen carry by T_{FH} cells are positively selected into the peripheral B cell compartment (57).

The BCR on a surface of the B cell can recognize both soluble and membrane-bound antigens, and this interaction provides the first signal for cell activation. The second co-stimulation signal is typically provided by T_{FH} cells in germinal centers of the spleen, where the B cells are clustered. CD40 ligand on T_{FH} interacts with the CD40 receptor on the B cell, which subsequently activates immature B cells. This contact induces polyclonal B cell proliferation, antibody affinity maturation, IgG class switching, and formation of memory B cells (61).

Activated B cells leave the GCs as high-affinity plasma and memory B cells. Plasma B cells are able to secrete antigen-binding antibodies for several weeks after activation. Memory B cells circulate throughout the body with high-affinity BCRs ready for quick response to the antigen and stopping the infection (62).

1.2.4 T cell-independent (T_I) carbohydrate antigens

Activation of B cells without the assistance of helper T cells is called T cell-independent activation (T_I) and occurs when BCRs interact with T-independent antigens, such as a polysaccharide of high molecular weight (T_I-2) or lipopolysaccharide (T_I-1). T_I-1, in comparison to T_I-2, can induce neonatal B cells and low-affinity antibody production in children below two years of age (3, 63). It was shown that CPSs are taken up by APC, engulfed into endosomes and fragmented into smaller carbohydrates by oxidative agents such as reactive oxygen species (ROS) and reactive nitrogen species. T cells are not able to respond to carbohydrates due to failure of these molecules to bind MHC class II, not to T cell inability to recognize presented glycans (64). Carbohydrates induce low- affinity IgM response. The lack of antibody class switching (from IgM to IgG), booster antibody response (no rapid increase in IgG titer after repeated contact with the antigen) and formation of memory T cells are typical features of the T cell-independent immune response (65, 66). The structure of capsular polysaccharides that contain many repeating units allows for cross-linking of multiple BCRs, providing the first signal for B cell activation. The second signal usually comes from other sources, such as interactions of Toll-like receptors with PAMPs, without the involvement of T cells (Figure 4a).

1.2.5 T cell-dependent (T_D) carbohydrate antigen

Zwitterionic polysaccharides (ZPS), containing both positive and negative charges in each repeating unit, are unique carbohydrates that can activate the T cell-dependent immune response. They are produced by *Bacteroides fragilis*, *Streptococcus pneumoniae* serotype 1, or *Staphylococcus aureus* type 5 and 8 (67). After the recognition of the antigen by APCs, ZPS is processed into smaller fragments and displayed on MHC class II through the electrostatic interaction with the peptide-binding groove. Thereby, a synapse with TCR on CD4+ T cells is

formed, leading to their activation and cytokine production (Figure 4b) (65, 66, 68). ZPS can activate adaptive immune responses in the absence of a carrier protein (69), leading to the possibility of carbohydrate only vaccines (70).

1.2.6 T cell-dependent recognition of glycoconjugate vaccines

Glycoconjugate vaccines contain bacterial capsular polysaccharide (CPS) chemically coupled to the T cell-dependent carrier protein or peptide. The concept was first described by Avery and Goebel in the early 1930s. This standard practice was finally introduced in the mid-1970 after the realization that Hib and meningococcus C vaccines are not effective in young children (57, 71). The development of technology for glycoconjugate production is considered one of the major milestones in vaccinology in recent decades. Polysaccharide protein conjugate vaccines, based on isolated CPS antigens attached to carrier proteins, also protect young children and the elderly, from deadly bacterial pathogens including Haemophilus influenza type b (Hib), Neisseria meningitides, and the encapsulated Gram-positive bacterium *Streptococcus pneumoniae* (Table 2). The covalent linkage of a carbohydrate antigen to a protein enables to evoke a longlasting T-cell memory response and polysaccharide specific plasma cells. After the boosting with a polysaccharide conjugate vaccine or contact with the bacteria, the plasma cells rapidly proliferate, maturate and produce high-affinity antibodies that can eliminate the pathogen responsible for the disease and the carriage of bacteria (71, 72).

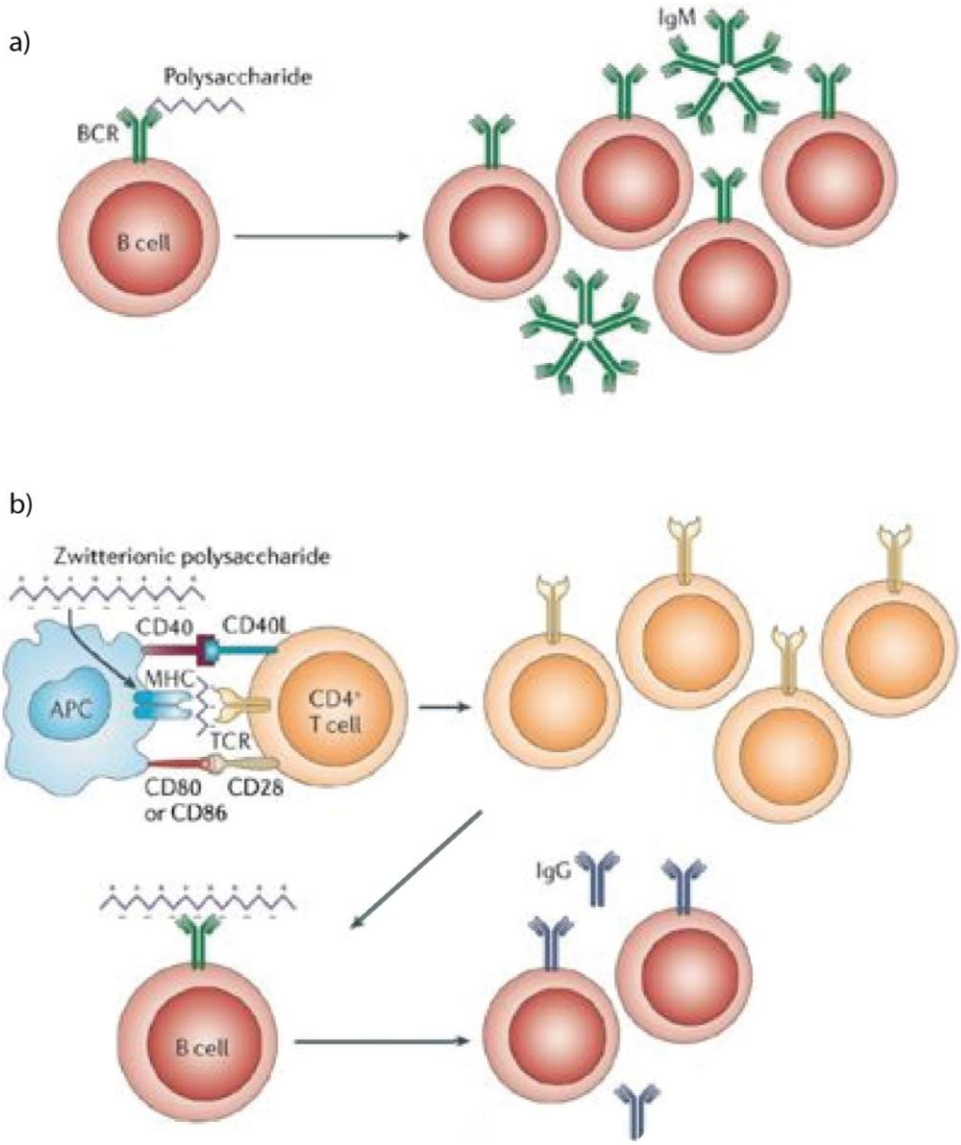

Figure 4. T cell-dependent and T cell-independent immune response to the polysaccharide. a) Bacterial polysaccharides are classic T cell-independent antigens. Interaction between the polysaccharide and the BCR induce the clonal expansion of B cells and the production of low-affinity IgM antibody but no immunological memory is generated. b) Zwitterionic polysaccharides are known to be a T cell-dependent antigen in a manner similar to protein antigens. These specific type of polysaccharides are presented to T cells, leading to their activation. Zwitterionic polysaccharides elicit high-affinity IgG production and B cell memory formation. Modified from *Mazmanian and Kasper*, 2006 (65)

Table 2. Licensed glycoconjugate vaccines.

Adapted from *Berti & Adamo* (71). TT - tetanus toxoid; OMPC - Outer membrane porin C; DT - diphtheria toxin; NTHi - Nontypeable *Haemophilus influenzae*; PD - Protein D of *Haemophilus influenzae*

Pathogen	Commercial trade name/manufacturer	Carrier protein	Saccharide chain length
Haemophilus influenzae **type B**	ActHIB/SanofiPasteur (monovalent)	TT	Native PS
	Hiberix/GSK	TT	Size-reduced PS
	Quinvaxem/GSK	CRM197	Depolymerized PS
	PedvaxHIB/Merck	OMPC	Native PS
Neisseria meningitidis **serotype C**	NeisVac-C/Pfizer	TT	Native PS
	Meningitec/Nuron Biotech	CRM197	
	Menjugate/GSK	CRM197	Depolymerized PS
	Menitorix/GSK (with Hib)	TT	Size-reduced PS
Neisseria meningitidis **serotypes CY**	MenHibrix/GSK (with Hib)	TT	
Neisseria meningitidis **serotypes ACWY**	Menactra/Sanofi Pasteur	DT	Depolymerized PS
	Menveo/GSK	CRM197	Depolymerized PS
	Nimenrix/Pfizer	TT	Size-reduced PS
Streptococcus pneumoniae **(serotypes 4, 6B, 9V, 14, 18C, 19F, 23F)**	Prevnar/Pfizer	CRM197	Native PS
Streptococcus pneumoniae **(serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F)**	Synflorix/GSK	NTHi PD, DT, TT	Size-reduced PS
Streptococcus pneumoniae **(serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F)**	Prevnar13/Pfizer	CRM197	Native PS

The classical hypothesis of immune activation by glycoconjugate states that the protein portion of the conjugate activates protein-specific CD4+ T cells to help carbohydrate specific B cells produce long-lasting IgG antibodies. Activation can be cytokine-mediated or through cognate interactions (73), suggesting that only peptide is presented to the T cells, ignoring the fact that glycan is chemically attached via a strong covalent linkage. However, the current theory strongly

indicates the contribution of glycan-specific CD4+ T cells, called Tcarb. The glycoconjugate is uptaken by APCs and depolymerized inside the endosome to the short glycan (size ~10kDa) still covalently linked to the peptide. Antigenic fragment of the polysaccharide is presented to the CD4 T cells in a context of MHC class II by the anchoring peptide from the carrier protein (Figure 5). The covalent linkage of carbohydrates to proteins in glycoconjugate vaccines is unlikely to be broken within the endosome (64, 72). It was shown that carbohydrate specific antibody levels were comparable when different carrier proteins conjugated to the glycan were used for priming and boosting. This observation suggests a similar level of B cell activation independent of the carrier (72). The glycoconjugate induces the Tcarbs that recognize the carbohydrate portion of the vaccine. However, the structure of the polysaccharide is critical for the final presentation of the antigen to $CD4^+$ T cells. Some oligosaccharides might be depolymerized into fragments too small or fragments not recognizable by TCRs and not able to trigger T cell-dependent immune response (74). A better understanding of differences in carbohydrate antigen processing, presentation, and recognition are essential factors in the design and optimization of glycoconjugate vaccines.

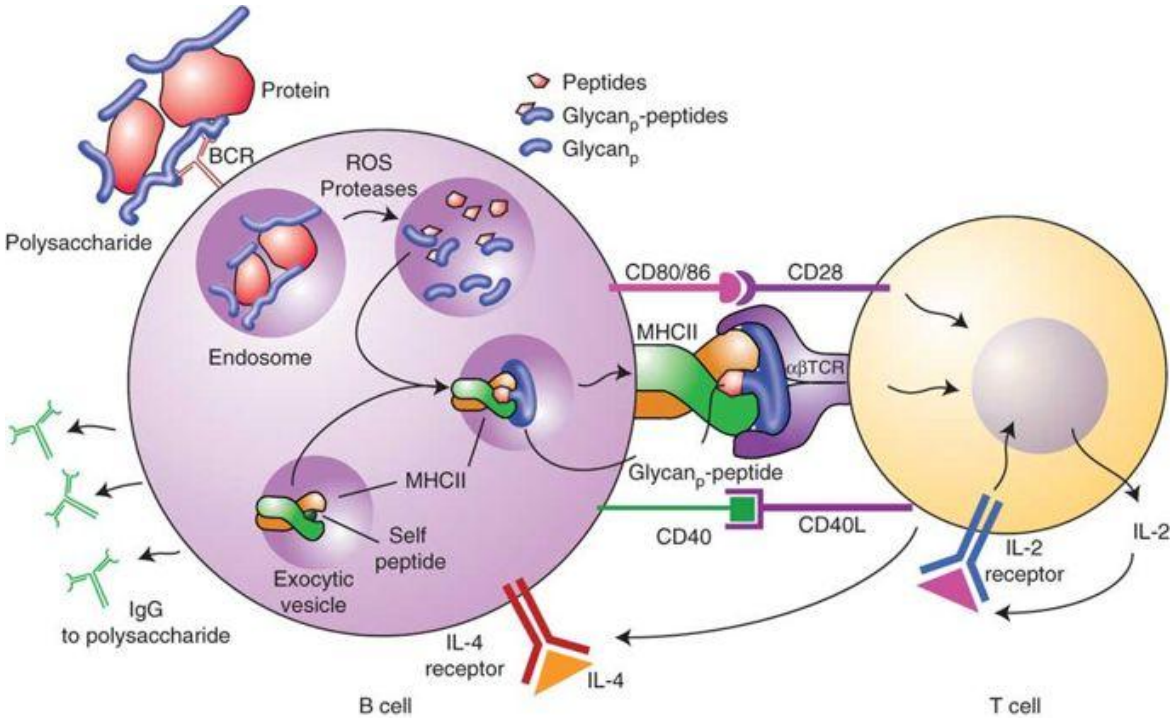

Figure 5. Processing and presentation of a carbohydrate vaccine by immune cells.

Glycoconjugate vaccine antigens allow for the glycan-specific B cell to present carrier protein peptides on MHCII to T cells, thus confers a stronger, T cell-dependent immune response, *Avci et. al*. (64).

1.2.7 Antibody production and their way of protection

Significant variations of response to glycoconjugate vaccines and following protection against bacteria have been observed between age groups (Figure 6). The antibody response of small children, which are in a high-risk infection group, fall rapidly after primary vaccination, raising the need to introduce booster immunization to maintain antibody levels. In young adults, natural nasopharyngeal colonization of encapsulated bacteria such as *S. pneumoniae*, *H.influenza B* and *N. meningitis* boost the memory immune response and play a significant role in sustaining immunity (75). This indicates that reactive memory, by itself is insufficient to protect against infection (57, 76).

In the absence of specific antibodies, capsular polysaccharide allows bacteria to evade innate host defenses by preventing complement deposition (no access to the regulators of complement activation (RCA) which are crucial determinants for complement function on self and non-self cells) on their surfaces and PRR activation (77).

Protection against encapsulated bacteria is mediated by antibodies *via* complement-mediated killing or opsonophagocytosis. The complement system can be activated mainly through three distinct ways: the classical pathway ($C1qr_2s_2$, C4 and C2), the alternative pathway (C3, factor B, factor D, and properdin), and the lectin pathway (MBL or ficolins /MASPs, C4 and C2). All of them finally lead to the ultimate goal – elimination of the target bacteria either by lysis or phagocytosis. Each of the three activation pathways leads to the formation of a C3 convertase, following the formation of the principal phagocytosis stimulating opsonins C3b and iC3b. The classical pathway requires immunoglobulins IgM and IgG for its activation. The antigen-antibody complexes containing IgG or IgM are formed, and they can be recognized by the first component of the complement system C1q. Shortly afterward, C1q triggers autoactivation of C1r, which then cleaves C1s. Active C1s cleaves C4 and C2 to a vital complex known as C3 convertase C4bC2a. The C3 convertase cleaves C3, the major complement protein in serum (78). Efficient phagocytosis of opsonized target cells is highly dependent on the recognition of the

target cell by IgG/IgM and complement components generated from C3. $C3b_2$–IgG/IgM complexes are significantly more effective than IgG antibody alone in inducing phagocytosis by professional phagocytes (neutrophils, monocytes, macrophages, DCs, osteoclasts, and eosinophils) (79, 80). Complement activation might continue with the terminal complement components (C5-C9) that accumulate and form a cell lysing membrane attack complex which kills gram-negative bacteria (e.g., *N. meningitidis* or *H.influenza* type b). However, gram-positive bacteria as *S.pneumoniae* resist the bactericidal action of C5-C9 (78, 81, 82).

Antibody-mediated opsonophagocytosis is the basic protection mechanism of the CPS-based vaccines. However, a high IgG titer is not the only factor to consider in potential vaccine efficacy. The epitope specificity, antibody affinity, avidity, subclass, and functionality have to be also evaluated (77).

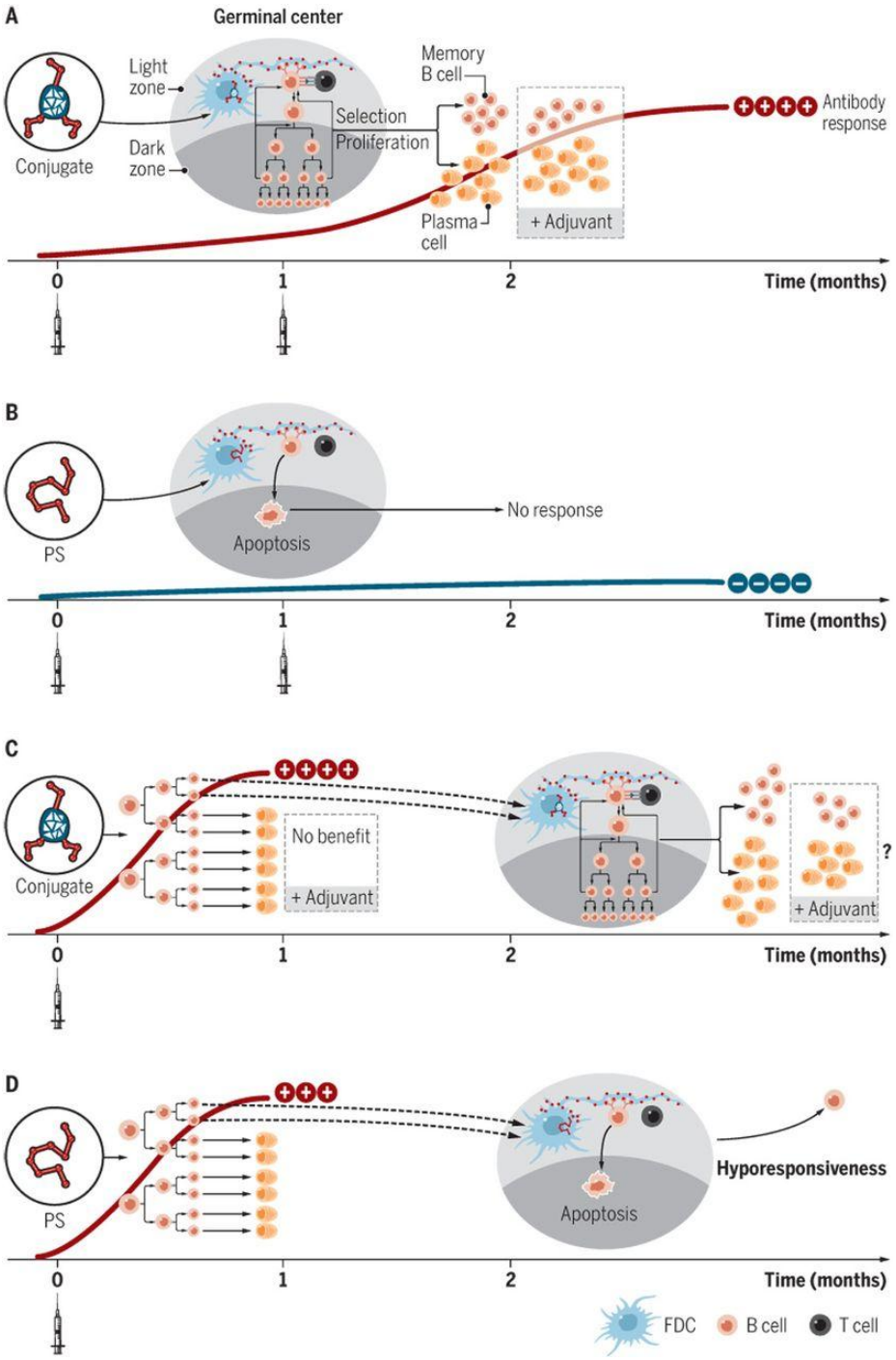

Figure 6. Mechanism of the immune response to polysaccharide and glycoconjugate vaccines in infants and adults.

Immune response to glycoconjugate differ in infants **(a)** and adults **(c)** and at least one additional boosting in children above the age of two is necessary to achieve the antibody titer equivalent to one immunization in adults. Polysaccharides in infants do not initiate proliferation of B cells but their apoptosis **(b)**. The preexisting low-affinity memory B cells in adolescents are responsible for a rapid immune response to polysaccharide already after one immunization **(d)**. (Adapted from *Rappuoli 2018*, (57)).

1.3 How To Improve Vaccine - Rational Vaccine Design

1.3.1 Commercial vaccines against *S.pneumoniae*

Immunization is the only method for preventing bacterial infections. Due to the high impact of *S. pneumoniae* on morbidity and mortality in adults and children, great efforts have been invested in reducing the rate of pneumococcal disease by vaccination over the past 30 years. The polysaccharide capsule is an essential factor responsible for pneumococcal colonization and virulence. Therefore, it is a perfect target as a vaccine antigen.

The vaccines against *S. pneumoniae* contained purified polysaccharide capsules as antigens. A first pneumococcal polysaccharide vaccine (PPSV23; Pneumovax 23®) has been developed in 1983 by an American physician Robert Austrian. PPSV23 protects against the following capsular serotypes: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. Capsular polysaccharides are purified from bacteria culture, and 25 μg of each CPS is formulated together into a polyvalent vaccine. It is recommended that all adults older than 65 years receive one dose of PPSV23. The vaccine contains T-cell independent antigen (unconjugated polysaccharide), that is why the Centers for Disease Control and Prevention (CDC) recommends immunization at least eight weeks after PCV13 administration (83). The vaccine did not generate an immune response in the group with the highest rate of pneumococcal disease burden and children younger than two years of age (84)

To protect the pneumococcal infection high-risk group and stimulate their protective immune response, vaccines containing the capsular polysaccharide conjugated to carrier proteins were developed. The first polysaccharide conjugate vaccine against *S. pneumoniae* was developed in 2000 and manufactured by Wyeth (later acquired by Pfizer) and covered 7 CPS from serotypes 4, 6B, 9V, 14, 18C, 19F and 23. Ten years later, additional serotypes 1, 3, 5, 6A, 7F, and 19A, were included to form 13- valent pneumococcal vaccine (PCV13, Prevnar13®). PCV13 is a mixture of 2.2 μg of each isolated CPS, except serotype 6B which is in the amount of 4.4 μg, conjugated to the CRM197 carrier protein. The vaccine is adsorbed onto 0.125 mg of Alum (Aluminium Phosphate). Currently, the CDC recommends that all children younger than two years of age undergo a series of Prevnar13® immunizations. All infants should be given the first

series of PCV13, at ages 2, 4, and 6 months with a booster at age 12 to 15 months (83). The WHO report from 2017 vendors all countries to adopt PCV and implement a schedule containing a minimum of three doses, which may be administered either as 3 primary doses without a booster (3p + 0) or as 2 primary doses with one booster (2p + 1). Some national immunization programs use a 3p + 1 schedule (85). Implementation of PCV13 into vaccination schedule has provided a substantial reduction of the incidence of invasive pneumococcal disease. Multisite population-based surveillance analyses revealed an overall reduction of 64% in invasive pneumococcal disease in children younger than five years of age (86). Synflorix® is another license conjugated polysaccharide vaccine. It contains ten CPS isolated from the same serotype as Prevnar13® except for serotype 3, 6A and 19A in an amount 1 μg or 3 μg. The CPSs are conjugated to either protein D from *H.influenzae*, tetanus toxoid or diphtheria toxoid and adsorbed onto 0.5 mg of Alum (Aluminium phosphate).

1.3.2 Synthetic carbohydrate vaccines

The procurement of polysaccharides for conjugate-vaccine production by the isolation of CPS from cultured bacteria is conceptionally simple but operationally challenging. Antigen heterogeneity, batch-to-batch variation, and poorly-defined conjugation to carrier proteins can be overcome when synthetic oligosaccharides are employed (87). The effective glycoconjugate vaccine QuimiHib, licensed in several countries to protect against *Haemophilus influenza* type b, is based on a synthetic oligosaccharide resulting from chemical polymerization (88). The medicinal chemistry approach to glycoconjugate vaccine development offers an alternative to CPS isolation for a variety of glycan antigens, including those for the hospital-acquired infection-causing bacteria *Clostridium difficile* and *Klebsiella pneumoniae* (89-92). Recent advances in the chemical synthesis of complex glycans including automated glycan assembly (AGA) have enabled the synthesis of a variety of diverse oligosaccharides resembling the CPS of different serotypes (93, 94). Active immunization of mice and rabbits with CRM197 conjugated with ST2 (95), ST3 (96), ST5 (97), ST8 (98) and ST14 (99, 100) elicited opsonophagocytic antibodies that were shown to be protective in infection models of the disease.

The production of effective semi-synthetic oligosaccharide-based glycoconjugate vaccines relies on the identification and synthesis of well-defined glycotopes. Glycan microarrays containing

isolated CPS as well as synthetic glycans enable the rapid screening and identification of protective glycotopes as a basis for the development of highly effective next-generation synthetic glycoconjugate vaccines. The medicinal chemistry approach has identified fragments containing disaccharides to tetrasaccharides as potential vaccine candidates (87). The immunogenicity of the CPS depends, among other factors, on rare sugars and labile functional groups (95, 97, 98). Stable synthetic oligosaccharide analogs can fix production problems such as those encountered for ST5 CPS due to the labile ketone present in the repeating unit (97).

The specific vaccine modification used here, such as synthetic antigen conjugated to the alternative carrier proteins, various adjuvant formulations and delivery system, as well as animal models, are investigated to generate relevant vaccines for clinical use. A key issue is whether the same adaptive immune response to these polysaccharides can be expected in humans (17).

1.3.3 *S.pneumoniae* serotype 3 (ST3) as an important vaccine target

S.pneumoniae serotype 3 (ST3) is an important cause of invasive pneumococcal disease, particularly pneumonia, in both children and adults (86). Serotype 3 conjugate was added to the formulation of 13-valent pneumococcal vaccine (PCV13) and is now included in the routine immunization schedule. However, this particular serotype remains a prominent cause of the invasive pneumococcal disease (IPD) in all age groups in most countries using PCV13. Many studies show no change in the incidence rate of serotype 3 after the introduction of PCV13 (Figure 7). *S. pneumoniae* serotype 3 produces a very thick mucoid capsule (101) which protects the bacteria from phagocytosis, inhibits opsonization by complement, helps to escape the neutrophil extracellular traps and prevents macrophage killing. Lack of clinical efficacy against serotype 3 after pneumococcal conjugate vaccination may be a result of reduced induction of immune memory (102). It was found that the levels of pre-existing ST3-specific antibody are negatively correlated with the B cell memory response to a booster dose of PCV13 containing ST3 glycoconjugate (103). Improvement by alternative approaches is needed to advance vaccines for this highly invasive pneumococcal serotype.

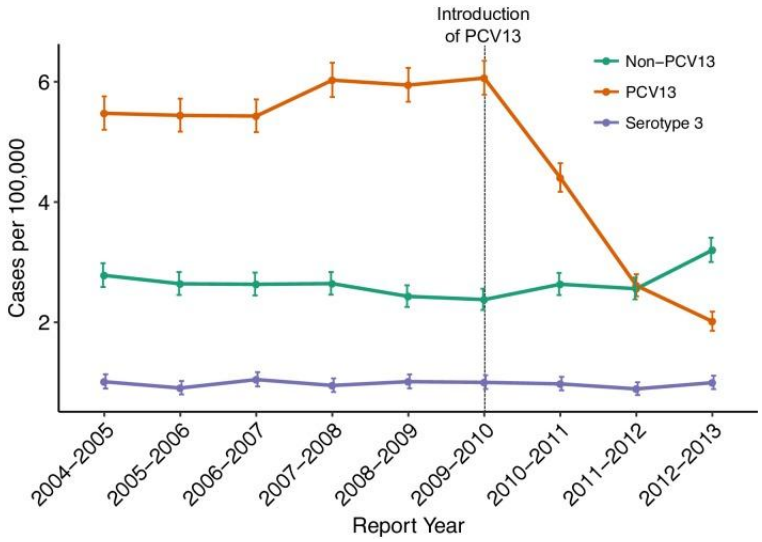

Figure 7. Changes in the incidence of invasive pneumococcal disease (IPD) caused by various *S.pneumoniae* serotypes among all ages.
The orange line represents the IPD rate caused by serotypes included in PCV13 (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F); green line represents the IPD rate for the five most common non-PCV13 serotypes; purple line the rate for serotype 3 only;. Rates of IPD expressed as cases per 100,000. (Based on Active Bacterial Core (ABC) surveillance data reported by the CDC. Adapted from *Moor et al*., 2015.

Synthetic oligosaccharides based on ST3 CPS repeating units have been shown to protect mice against lethal systemic challenge with ST3 pneumococci (104). A highly immunogenic tetrasaccharide glycotope based on the disaccharide repeating unit of *S.pneumoniae* serotype 3 was synthesized (Figure 8). The semi-synthetic oligosaccharide-based glycoconjugate vaccine candidate shows an immunoprotective effect against experimental pneumonia caused by transnasal infection with ST3 strain PN36. However, the vaccine needs further enhancement to achieve long-lived immune memory (105).

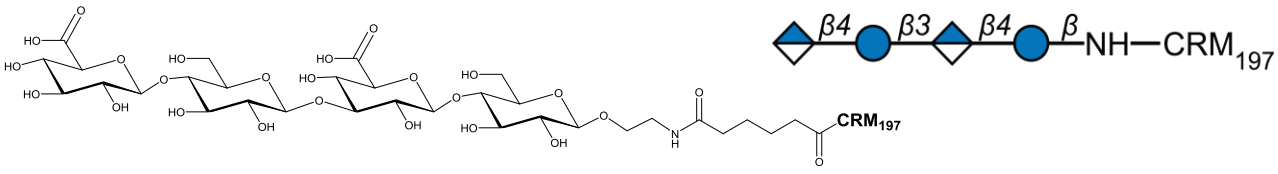

Figure 8. The synthetic repeating unit of *S.pneumoniae* serotype 3 (ST3-tetrasaccharide) conjugated to CRM197 carrier protein used as a vaccine candidate.

1.3.4 Alternative carrier proteins from *S.pneumoniae*

It is a general rule that organisms with overlapping ecological niches, such as different serotypes of *S.pneumoniae*, compete with each other for resources, resulting in multifaceted interactions and dynamics. Replacement of specific serotypes included in the vaccines by the serotypes not included occurs frequently and is called serotype replacement. Development of non-vaccine serotypes is a major challenge in controlling of pneumococcal diseases. This problem has been a major reason for intensive research in the direction of developing a 'universal' pneumococcal vaccine immunogenic in all age groups and broadly cross-protective against all serotypes. Several studies reported a protein-based serotype-independent vaccine to prevent pneumococcal infections (36). Targeting multiple membrane proteins based on their roles in bacterial pathogenicity and physiology seems to be a promising approach to achieve additive protection against pneumococci in mice (106).

The nature of the carrier protein is critical in defining the magnitude and quality of vaccine-induced immune responses, and the duration of protection. Different aspects, such as competition with anti-carrier antibodies elicited by related vaccination against tetanus and diphtheria, an overload of carrier protein or carrier-mediated epitope suppression, may influence both T- and B-cell responses to glycoconjugate vaccines. Additionally, differences in extent and persistence of protective antibodies triggered by primary vaccination with bacterial polysaccharides conjugated to various carrier proteins have been observed in infants (50, 107). Polysaccharides conjugated to a synthetic multi- or hybrid protein carrier (multiple T-cell epitopes) might be capable of broad binding to different types of MHC class II molecules resulting in the faster and stronger immune response to polysaccharides compared with licensed glycoconjugate vaccines (107). Many studies utilize highly conserved *S.pneumoniae* proteins such as pneumolysin toxoid (Ply) (108, 109) or Pneumococcal surface protein A (PspA) (110, 111) to provide broader protection against pneumococcal disease.

1.3.5 Adjuvants influence vaccine immunogenicity

Adjuvants are substances that modulate or strengthen an effective immune response against the vaccine antigens in different ways (Figure 9). They can support antigen immunogenicity by

increasing local inflammation and antigen recognition by PRR, antigen uptake by APCs, and cell migration to lymph nodes. Adjuvants select or modulate cell-mediated and humoral immunity, moderate antibody isotype, subclasses, quantity, and specificity as well as broad antibody cross-reactivity. Additionally, adjuvants can reduce the amount of antigen or the number of immunizations needed for vaccination, so-called antigen dose sparing (112-114). Modern vaccines that use better defined, or even synthetic antigens, are generally less immunogenic than crude, whole organism vaccines. Research is ongoing in advancing the efficacy of adjuvant systems for vaccines (112). Different modes of action of antigen are summarized in Table 3.

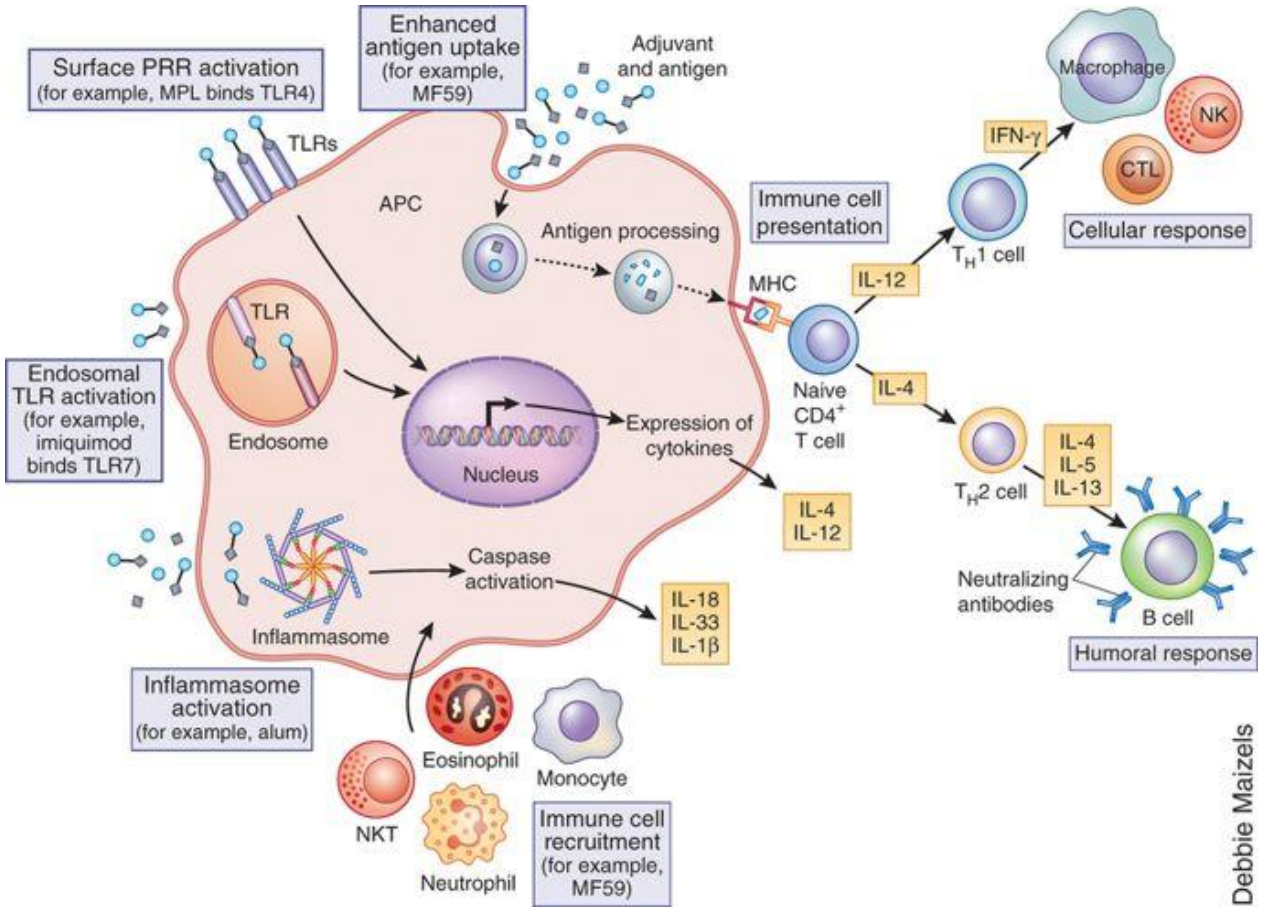

Figure 9. Mechanisms through which adjuvants mediate their activity.
Many adjuvants can act as ligands for PRRs that activate an innate immune response. Receptor signaling trigger transcription factors that induce the production of cytokines. The cytokines help to direct a specific T_h1 or T_h2 type immune response, as well as influence the immune cells that are recruited to the site of injection. Some adjuvants also influence antigen presentation by MHC molecules (MHC class I or II), affect antigen uptake, PRR signaling, inflammasome activation and recruitment of immune cells. Adapted from *Reed et al*. 2013 (115)

Table 3. Modes of action of adjuvants.

Antigen uptake	• Recruitment of APCs • The antigen recognition through pathogen recognition receptors (PRRS) • APCs maturation • Local inflammation at sites of injection (creating the local environment)
Activation of immune cells	• Efficient antigen presentation • The antigen protection
Co-stimulation	• Cytokines production
Effector messages	• Downstream modulations • Differentiation of T helper cells

1.3.5.1 Aluminum-based adjuvants

Aluminum-based mineral salts (Alum) have served as the only adjuvants for human vaccines for many years (116). They are used for a wide range of vaccines that predominantly aim to induce antibody-mediated immune responses (117). Despite its universal use, research on its mechanism of action is not completely clear (118). The antigen adsorbs onto Alum particles based on electrostatic attraction, ligand exchange (depending on substitution of surface hydroxyl group) and other mechanisms such as Van der Waals interaction or hydrophilic interaction. Several Aluminium compounds have been investigated as adjuvants, but the most popular are Aluminium Phosphate ($AlPO_4$) and Aluminium Hydroxide ($Al(OH)_3$) gels. $AlPO_4$ and $Al(OH)_3$ particles have negative and positive charges respectively so different antigens (based on their overall charge) preferentially get adsorbed onto them (119).

Several mechanisms of action of aluminum adjuvants have been proposed, such as delay in clearance from the injection site, attraction, and stimulation of cells of innate immune (through Toll-like receptors), presentation of adsorbed soluble proteins as ”particulate” antigens or improving antigen targeting towards APCs (108-110). Aluminium-based adjuvants also modulate the production of co-stimulatory molecules of Th-2 associated cytokines (IL-4, IL-10, IL-13, and possibly others), increased Ag-specific CD4+ T cell proliferation, stimulate Th_2 associated antibodies/isotypes (IgG_1 and IgE) as well as the NALP3 inflammasome leading to the release of IL-1ß and IL-18 (120-122).

1.3.5.2 Oil-in-water emulsion

Oil-in-water (O/W) emulsions have been utilized to vaccinate animals for many decades. Mineral (paraffin) oil combined with mycobacterial cells, known as Freund's adjuvant, is very well known but not approved for human purposes. MF59^{TM} (Novartis) is a new licensed O/W emulsion adjuvant already used in over 20 million individuals as a part of the influenza vaccine (123). It contains squalene, the naturally occurring triterpene hydrocarbon with a simple composition ($C_{30}H_{50}$) but a complex structure, found in many plants and animals, including humans. MF59^{TM} also contains two plants-derived nonionic surfactants, Tween 80 and Span 85, which are commonly used in a range of pharmaceutical products. (124). Two other well-known O/W licensed adjuvants are AS03 (GSK) containing squalene, Tween 80 and tocopherol, as well as AF03 (Sanofi Pasteur) with squalene, cetostearyl ether and sorbitan oleate (125).

O/W adjuvants induce a transient NF-κB, cytokine and chemokine response, increase recruitment of innate immune cells to the site of injection, as well as activate APCs loaded with the antigen and their migration to draining lymph nodes. The adjuvants also enhance recruitment of innate immune cells at the local draining lymph nodes and activate CD4+ T cells interacting with antigen-specific B cells, inducing high numbers of memory cells (126, 127). MF59^{TM} induced a vast expansion of vaccine-specific CD4 T cells but did not modify the cytokine profile of the stimulated T cells (characterized by the production of IL-2 and TNF-α). Thus, MF59^{TM} does not bias the response toward either Th_1 or Th_2 (128, 129).

1.3.5.3 Toll-like Receptors (TLRs) Ligands (TLRLs)

Toll-Like Receptors Ligands (TLRLs) are natural or synthetic molecules that bind TLRs and stimulate innate immune responses (by the production of inflammatory cytokines and type I or II interferons) which further influence the shape of adaptative B- and T-cell response. The first attenuated vaccines, a Rabies vaccine invented by Pasteur in 1885, already contained TLRL, e.g., ssRNA in Rabies vaccine activates TLR7/8; LPS and DNA in Typhoid vaccine trigger TLR4, TLR5, and TLR9, as well as lipoprotein and DNA in BCG vaccine, activates TLR2 and TLR9 (130).

1.3.5.3.1 Monophosphoryl lipid A (MPLA) – TLR4 ligand

Monophosphoryl lipid A (MPLA) is a derivative of lipopolysaccharide (LPS), the component of Gram-negative bacterial cell membranes and a strong activator of TLR4 (Figure 10). It was discovered the 1970s by Edgar Ribi who chemically modified LPS to exclude the endotoxic effect. He noticed that the product maintained desirable immunostimulatory activities and could be used as an immunomodulator. The less cytotoxic effect of MPLA was achieved by successive acid-base hydrolysis of LPS. Additionally, the number, structure, position of lipids and the degree of phosphorylation are important factors influencing the bioactivity of lipid A (131). MPLA is currently approved for use as an adjuvant in a pollen vaccine (Pollinex®) and in combination with $Al(OH)_3$ as (AS04) in human papillomavirus vaccine Ceravix™ and hepatitis B virus Fendrix™ vaccines (132). In the shingles vaccine (Shingrix®) MPLA is formulated together with purified fat-like substance and QS-21 from the bark of the Quillaja *Saponaria* tree (133).

MPLA is an effective stimulator of Th_1 cells and antibody responses. Administration of the adjuvant stimulates high serum titers of both IgG_2 and IgG_3 antibodies isotypes and increasing the surface expression of MHC and the co-stimulatory molecules on the APCs thus increasing the stimulatory properties of DCs, macrophages, and B lymphocytes (134). MPLA induced tumor necrosis factor (TNF) and interleukin-1β (IL-1β) (135).

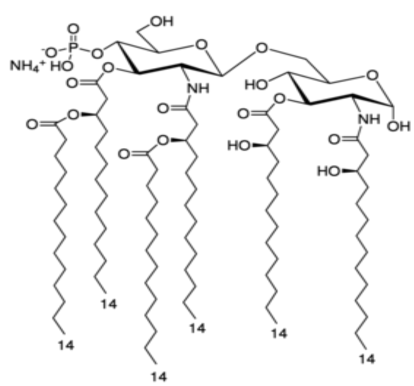

Figure 10. Monophosphoryl lipid A (MPLA) structure.

1.3.5.3.2 TLR-7/8 agonist - Resiquimod

TLR7/8 are predominantly expressed by important antigen-presenting cells, e.g., plasmacytoid DCs (pDCs) and are involved in the robust expression of IL-6, IL-12, TNF-α, and IFN-α both in humans and mice (136). It has been proven that the stimulation of IFN-α secretion is an essential driving force of TLR-mediated Th_1 immune responses and IFN-γ-dependent production of Th_1-specific IgG_2/IgG_3 (137). TLR7 and TLR8 recognize single-stranded RNA molecules (ssRNA) and are also activated by synthetic agonists, such as imidazoquinoline derivatives: Imiquimod and Resiquimod. Imiquimod was the first commercially available imidazoquinoline used for anogenital warts, actinic keratosis, and superficial basal cell carcinoma (138).

Resiquimod (RQ-848), 4-amino-2-ethoxymethyl-α,α-dimethyl-1*H*-imidazo[4,5-c]quinolin-1-ethanol, is a more potent and soluble analog of imiquimod (Figure 11). R-848 produces a 50- to 100-fold cytokine response compared to imiquimod by induction of immune cells to produce cell-mediated or Th_1 cytokines (138). Resiquimod has been known as a powerful immunostimulator for the treatment of cutaneous cancers in various clinical trials (139).

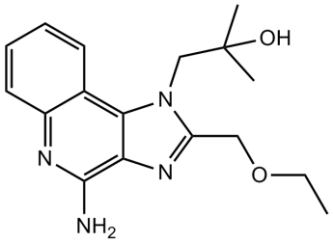

Figure 11. The structure of Resiquimod (R-848).

1.3.5.3.3 Bacterial RNA – *vita*-PAMP

The innate immune system can discriminate between selves and non-self components *via* recognition of pathogen-associated molecular patterns (PAMPs). The additional differentiation between live and dead microorganisms occurs through a uniquely associated with live microorganisms sets of PAMPs called *vita*-PAMPs. Bacterial messenger RNA (mRNA) has been recognized as one of the *vita*-PAMPs. Innate recognition of bacterial RNA occurs through endosomal TLR8 on human monocytes while both human and murine plasmacytoid DC do so through TLR7 (140). Detection of live bacteria caused the activation of the NLRP3

inflammasome complex and the subsequent release of IL-1β and increased production of type-I Interferons (IFN) (141). The idea to incorporate vita-PAMPs into vaccine formulations combines the excellent protection of live vaccines with the safety of synthetic vaccines (142).

1.3.5.3.4 TLR synergy

Pathogens express various PAMPs, and in natural infections, immune responses are amplified by multiple PAMPs presented all together on particulate pathogens (e.g., viruses or bacteria) (143). The experience with live attenuated vaccines, such as BCG, confirm the use of vaccines containing several compounds activating multiple innate pathways (50). Combining multiple PAMPs and triggering multiple PRRs at the same time, has been an essential strategy for adjuvant design in modern vaccine research. Synergic activation and TLR-TLR cross-talk can lead to enhanced cytokine secretion, more efficient T cell activation, and better antibody response (143).

Numerous studies have documented the importance of simultaneous stimulation of different PAMPs, such as TLR4 and TLR9 ligands (formulation of MPLA and CpG (130, 144)) or TLR4 and TLR7 (a nanoparticle-based vaccine combining antigen, MPL + Imiquimod (145)); which gives a much better immune response. The levels of TNF production are much greater than that observed for each of the ligands alone. In a mouse model, analysis of neonatal innate immune responses proved that the simultaneous activation of multiple TLR improves pro-inflammatory signaling and triggers DC maturation, comparable to an adult-like antigen presentation capability (50). However, PRRs may also negatively regulate each other, especially when different types of pathogens are involved (146).

1.3.6 Formulation: vaccine delivery systems

Optimal vaccine design requires both the antigen and the adjuvant to be delivered together to the appropriate immune cells. The biochemical properties of some antigens and adjuvants do not allow for their direct chemical conjugation. The co-administration of antigen and adjuvant on delivery vehicles such as nanoparticles have been intensively studied for their feasibility, varying

from sutures to bone reconstruction, and in implants as well as particles for sustained drug delivery and vaccine formulations.

There are already four vaccines in the market using delivery vehicles such as virus-like particles (VLPs) with the major immunogenic antigens on the surface. Those are two anti-hepatitis B vaccines: Engerix-B® (GSK) and RECOMBIVAX HB® (Merck) and two papillomavirus vaccines: Cervarix® (GSK) and GARDASIL® (Merck) (147).

Polylactic acid (PLA) and poly(lactic-co-glycolic acid) (PLGA) are copolymers of hydroxy acid monomers, D-lactic, L-lactic, and/or glycolic acid can entrap antigens for their efficient delivery to cells (148). PLGA or PLA have been used for encapsulation of antigens due to their homogenous distribution on the matrix (149). They form biodegradable and biocompatible platforms and have been widely approved by the US Food and Drug Administration (FDA) and European Medicines Agency (EMEA) for humans and veterinary use. The slow release of antigen and adjuvant molecules for several weeks to months prolongs and enhances antibody response to the antigen and overall stimulation of the immune system by cytokine production (147). For the formulation of particles, mostly amorphous d,l-PLGA is used, which differ in L-lactic: glycolic acid monomer ratio, molecular mass, and end-group chemistry. These parameters determine the hydrophobicity and degradation kinetics of the materials, equivalent to the microencapsulation efficiency and release rate of drugs and antigen. The hydrophobicity has a high impact on the degradation of the polymer by hydrolysis (149).

PLA/PLGA particles have been used to deliver antigens from various pathogens, such as hepatitis B virus (HBV) (150, 151), malaria protein (152), HIV antigens (153) or tumor-associated antigens (154). Additionally, the vehicles system also allows for the simultaneous use of multiple co-stimulants (137). Particles promote immunogenicity through (1) stabilization and protection of the antigen from chemical or enzymatic degradation; (2) controlled antigen release which increases antigen exposure to the professional APCs and prolong antigen presentation; (3) facilitating the antigen uptake by DCs by mimicking the size and shape of the pathogen; (4) targeted delivery through additional costimulatory molecules; (5) parallel delivery of multiple components and (6) regulation of the type of immune response (by particles size, charge and costimulatory molecules) (155-158). In general, the use of particles in the vaccine formulations

improved not only the antigen delivery (antigen depot and targeting APCs) but might also act as an adjuvant.

1.4 Objectives of this thesis

High mortality rates of bacterial pneumonia and increased antibiotic resistance are major reasons to develop novel vaccine strategies against *Streptococcus pneumoniae.* An ideal vaccine has to be affordable, broadly available, easy to administer and induces long-lived protection. This requires activation of both innate and adaptive immunity. Vaccine efficiency depends on the quality and way of recognition of the antigen, which is in the case of carbohydrate vaccines associated with the antigen characteristic, carrier protein, or proper adjuvant formulation.

S. pneumoniae serotype 3 (ST3) is one of the most frequent serotypes isolated from patients with invasive pneumococcal diseases, even though it is included in the routine immunization schedule. Preliminary results from our group showed protective effects of a vaccine candidate composed of ST3-tetrasaccharide the synthetic repeating unit of the *S. pneumoniae* serotype 3 capsular polysaccharide conjugated to the CRM197 carrier protein in mice. However, induction of long-term immunity required optimization (105).

The first step taken to improve the semisynthetic ST3-tetrasaccharide conjugated vaccine was to optimize the dose of antigen. The previous study from Seeberger group immunized mice with a dose of conjugate equivalent to 5 μg of tetrasaccharide (96) while dosing for the human is 2.2 μg of CPS. Taking into account the size of the human and mouse model and the high purity of the oligosaccharide, the antigen dose was extremely high. To avoid vaccine overload and weakening the immune system, the ST3-tetrasaccharide amount was profoundly decreased.

The immunization against pneumococcal polysaccharides can lead to the replacement of vaccine-type pneumococcal serotypes with new pathogenic serotypes (159). Due to the large serotype diversity and serotype-replacement, efforts have been undertaken to develop alternative pneumococcal vaccine candidates. Membrane proteins originating from the same pathogen can induce an additional response, so-called “additional valency”. Conserved among all *S.pneumoaniae* serotypes pneumolysin and PspA were chosen as a proof-of-concept to broaden the spectrum of protection of the pneumococcal vaccine.

It was proven that an adjuvant can significantly change the immune response induced by the vaccine. The selective activation of cell-mediated/humoral and T_h1/T_h2 immunity is possible by the adjustment of the adjuvant formulation. In the thesis, different adjuvant formulations were

used to improve the efficiency of the semisynthetic ST3-tetrasaccharide conjugate vaccine. The aluminum-based adjuvant used in Prevnar13® induces Th_2-balanced response. Comparison of Th_2 adjuvants Alum and water-in-oil squalene-based emulsion MF59™ was investigated. A separate group of TLR ligands-based adjuvants selectively induce innate signals that promote Th_1 and T_{FH} differentiation and humoral immunity. Encapsulation of Toll-like receptors (TLRs) ligands, such as MPLA, Reiquimod, and PAMPs (bacterial mRNA) in poly(lactic acid) particles (PLGA), are used to improve the immunoprotective effect of the vaccine. The particles might also serve as a delivery system for the vaccine. The *in-vivo* mouse model (C57BL/6 mice) was employed to study different formulations of ST3-tetrasaccharide vaccine and long term immunity directed against bacterial pneumonia.

The mouse model has been unquestionably the most widely used animal model for research mostly due to easy handling and simple genetic manipulation of rodents. However, the suitability of the models to actually human physiology and disease is not optimal. Substantial analogies between human and porcine physiology and especially a strong overlap in the immune system including expression and function of TLR makes domestic pigs an increasingly interesting model animal to obtain reliable pre-clinical data. Drastically increasing rates of antibiotic resistance and frequent emergence of veterinary pathogens in industrial animal farming further add to the relevancy of this animal model as a relevant target population for vaccine studies. In this work, for the first time, the swine model has been employed in the field of the synthetic carbohydrate vaccine development.

Work presented in this thesis follows the pipeline of vaccine development (Figure 12). Glycan arrays containing well-defined synthetic glycans are used as the main platform for the high throughput screening of various serum samples and identification of protective glycotopes.

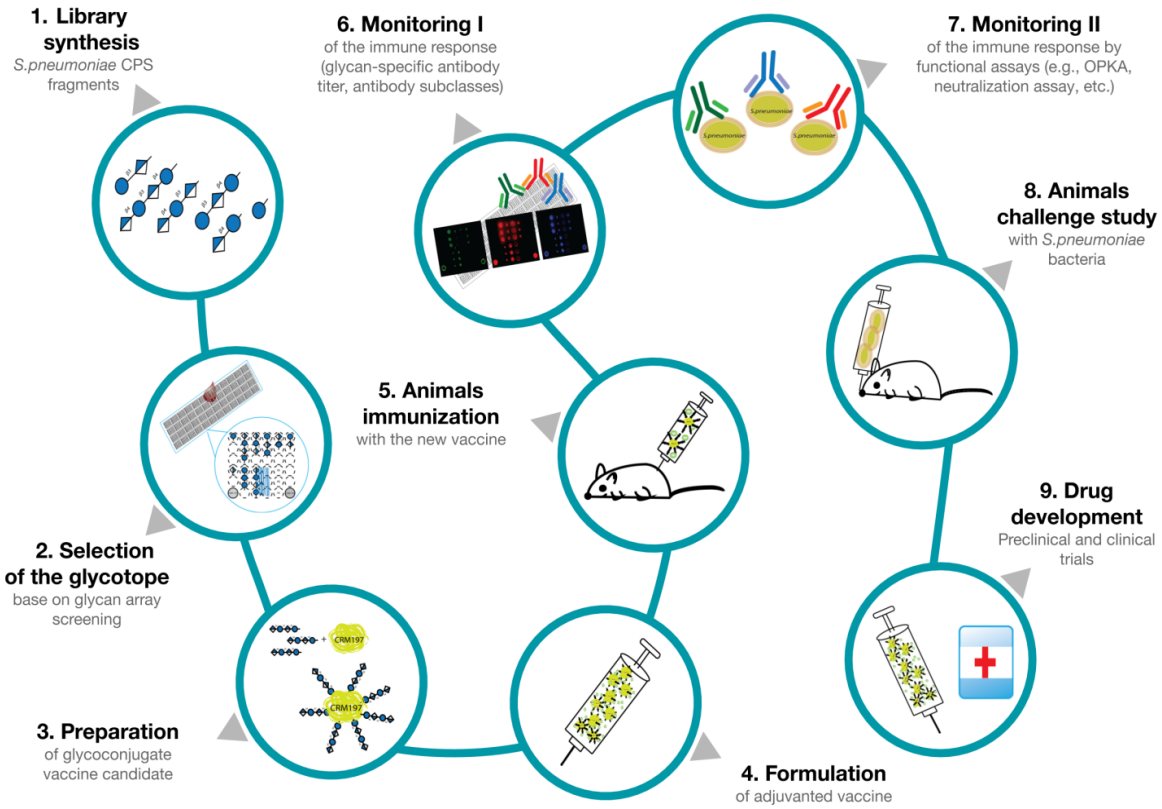

Figure 12. *Streptococcus pneumoniae* semisynthetic glycoconjugate vaccine development pipeline.

2 Experimental Section

2.1 Immunization

Mice were treated according to German (Tierschutz-Versuchstierverordnung) and European Law (Directive 2010/63/EU). Recommendations of the Society for Laboratory Animal Science (GV-SOLAS) and of the Federation of European Laboratory Animal Science Associations (FELASA) were followed. The permits used in the study (Permit Number: G0135/14, A0103/12, H0184/09) were approved by the Office for Health and Social Affairs Berlin (LAGeSo). Animal suffering was minimized, and mice were euthanized as necessary according to humane experimental endpoints.

Mice were housed in individually-ventilated cages under specific pathogen-free conditions in the animal facility of the Federal Institute for Risk Assessment (BfR, Berlin, Germany). Mice were provided with food and water *ad libitum*. Mice used for immunizations were female C57BL/6. Mice were either from in-breed strains at the BfR animal facility or sourced from Janvier Labs (Saint-Berthevin, France) and Charles River (Sulzfeld, Germany).

Mice were immunized subcutaneously (s.c.) with an amount of conjugate corresponding to a particular amount of glycan antigen per injection. The glycoconjugates, together with an adjuvant, were diluted in sterile PBS to a final volume of 100 µL per dose (formulation depends on adjuvant used). The animals were immunized on day 0 (primary immunization) and boosted with the same formulation on days 14, 28, and final boosting depending on the character of an experiment (see a general timeline on Figure 13 and Table 4).

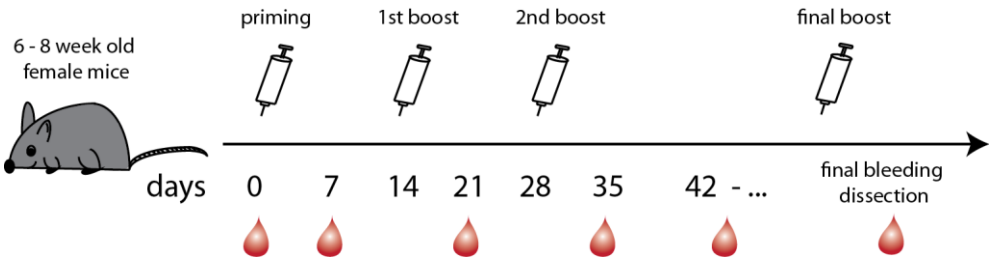

Figure 13. General immunization regime for glycoconjugate vaccine development study. Six to eight week old female C57Bl/6 mice were s.c. immunized with a final volume of 100 µL of glycoconjugate vaccine in PBS according to the prime + boost + boost + final boost schedule.

2.2 Sera collection

To obtain sera, blood was collected either by cutting the tail-tip or through mandibular bleeding via facial vein puncture with 4-5 mm lancet (Goldenrod Animal Lancet) according to the general schedule (shown in Table 4). Three to four drops of blood (a total of about 60-80 µL) were collected, and the puncture site was immediately compressed with a cotton swab for a few seconds. The blood was left to coagulate for at least 30 min at RT and then centrifuged for 15 minutes at 2000 rcf to pellet the blood cells and separate the serum. The serum was aspirated, transferred to the sterile Eppendorf vials and an additional centrifugation step was repeated to clean the serum from remaining blood cells. The serum was frozen at -20°C until further use.

Table 4. A general timeline of the immunization regime.

Week	Procedure
0	Bleeding, prime immunization
1	Bleeding
2	Bleeding, 1st boost
3	Bleeding
4	Bleeding; 2nd boost
5	Bleeding
6-20	Bleeding every 2-4 weeks
22	Bleeding, final boosting
23	Bleeding
25	Experiment termination: final bleeding/dissection

2.3 Antigen

2.3.1 ST3-tetrasaccharide conjugation to a CRM197 carrier protein

S.pneumoniae tetrasaccharide (ST3-tetrasaccharide) **(1)** and bis (4-nitrophenyl) adipate **(2)** were combined at 1 to 6.5 equivalent in DMSO and pyridine in v/v ratio 1.6 (Figure 13). Then 180 equivalents of triethylamine were added, and the solution was stirred for three hours at room temperature. The resulting solution was frozen in liquid nitrogen and subsequently lyophilized to give a crude white solid. Excess bis (4-nitrophenyl) adipate was removed by washing the crude solid with chloroform (3 x 1 mL) and dichloromethane (3 x 1 mL) until no more bis (4-nitrophenyl) adipate was observed by the Thin-layer Chromatography (TLC). The resulting compound (3) was taken forward to the next reaction without additional purification. To a pre-weighed vial of CRM197 (4; Pfenex, California, USA) was added autoclaved water (750 µL). The solution was transferred to an Amicon 10K filter and centrifuged for 8 min at 10,000 rpm. The filtrate was discarded, and the remaining solution was added to the filter and centrifuged for 8 min at 10,000 rpm. The CRM197 vial was washed with autoclaved water (~350 µL), transferred to the filter and centrifuged for 8 min at 10,000 rpm. The CRM197 vial was washed with phosphate buffer (350 µL, pH 8) **(4)**, transferred to the filter and centrifuged for 8 min at 10,000 rpm. Subsequently, the filter was removed, turned upside down into a clean vial and centrifuged for 2 min at 1,000 rpm, giving a colorless filtrate containing CRM197. The filtrate was added to a vial containing the tetrasaccharide-linker construct **(3)**. The resulting solution **(5)** was stirred for 18 h, after which the solution was transferred to an Amicon 10K filter and washed with sodium phosphate buffer pH 8 (2 x 400 µL). The sample was centrifuged for 8 min at 10,000 rpm, followed by additional washing with autoclaved water (3 x 400 µL). Prior to the third wash, autoclaved water (200 µL) was added to the filter with thorough mixing. A small sample (10 µL) was taken for MALDI-TOF-MS analysis. Autoclaved water (200 µL) was added to the filter and centrifuged for 8 min at 10,000 rpm. The sample was washed with PBS (400 µL). Afterward, the filter was removed and turned upside down into a clean vial and centrifuged for 2 min at 1,000 rpm. The filtrate was diluted with PBS (350 µL) and stored at 5 °C.

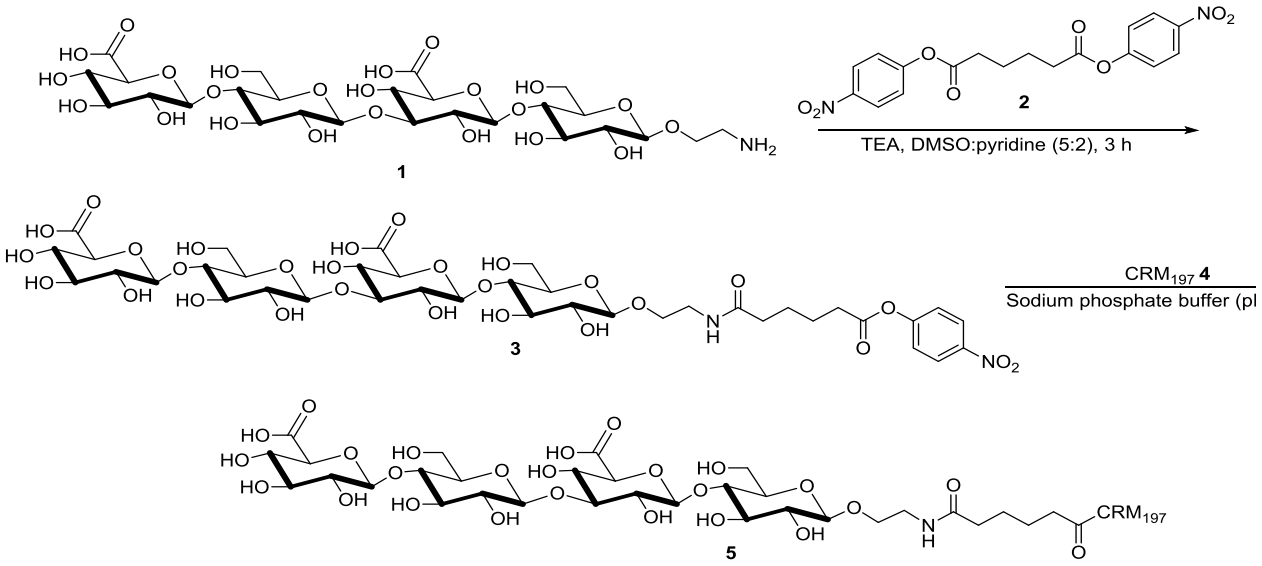

Figure 14. The ST3-tetrasaccharide and CRM197 conjugation reaction.
ST3-tetrasaccharide was covalently conjugated with CRM197 using the p-nitrophenyl adipate ester as a coupling reagent**.**

2.3.2 Conjugate characterization

2.3.2.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis of glycoconjugates was performed according to the standard protocol. Discontinuous SDS-PAGE was prepared according to Lämmli's protocol, using a MiniProtean system (Bio-Rad, Hercules, USA). An alkaline separating gel (375 mM Tris/HCl pH 8.8, 10 to 12% (w/v) of a 29:1 acrylamide/*N*,*N'*-methylenebisacrylamide mixture) and an acidic stacking gel (100 mM Tris/HCl pH 6.8, 4.5% (w/v) of a 29:1 acrylamide/*N*,*N'*-methylenebisacrylamide mixture), polymerized by the addition of TEMED and 10% (w/v) ammonium peroxodisulfate, were used. The glycoconjugates samples were dissolved in SDS-PAGE loading buffer (200 mM Tris-Cl (pH 6.8), 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% (v/v) glycerol). An amount of 1-2 μg of glycoconjugate and CRM197 (as a positive control) was loaded per lane and PageRuler Plus Prestained Protein Ladder 10 to 250 kDa

(Thermo Scientific) was used as a size marker (2 μL per lane). All samples were run at 120 V and 25 mA for 90 min and stained with 0.5 % (w/v) Coomassie Brilliant Blue R-250 in 50 % (v/v) methanol and 10 % (v/v) acetic acid for 30 min. Stained gels were destained with 50 % (v/v) methanol and 10 % (v/v) acetic acid.

2.3.2.2 Matrix-assisted laser desorption/ionization with time-of-flight detection mass spectrometry MALDI-TOF MS.

Mass spectra were acquired with an Autoflex Speed MALDI-TOF system (Bruker Daltonics; Bremen, Germany). Samples were spotted using the dried droplet technique with 2,5-dihydroxyacetophenone (DHAP) as matrix on MTP 384 ground steel target plates (Bruker Daltonics). Samples were prepared by mixing protein sample with DHAP matrix and 2% (v/v) trifluoroacetic acid (TFA) before spotting. The mass spectrometer was operated in linear positive mode. Mass spectra were acquired over an m/z range from 30,000 to 210,000 and data was analyzed with the FlexAnalysis software provided with the instrument.

2.4 Dose-dependent study

2.4.1 Vaccine preparation and immunization

An amount of conjugate corresponding to a dose of ST3-tetrasaccharide antigen from 2.2 μg to 0.01 μg per injection was mixed with 125 μg Aluminum Hydroxide ($Al(OH)_3$) from Alum gel (Alhydrogel, Brenntag, Denmark) in total volume of 100 μL of phosphate buffer (PBS pH 7.4, PAN-Biotech, Germany). The ST3-tetrasaccharide CRM197 conjugate was provided by Vaxxilon (Vaxxilon Deutschland GmbH, Berlin, Germany). Different antigen doses were calculated based on a glycan:protein ratio evaluated by MALDI-TOF-MS (Figure 15). The vaccine was rotated overnight at 4°C. The conjugates were filter sterilized using 0.22 μm small volume syringe filters. Prior to the formulation, the adjuvant was aliquoted and kept sterile, every time a fresh aliquot was used.

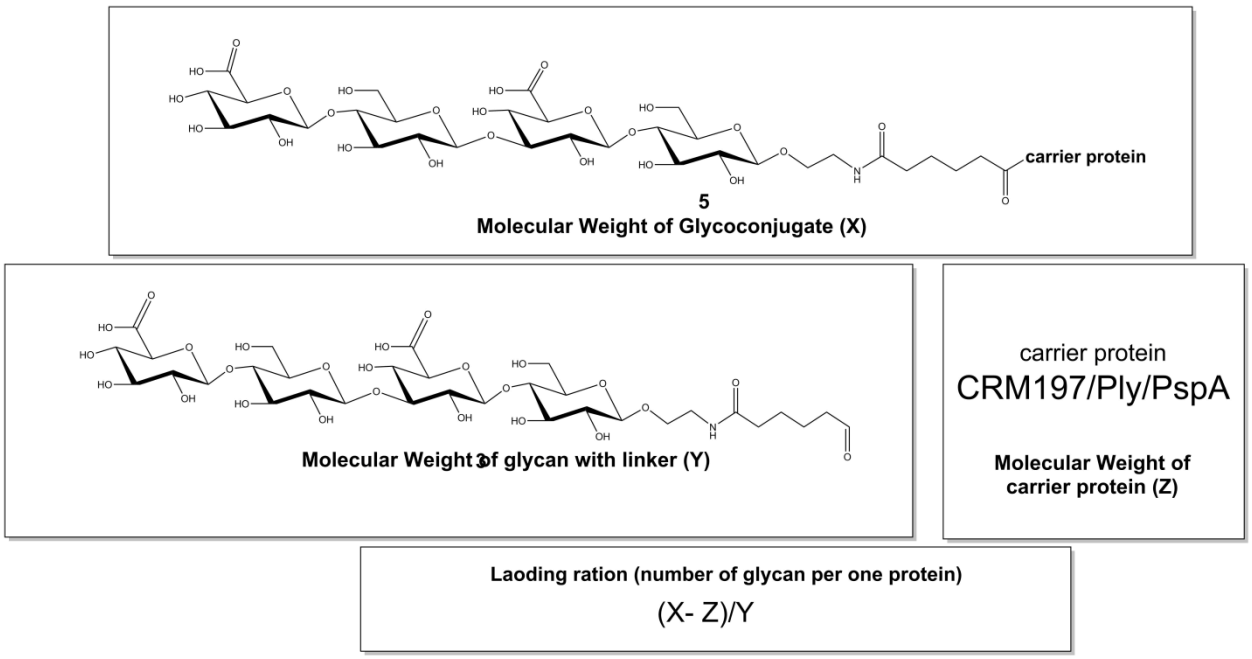

Figure 15. Calculation of glycan loading ration per protein base on the mass of compound evaluated by MALDI-TOF-MS.

Mice (six animals per group) were immunized with 100 µL of glycoconjugate vaccines containing different amounts of SP3-tetrasaccharide antigen listed in Table 5. A total of four injections, prime + boost + boost + final boost regime, were administered s.c. in order to generate the antigen-specific antibody response.

Table 5. Experimental groups for the dose-dependent study.

Group	ST3-tetrasaccharide dose	Adjuvant Aluminum Hydroxide	Vaccine preparation
1	2.2 µg	125 µg	Adjuvant/glycoconjugate preparation just before injection, 4°C, rotation O/N
2	0.4 µg	125 µg	Adjuvant/glycoconjugate preparation just before injection, 4°C, rotation O/N
3	0.1 µg	125 µg	Adjuvant/glycoconjugate preparation just before injection, 4°C, rotation O/N
4	0.1 µg	125 µg	Adjuvant/glycoconjugate – the same batch for all injections, 4°C, rotation O/N, aliquots were stored at 4°C
5	0.05 µg	125 µg	Adjuvant/antigen preparation just before injection, 4°C, rotation O/N
6	0.01 µg	125 µg	Adjuvant/antigen preparation just before injection, 4°C, rotation O/N

2.4.2 Determination of non-adsorbed antigen on Alum particles - the Bicinchoninic acid assay (BCA assay)

The adsorption of antigen was evaluated by determination of the free glycoprotein level in the supernatant by the Bicinchoninic Acid assay (BCA assay) performed according to the instruction included into Micro BCA Protein Assay Kit (Thermo Fisher Scientific GmbH, Berlin, Germany). The vaccines were prepared as mentioned above (2.4.1) and rotated overnight at 4°C to allow adsorption of the antigen on alum. The samples were centrifuged for 10 mins at 3000 x g, and non-adsorbed supernatants were carefully transferred to new Eppendorf tubes. No-absorbed protein concentrations in the supernatants and control samples without adjuvant were analyzed immediately by BCA assay. Briefly, the round bottom non-treated 96 wells plate (Costar 3788, Sigma Aldrich, St. Luis, USA) was used for the assay. Samples and a calibration curve (CRM197 or Albumin standard at concentration of 1 mg/mL, 0.8 mg/mL, 0.6 mg/mL, 0.4 mg/mL, 0.2 mg/mL, 0.1 mg/mL) were added to the respective wells. The BCA Working Reagent

(WR) containing reagent B + C + A in ratio 24:1:25 was prepared and mixed with each control or samples well at ratio 1: 20 (sample to WR). The plate was incubated at 37°C for 30 min in the dark while shaking. The absorbance was measured at 562 nm. The percentage of non-adsorbed protein was calculated relative to control without adjuvant, as % adsorbed = 100% − % non-adsorbed. All samples were measured in triplicates.

2.4.3 Glycan array serum screening

Individual synthetic oligosaccharide fragments of *S.pneumoniae* CPS, native CPS and related proteins were dissolved in sterile printing buffer (50 mM sodium phosphate buffer, NaPi, pH 8.5) to the final concentration of 100 – 200 μM (volume around 20-50 μL). Compounds were spotted onto CodeLink N-hydroxysuccinimide-activated glass slides (SurModics Inc., Eden Prairie, USA) in two or three replicates using a contact-free piezoelectric microarray spotter (Scienion, Berlin, Germany). After the spotting was finished, slides were incubated for 16 to 24h in a humidity box at room temperature to allow the completion of the coupling reaction and quenched for 1 h at room temperature (using 100 mM ethanolamine in 0.1 M NaPi pH 9) to suppress the free reactive groups on the microarray surface. The slide was washed with water ddH_2O, dried by centrifugation (5 min at 300x g) and stored in at 4°C until use.

Directly before the assay, the slide was blocked with the blocking buffer (1% (w/v) BSA in PBS) for one hour at room temperature or overnight at 4°C. A FlexWell grid (FlexWell 64, Grace Bio-Labs, Bend, US) was attached and 20-40 μL of the serum samples were applied into wells. The slide with serum samples was incubated for 1h at 37°C in a light-protected humidified box. The microarray was washed three times by applying 50 μL of washing buffer (PBS + 0.1% Tween-20) into every well. The secondary fluorescently labeled antibodies diluted in sample buffer (1% BSA (w/v) in PBS) were added (see secondary antibodies dilution in Table 6)

) and incubated for 1h at 37°C. The slide was washed three times with 50 µL PBS + 0.1% Tween-20 and ddH_2O and dried by centrifugation (5 min at 300x g)

The fluorescence read-out was performed using an Axon GenePix 4300A microarray scanner and GenePix Pro 7 software (Molecular Devices, Sunnyvale, CA, USA). Image analysis was carried out with the GenePix Pro 8 software (Graphpad Software Inc., La Jolla, USA). The photomultiplier tube (PMT) voltage was adjusted such that scans were free of saturation signals.

Table 6. Fluorescently labeled antibodies used in study

Antibody	Provider	Catalog number	Dilution
Anti-mouse IgG (H+L) FITC	Life Technologies, Carlsbad, CA, USA	A-31574	1:400
Anti-mouse IgG1 Alexa Fluor® 594	Life Technologies, Carlsbad, CA, USA	A-21125	1:400
Anti-mouse IgG2a (γ2a) Alexa Fluor® 647	Life Technologies, Carlsbad, CA, USA	A-21241	1:400
Anti-mouse IgG3 (γ3) Alexa Fluor® 488	Life Technologies, Carlsbad, CA, USA	A-21151	1:200
Anti-mouse IgM (µ chain) Alexa Fluor® 546	Life Technologies, Carlsbad, CA, USA	A-21045	1:200

2.4.4 Enzyme-Linked Immunosorbent Assay - ELISA

ELISA was performed using high-binding 96-well polystyrene microtiter plates (Corning, USA) coated with different CPSs (SSI Diagnostica, Kopenhagen) at a concentration 10 µg/mL (50 µL per well) in PBS (overnight incubation at 4°C). The plates were washed three times with PBS + 0.1% Tween-20 and blocked with 1% BSA-PBS at RT for 1 h. After three washing steps with PBS + 0.1% Tween-20, plates were incubated with serial dilutions of serum in duplicate or triplicate for 1 h at 37°C. The plates were washed with PBS + 0.1% Tween-20 and treated with horseradish peroxidase (HRP)-labeled secondary antibody diluted accordingly to Table 7 in 1% BSA-PBS followed by incubation for 1 h at 37°C. The plates were washed three times with PBS + 0.1% Tween-20 and the color was developed using HRP substrate 3,3',5,5'-tetramethylbenzidine (TMB substrate; BD Biosciences, San Jose, USA). The reaction was stopped by quenching with 2% H_2SO_4. The absorbance was recorded at 450 nm using a standard ELISA plate reader.

Table 7. Enzyme-linked antibodies used for ELISA

Antibody	Provider	Catalog number	Dilution
Anti-mouse IgG (Fc-specyfic) HRP	Dianova, Hamburg, Germany	115-035-164	1:10,000
Anti-mouse IgM (μ chain) HRP	Life Technologies, Carlsbad, CA, USA	M-31507	1:3000

2.4.5 *In-Vitro* Opsonophagocytic Killing Assay – OPKA

The *in-vitro* opsonophagocytic killing assay was performed as described previously by *Romero-Steiner et al.* (160). Briefly, the effector HL-60 cells (a human origin leukemia cell line) at the concentration of approximately 4×10^5 cells/mL in a complete RPMI cell culture medium (90% RPMI 1640, 10% FCS, 1 mM L-glutamine and penicillin-streptomycin solution; PAN Biotech, Germany) were differentiated with 0.8% Dimethylformamide (DMF; 99.8% purity; Fisher Scientific, Fair Lawn, N.J., USA) for 5-6 days at 37°C in the presence of 5% CO_2. After differentiation, the cells were harvested by centrifugation (300 × g, 5 min) and then viable cells were counted by using 1% trypan blue exclusion and resuspended in opsonophagocytic buffer (HBSS with Ca^{2+} and Mg^{2+}, 0.1% gelatin, and 10% FBS; HyClone) at a density of $1x10^7$ cells/mL directly before use.

The frozen stock of *Streptococcus pneumoniae* previously grown to mid-log phase (in a growth medium at 37°C / 5% CO_2 to log phase OD_{600}= 0.3 – 0.4) was diluted in the opsonophagocytic buffer to a final density of 10^6 CFU/ml (1000 CFU in 20 μL). Individual or pooled sera samples (10 μL per well) were heat-inactivated (56°C, 30 min) and aliquoted in round bottom non-treated 96-well plates at four-fold dilution intervals (native serum follow by 1:8 to 1:8192 dilutions) and treated with the bacterial suspensions (20 μL per well) to initiate opsonization (incubation for 15 min at 37°C). After preopsonization, 10 μL of external complement source (baby rabbit complement, CedarLane, Ontario, Canada) as well as 4×10^5 differentiated HL-60 cells in a volume of 40 μL were added to each well (phagocyte/bacteria ratio 400:1) and plates were incubated for 45 min at 37°C in 5% CO_2 environment (preferably with shaking at 220 rpm). The phagocytic reaction was stopped by putting the plate on ice for 20 min. Viable extracellular pneumococci were determined by plating aliquots (5 μL) from each well on Columbia Agar plates with 5% (v/v) sheep blood (BD, New Jersey, USA) and incubating at 37°C in 5% CO_2, for

several hours to allow bacteria growing (6-8 in case of *S.pneumoniae* serotype 3). Visible colony forming units (CFU) were counted. Negative controls lacking either antibody, HL-60 or complement, as well as standard control WHO 007sp typing serum (Human Anti-Pneumococcal capsule Reference Serum, NIBSC, Herts, UK) were used. The assay was repeated two to three times independently. Percentage killing of bacteria was calculated as CFU reduction relative to negative control wells. Serum dilution responsible for 50% killing of bacteria was estimated through non-linear interpolation of the dilution-killing OPKA data.

2.5 Carrier proteins

2.5.1 Pneumolysin (Ply) as an alternative carrier protein

2.5.1.1 Transformation of BL21 cells with Ply plasmid

BL21 competent *E. coli* cells were used for transformation with the plasmid expression vectors for the *S. pneumoniae* Ply mutants based on the pET101/D-TOPO backbone containing Polyhistidine-tag (PlyW460E, PlyW433E – Trp460/433-->Phe mutation received from L.S. McDaniel, University of Mississippi Medical Center, USA). BL21 cells were slowly thawed on ice and gently mixed to ensure the even suspension. The purified plasmid (1 μL, in a concentration range 1-10 ng/μL) was added directly to the BL21 cells, stirred and incubated on ice for 5 min. Next, the tubes were heated for precisely 30 s in a 42°C water bath (avoid shaking) and place on ice for 2 min. The room temperature SOC medium (Super Optimal broth with Catabolite repression, Merck, Darmstadt, Germany) in 250 μL volume was added to each tube and incubated for 60 min at 37°C while shaking (250 rpm). Cells were plated on selective media (LB agar; 50mg/mL filter-sterilized carbenicillin).

2.5.1.2 Protein expression

A single clone of Ply transformed BL21 cells were picked and grown in selective LB medium (10 g/L Tryptone, 5 g/L Yeast Extract, 5 g/L NaCl plus, 50 mg/mL carbenicillin to an OD_{600} =

0.5. Isopropyl-b-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM to induce protein expression. The culture was grown for an additional 4 – 5 hours and bacterial cells were collected by centrifugation for 10 min at 8,000 x g in 4°C. Cells pellet was resuspended in PBS with 1 mg/mL of lysozyme and incubated for 1 hour at 30°C. The cells were cooled for 30 mins, lysed by sonication (10 intervals; 30s per interval, with alternating 30s rest periods) and centrifuged for 20 min at 20,000 x g in 4°C. The supernatant containing protein was collected.

2.5.1.3 The Pneumolysin (Ply) purification

The recombinant Ply protein with Polyhistidine-tag (His-tag) was purified using the Protino® Ni-NTA Column (Macherey-Nagel, Germany) and ÄKTA pure chromatography system (GE Healthcare Life Science, Chicago, USA). The column was equilibrated with equilibration buffer and cell lysate with recovered recombinant Ply was loaded onto a 1 mL prepacked Ni-NTA column (1mL/min flow rate, 0.3 MPa pressure limit). The column was extensively washed with washing buffer (PBS, 25mM imidazole pH 7.4). Next, the His-tagged Ply protein was eluted with elution buffer (PBS, 250mM imidazole, pH 7.4) and subsequently dialyzed against PBS (pH 7.4). Before using for biological assays, the protein was concentrated using Amicon® Ultra 10K filters (Merck Millipore, Massachusetts, USA). A summary of all buffers used for the purification is shown in Table 8.

Table 8. Buffers used for affinity chromatography purification.

Extraction buffer (PBS)	50mM Sodium phosphate 300mM Sodium chloride
Equilibration buffer (pH 7.4)	PBS 10mM Imidazole
Washing buffer (pH 7.4)	PBS 25mM Imidazole
Elution buffer (pH 7.4)	PBS 250mM Imidazole

Pneumolysin purity was checked by Coomassie Blue staining of SDS-polyacrylamide gels and a concentration was determined by NanoDrop 1000 Spectrophotometer (A_{280} absorbance was automatically translated to protein concentration based on an intrinsic standard).

2.5.1.4 Protein conjugation to the synthetic oligosaccharide

Pneumolysin PlyW433E was conjugated to *S. pneumoniae* serotype 3 tetrasaccharide (ST3) using the standardized protocol described previously (see section 2.3.1). The conjugation conditions were optimized by using PBS pH 7.4 as a washing solution and the sugar amount corresponding to the 1:70 equivalents (m/m protein: sugar).

2.5.1.5 Mouse immunization and serum collection

C57BL/6 six to eight week old female mice were immunized subcutaneously (s.c.) with the dose of conjugate corresponding to 0.4 µg of SP3-tetrasaccharide and Aluminium hydroxide as an adjuvant (0.125 µg of Alum, Alhydrogel, Brenntag, Denmark) in a total volume of 100 uL of PBS. The immunization followed the standard schedule of prime + boost + boost as showed in Figure 16. Blood was collected via the tail-tip bleeding method and processed as described in paragraph 2.2

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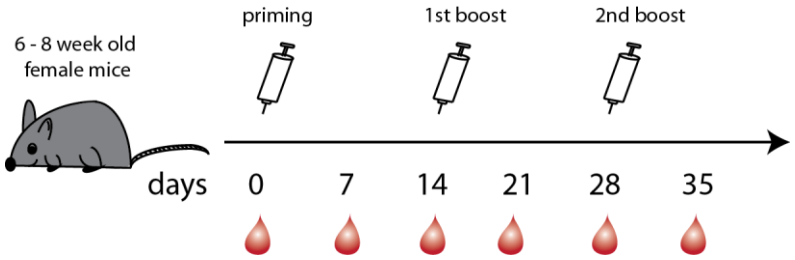


Figure 16. Short-term prime + boost + boost immunization schedule.

2.5.1.6 Evaluation of antibody response

The carbohydrate- and protein-specific antibody response was measured by glycan array and ELISA (as described previously in section 2.4.3 ad 2.4.4). ELISA plates were coated with purified Pneumolysin (PlyW433E) and CPS of *S.pneumoniae* serotype 3 (SSI Diagnostica, Kopenhagen) at concentration 10 µg/mL in PBS (volume of 50 µL per well).

2.5.1.7 Red blood cell (RBC) lysis assay

The neutralizing ability of Pneumolysin-specific antibodies was tested using the red blood cell lysis assay. A 96-well non-treated round-bottom plate was used for the test. Serum samples were serially diluted in 1% BSA/PBS to a final volume of 25 µL. The full-length native pneumolysin was diluted to the concentration of 1 µg/mL and added to each well in a volume of 25 µL. The plate was incubated for 30 min at 37°C with shaking. Human red blood cells (hRBC) were prepared at 1% concentration in PBS and distributed to each well in a volume of 50 µL. The plate was incubated for 60 min at 37°C without shaking and centrifuged for 10 min at 1,000 rpm. 80 µL of supernatants from each well were transferred to a new 96-well flat-bottom plate. The absorbance was measured at 414 nm using an ELISA plate reader. Control wells containing nonlysed 1% hRBC were used to determine the average maximum absorbance value at A_{414}. The hemolytic titer was defined as the reciprocal dilution of serum that corresponded to a 50% decrease in the maximum absorbance value at A_{414}.

2.5.1.8 RBC lysis assay with a whole bacteria cells lysates

Bacterial culture (2 mL of *S.pneumoniae* serotype 3 (ST3), *S.pneumoniae* serotype 2 (ST2) and *S.pneumoniae* serotype 8 (ST8)), were cultured in brain heart infusion broth (at 37°C in 5% CO_2 environment) to log phase (OD_{600} = 0.6). Cells were centrifuged, washed with PBS and re-suspended in 100 µL of cell wall digestion buffer (10 mM Tris pH 7.5, 30% Sucrose, 1x protease inhibitor, 1 mg/mL lysozyme) for 3 h at 37 °C. The protoplasts were additionally lysed by sonication (samples on ice, sonication output control 4, 40W, 5 min). The lysates were centrifuged, and the supernatants were filter sterilized. Samples were filled to 1 mL a volume with F-12 K medium (Ham's F-12K (Kaighn's) Medium, Thermo Fisher Scientific, Massachusetts, USA). The supernatant was plated at blood agar plates to confirm the absence of bacterial colonies.

2.5.1.9 Electric Cell-substrate Impedance Sensing - ECIS

The A549 adenocarcinomic human alveolar basal epithelial cell line (received from Sander Group, Department of Infectious Diseases and Respiratory Medicine at Charité

Universitätsmedizin Berlin) was maintained in complete DMEM (Dulbecco Modified Eagle Medium, PAN Biotech, Germany) with 20% FCS (PAN Biotech, Germany) at a cell concentration between 6 x 10^3 and 6 x 10^4 cells/cm^2.

The ECIS® Zθ Array station (Applied Biophysics Inc, New York, USA) was used for the assay. 1 x 10^5 A549 cells in a volume of 400 µL of complete DMEM with 20% FCS were added to each well of 8W10E ECIS Cultureware™ Disposable Electrode Array (Applied Biophysics Inc, New York, USA) and cultured for 24h at 37°C/5% CO_2. On the next day, the medium was exchanged to DMEM without FSC (100 µL), and the control resistance was measured for one hour. The whole bacteria cell lysates of *S.pneumoanie* serotype 2, 3 and 8 were pre-incubated for 1h in 37°C on a shaker with serum from mice immunized with ST3-tetrasaccharide pneumolysin conjugate (see 0) collected on day 35. Pre-incubated bacteria lysate and serum samples were added to the respective wells (see an example in Figure 17). The assay was performed overnight. The electrode system measured the resulting voltage (V) across the electrodes. The impedance (Z) was given by the AC equivalent of Ohm's law: Z=V/I, where I was corresponding to a current. The pure resistive (R), as well as the capacitive portions (C) of the impedance, were also recorded.

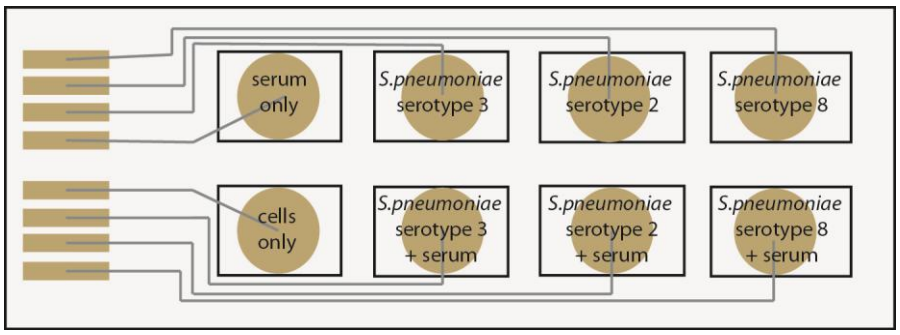


Figure 17. The exemplary setting of the Electric Cell-substrate Impedance Sensing assay. The whole bacteria cell lysates of *S.pneumoanie* serotype 2, 3 and 8 with and without serum samples were added to the wells containing A549 adenocarcinomic human alveolar basal epithelial cell line. The ECIS® Zθ Array Station measured the resulting voltage (V), impedance (Z) and pure resistive (R).

2.6 Pneumolysin challenge study

2.6.1 Immunization

Six to eight week old female C57BL/6 mice (purchased from Charles River; Sulzfeld, Germany) were immunized according to the prime + boost + boost schedule as shown in Figure 16. In total, 64 animals were divided into two infection group accordingly with *S.pneumoniae* serotype 2 (32 mice) and *S.pneumoniae* serotype 3 (32 mice). Each infection group was vaccinated s.c. with: (1) conjugate of *S.pneumoniae* serotype 3 tetrasaccharides and PlyW433E (ST3-Ply); (2) conjugate of *S.pneumoniae* serotype 3 tetrasaccharide and CRM197 (ST3-CRM197); (3) positive control of Prevnar®13 and (4) negative control as PBS only (see Table 9).

Table 9. Experimental groups used for SP3-tetrasaccharide Pneumolysin conjugate immunization study.

Group #	Vaccine	Infection with S.pneumoniae serotype 2 (ST2)	Infection with S.pneumoniae serotype 3 (ST3)
1	ST3-Ply	8	8
2	ST3-CRM197	8	8
3	Prevnar13®	8	8
4	PBS	8	8
	In total	32	32
			Σ64 animals

The vaccines were prepared as described previously (2.4.1). Briefly, groups 1 and 2 containing an amount of conjugates (both SP3-Ply and SP3-CRM197) corresponding to a dose of 0.4 µg of SP3-tetrasaccharide in formulation with 0.125 µg of Alum (Aluminium hydroxide; Alhydrogen, Brentage, Denmark) in a total volume of 100 µL PBS. Group 3 contains 100 µL of Prevnar13® and group 4, 100 uL of PBS.

2.6.2 *S.pneumoniae* serotype 2 (ST2) and serotype 3 (ST3) challenge model

S.pneumoniae serotype 2 and serotype 3 were plated from frozen stocks on Columbia Agar plates with 5% (v/v) sheep blood, grown for approximately nine hours at 37 °C/5% CO_2 and inoculated as single colonies in Todd Hewitt Broth with 0.5% (w/v) yeast extract (growth

medium). Cultures were cultured at 37 °C/5% CO_2 to mid-log growth phase (OD_{600} = 0.3 – 0.4) and harvested by centrifugation (5000 rpm / 10 min).

Mice were anesthetized by intraperitoneal (i.p) administration of ketamine (80 mg/kg, Ketavet®, Pfizer, Berlin, Germany) and xylazine (25 mg/kg, Rompun®, Bayer, Leverkusen, Germany). Infection with *S. pneumoniae* serotype 2 (5 x 10^7 CFU) and *S. pneumoniae* serotype 3 (5 x 10^6 CFU) was performed intranasally in a total volume of 20 µL bacteria diluted in PBS. Disease severity was evaluated at 12-hour intervals (more often if animals were severely ill) for 36 - 48 h after bacterial infection to assess appearance, behavior, grooming, respiration and body weight. Measurement of rectal temperature was made at every time point (BAT-12 Microprobe Thermometer, Physitemp Instruments, Clifton, USA).

Mice were sacrificed 36h (ST2 infection) and 48h (ST3 infection) after bacteria inoculation or when they reached at least one of the predefined human endpoints criteria (body temperature <30°C; bodyweight loss >20%; cumbersome breathing; accelerated breathing in combination with staggering, pain or paleness) by exsanguination via the caudal *Vena cava* after i.p. injection of ketamine (160 mg/kg body weight) and xylazine (75 mg/kg). Blood was drawn from the *Vena cava caudalis,* and lungs as well as spleen were removed.

After blood collection, serial dilutions of blood were plated on Columbia agar plates with 5% (v/v) sheep blood and incubated overnight at 37°C in 5% CO_2 environment to estimate bacteria recovery (number of CFU in the blood). Blood antibody levels were monitored by glycan microarray and ELISA analysis. Lungs were homogenized by passage through a cell strainer (100 µm pores, BD Bioscience, Heidelberg, Germany) to assess the residual bacterial burden. Serial dilutions of lung homogenates were assessed for bacteria growth (CFU level) as described above.

2.6.3 *S.pneumoniae* serotype 2 challenge study

2.6.3.1 Conjugation of ST2-hexasaccharide and ST3-tetrasaccharide to Pneumolysin and PspA carrier proteins and conjugates characterization.

S. pneumoniae serotype 3 tetrasaccharide (ST3) and serotype 2 hexasaccharide (ST2) were conjugated to pneumolysin PlyW433E (Ply), and PspA using the standardized protocol described previously (see section 2.3). Proteins were received from Dr. Thomas Kohler from the Department of Molecular Genetics and Infection Biology, Universität Greifswald. The conjugation conditions were optimized by using PBS pH 7.4 as a washing solution for proteins and the sugar amount corresponding to the 1:70 equivalents (m/m protein: sugar). Conjugates were characterized by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization with time-of-flight detection mass spectrometry MALDI-TOF MS.

2.6.3.2 Immunization

The animal experiment was conducted in accordance to the guideline of the ethic committee at the University of Greifswald, the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). The trial was approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern (LALLF M-V, Rostock, Germany) and the LALLF M-V ethical board (LALLF M-V permit no. 7221.3-1-061/17). All efforts were made to minimize the discomfort of the animals and ensure the highest ethical standards.

Six to eight week old female inbred C57BL/6 mice (n = 12, Charles River Laboratories, Sulzfeld, Germany) were immunized intranasally a two-week interval according to the prime + boost + boost regime. Conjugates of ST2-hexasaccharide and ST3-tetrasaccharide with PspA and Pneumolysin (dose equal to 2.5 µg of protein) were formulated with 4 µg Cholera toxin subunit B (CTB; Sigma-Aldrich, Missouri, United States) as an adjuvant in a total volume of 10 µL. PBS and ST3-tetrasaccharide CRM197 conjugate with CTB were administered as a negative control.

Blood samples were taken before each immunization step (n = 6) and two weeks after the last immunization (n = 6) for antibody measurements. Therefore, mice were punctured in the facial vein following gentle anesthesia with 2.5% isoflurane/oxygen mixture (Baxter, Illinois, United States).

2.6.3.3 Intranasal infection of mice with *S.pneumoniae* serotype 2

Three weeks after the final immunization, mice were intranasally infected with *S. pneumoniae* D39 (serotype 2). Pneumococci were cultured in advance in liquid Todd Hewitt Broth (THY, Sigma Aldrich, Missouri, USA) supplemented with 10% heat-inactivated (h.i.) FCS until mid-log growth phase (OD_{600nm} 0.35) and stored as glycerol-supplemented aliquots at -80 °C. Appropriate amounts of aliquots were thawed on the day of infection followed by adjustment of the infection dose to 3.5×10^6 CFU in 5 µL of PBS per mouse, verified by plating on blood agar plates. For the intranasal application, mice were anesthetized by intraperitoneal injection of ketamine/xylazine (50 mg/kg weight of mouse ketamine (KetanestS, Pfizer Pharmaceuticals; Provet AG, Berlin, Germany) + 5 mg/kg weight of mouse xylazine (Rompun, Provet AG)). Three days post-infection, mice were euthanized, followed by a collection of nasal tissue, bronchoalveolar lavage (BAL), blood and spleens. BAL samples and homogenates of nasal tissue were serially diluted to determine the bacterial recovery (Log_{10} CFU/mL). Remaining BAL and the nasal homogenate were snap-frozen and stored at -80 °C for further analyses. Post-challenge blood samples were collected from the heart by puncture of the *Ventriculus cordis dexter* followed by centrifugation at 1000 × g for 20 min and storage of the collected sera at -20 °C. Isolated spleens (n = 6) were transferred to a culture medium (RPMI 1640 + 10% h.i. FCS + 1% Pen/Strep) and kept on ice until further processing. Single-cell suspensions of harvested spleens were prepared in 15 mL cell culture medium (RPMI 1640 + 10% h.i. FCS + 1% Pen/Strep) followed by the removal of red blood cells (RBC) by incubation with 5 mL RBC lysis buffer for 5 min. The reaction was stopped by the addition of 10 m cell culture medium. After centrifugation, the cells were resuspended in a 1 mL storage medium (h.i. FCS + 10% DMSO) and stored at -80 °C.

2.7 Adjuvants

2.7.1 Vaccine formulation and immunization

Six to eight week old female C57BL/6 mice (purchased from Janvier Labs, Saint-Berthevin, France) were immunized s.c. with 100 µL volume of ST3-tetrasaccharide CRM197 conjugate vaccines in a different adjuvant formulation, according to the long-term prime + boost + boost + final boost vaccination regime (as shown in Figure 13 and Table 4). In total, 60 animals were divided into eight experimental groups (Table 10).

Table 10. Adjuvant formulation study experimental groups.

	Group name	Adjuvant	Adjuvant dose	Adjuvant provider	Formulation
1	SP3-AlH	Aluminium hydroxide	0.125 mg of Aluminium/dose	10% Alhydrogel (Brenntag, Denmark)	adsorption
2	SP3-MF59	MF59™	1:1 (v/v)	AddaVax™ (InvivoGen, San Diego, USA)	emulsion
3	SP3-MPLA	Monophosporyl lipid A (MPLA, PHAD®)	10 µg/dose	Avanti Polar Lipids (Sigma Aldrich, St. Louis, USA)	PLGA particles
4	SP3-R848	Resiquimod (R848)	10 µg/dose	InvivoGen, San Diego, USA	PLGA particles
5	SP3-MPLA + R848	Monophosporyl lipid A (MPLA, PHAD®) + Resiquimod (R848)	10 µg + 10 µg/ dose	Avanti Polar Lipids (Sigma Aldrich, St. Louis, USA) InvivoGen, San Diego, USA	PLGA particles
6	SP3-RNA	Isolated bacterial RNA	5 µg/dose	Sander Group (Charité – Universitätsmedizin Berlin)	PLGA particles
7	Prevnar®13	Aluminum hydroxide	1/5 of the human dose	Pfizer, New York, USA	solution
8	Empty particles	-	-	-	PLGA particles

All groups contained an amount of *S.pneumoniae* serotype 3 tetrasaccharide CRM197 conjugate corresponding to a dose of 0.4 µg of ST3-tetrasaccharide (conjugate prepared as described previously in the chapter 2.3.1).

The ST3-tetrasaccharide formulated with Aluminium hydroxide was prepared as described previously in method 2.4.1. Group 2 – ST3-tetrasaccharide - MF59 was formed by

emulsification of the ST3-tetrasaccharide CRM197 conjugate with MF59 AddaVax™ (InvivoGen, San Diego, USA) in 1:1 volume ratio. The water-in-oil emulsion was created by the "two-syringe" technique directly before injection. Briefly, two glass syringes containing antigen and adjuvant were connected with a double-ended syringe connector. The emulsion was formed by forcing a mixture of oil-in-water adjuvant and the antigen back and forth until the material became homogeneous and viscous. The syringes were carefully disconnected, and the antigen-adjuvant emulsion was transferred to the plastic immunization syringe.

The preparation of the vaccines in the form of PLGA particles is described in the next paragraph.

2.7.2 Particle preparation

Polylactic acid particles (PLA) were fabricated using a water/oil/water (W1 /O / W2) double emulsion solvent evaporation technique. A two-step approach was employed while 100 µL of the internal aqueous phase containing cargo (IAP or W1) was emulsified into 2 mL of the organic phase (OP or O; 50 mg/mL PLA-PDL 50, 0.49 dL/g in Dichloromethane) in a 10 mL glass vial using a sonicator fitted with a microtip (output control 4, 40 W, 1 minute), resulting in the primary emulsion (W1 / O). Microparticles were fabricated by emulsifying the primary emulsion, to the external aqueous phase (EAP or W2 8 mL (2% Polyvinylalcohol-PVA, Mw. 31 - 50K, 98-99 % hydrolysis in 10% sucrose) by using a homogenizer with S 18 N-10G probe (10000 rpm, 8 min) resulting in the secondary emulsion (W1 /O / W2). The resulting secondary emulsion was poured into a glass beaker, covered with an aluminum foil punched with holes and stirred overnight at 400 rpm under a sterile bench to allow solvent evaporation and particle precipitation. The particles were collected by centrifugation at 13000 x g for 15 min, washed with nanopure water and lyophilized for 48 - 72 hours to obtain a fine powder. The vials were hermetically sealed and stored at 4°C until further use. The supernatant from washing steps was collected, and protein concentration was measured to assess the amount of non-capsulated antigen.

2.7.3 Particle characterization

2.7.3.1 Flow cytometry analysis (FACS) of antigen-loaded PLA particles

Lyophilized particles were re-suspended in PBS and washed by centrifugation (3000 x g, 5 min, 4°C). Monoclonal antibodies recognizing SP3-tetrasaccharide (7A9 and 5F6 received from Pirofski's group, New York, USA) and CRM197 (produced in-house) diluted in 1% BSA/PBS were added to the particles and incubated overnight at 4°C while rotating. The particles were washed three times with PBS and stained with anti-mouse IgG FITC (BD Biosciences, Heidelberg, Germany) and IgM- FITC (BioLegend, California, USA) diluted 1:100 in PBS for one hour. After three washing steps with PBS, the particles were subjected to flow cytometry using a FACS Canto II instrument (BD Biosciences, Heidelberg, Germany). 2,000 events were counted for each measurement.

2.7.3.2 Confocal microscopy analysis of antigen-loaded PLA particles

The encapsulation of antigen on the PLGA particles was checked by confocal microscopy analysis. Briefly, 10 mg of the particles were washed three times with PBS and incubated with the corresponding primary and secondary antibody as described in the chapter above (2.7.3.1). The samples were washed three times with PBS, placed onto a glass microscope slide and covered with a coverslip. Images were acquired using a confocal microscope (LSM-700 Carl Zeiss AG).

2.7.3.3 Estimation of encapsulated antigens onto PLA particles

The supernatant collected after washing the particles with PBS was used to evaluate the concentration of non-capsulated glycoconjugate by BCA assay (previously described in 2.4.2) and NanoDrop.

2.7.3.4 Transmission Electron Microscopy (TEM) of particles containing ST3-tetrasaccharide CRM197 conjugate and different adjuvants

High-resolution imaging was performed using a Tecnai F20 X-Twin transmission electron microscope at GFZ Potsdam. The TEM is equipped with a field emission gun as an electron emitter. TEM bright-field images were acquired as energy-filtered images using a Gatan imaging filter GIF. A 20 eV window was applied to the zero-loss peak. Data were evaluated with the Gatan Digital Micrograph software package.

2.7.3.5 Dynamic Light Scattering (DLS) Analysis

The size distribution of nanoparticles was analyzed by dynamic light scattering (DLS) measurement technique using the Zetasizer μV Nano (Malvern). Vesicles (10 μL) or 1 mg/mL nanoparticle suspension (10 μL) was diluted in 1 mL PBS or water, and three measurements were performed at 25° C, each measurement comprising of 15 scans for 14 min. Microparticle size distribution was analyzed using a Mastersizer with each measurement performed in triplicates with a size range of 0.02-2000 μm.

2.7.4 Analysis of the immune response of mice immunized with different adjuvant formulations

2.7.4.1 FACS analysis of spleen and bone marrow

Mice were sacrificed two weeks after the final boost. Blood was collected by cardiac puncture and spleen and bone marrow from the tibiae and femurs were isolated. Spleen was cut into four pieces and preserved in: (1) 4% formalin for immunohistochemistry staining; (2) 4% formalin for an immunofluorescence staining; (3) dry ice for an immunofluorescence staining; and (4) PBS for FACS analysis.

The last piece of each spleen was weight; splenocytes cell suspension was prepared by pressing the organ through a 40 μm cell strainer using a 10 mL syringe plunger and washed with PBS. Splenocytes were pelleted at 1300 rpm, 4°C for 10 min and resuspended in 3 mL of RBC lysis

buffer (0.01M $KHCO_3$, 0.155M NH_4Cl, 0.1 mM EDTA) for 10 min at RT. The reaction was stopped by adding 10 mL of PBS, and the pellet was washed twice with PBS. The splenocytes were counted using Trypan blue to exclude the dead cells.

The tibiae and femurs were flushed with complete PBS and pipetted vigorously to disentangle the cells. The cell suspension was filtered with a 70 μm cell strainer, centrifuged at 1300 rpm, 4 ^{o}C for 5 minutes, resuspended in PBS and counted.

Finally, both spleen and bone marrow cells were resuspended in 3 mL of PBS and divide into FACS tubes for the following staining: All antibodies used for staining were listed in Table 11, Table 12, and Table 13.

2.7.4.1.1 Surface staining (B cells)

The surface block master mix (0.5 mg/mL of αCD16/32, clone 93; Biolegend, San Diego, USA) was added to each tube and incubated for 5-10 minutes on ice. Cells were centrifuged at 1300 rpm, 4 ^{o}C for 5 minutes and incubated with a surface stain master mix for 30 minutes at 4^{o}C in the dark. Cells were washed once with PBS and analyzed on CANTO II (BD Bioscience FACSCanto™)

2.7.4.1.2 Surface staining (T follicular (T_{fh}) cells)

The surface block master mix (0.5 mg/mL of aCD16/32, clone 93; Biolegend, San Diego, USA) was added to each tube and incubated for 5-10 minutes on ice. Cells were centrifuged at 1300 rpm, 4 ^{o}C for 5 minutes and incubated with CXCR5-Bio/buffer for 45 minutes to 1 hour on ice. Cells were washed once with PBS and analyzed on CANTO II (BD Bioscience FACSCanto™).

2.7.4.1.3 Intracellular Cytokine Staining (ICS) and Intranuclear Staining (IS)

- Stimulation

Cells were resuspended at a concentration of 1 - 5 x 10^6/mL in T cell medium + PMA (phorbol. 12-myristate 13-acetate; ThermoFisher, Waltham, USA) + Ionomycin (calcium ionophore, ThermoFisher, Waltham, USA) to stimulate the intracellular production of the cytokines. Cells in 200 µL volume were added to the 96-well round plate and incubated for 5 hours at 37^{o}C. Brefeldin A (10 µL) and Monensin (final volume of 210 µL per well) were added to block extracellular proteins 2.5 hours post-stimulation transport and incubated for remaining 2.5 hours.

- Surface staining

Cells were centrifuged at 1300 rpm, 4^{o}C for 5 minutes, resuspended in Surface Block Master Mix (50 µL) and incubated 5 - 10 minutes on ice. 50 µL of a Surface Staining Intra Antibody Mix (surface) was added to each well and incubated 10 to 15 minutes on ice in the dark. Cells were centrifuged at 1300 rpm, 4^{o}C for 5 minutes and supernatant was discarded.

- Fixation (the FoxP3 staining buffers kit)

Cells were resuspended in 50 µL of Antibodies Mix with FoxP3 Fixation/Permeabilization solution (eBioscience, ThermoFisher, Waltham, USA) and incubated 60 minutes at RT while shaking in the dark. Wells were filled up to 200 µL with 1x Permeabilization buffer and centrifuged at 1300 rpm, 4 ^{o}C for 5 minutes. Cells were washed once with PBS and analyzed on CANTO II (BD Bioscience FACSCanto™)

Table 11. Staining panel for plasma cells, germinal center, and memory B cells.

Antibody	Color	Clone	Provider
CD138	PE	281-2	Biolegend, San Diego, USA
GL7	PerCP/Cy5.5	GL7	Biolegend, San Diego, USA
CD19	PE-Cy7	ID3	ThermoFisher, Waltham, USA
FAS	APC	15A7	ThermoFisher, Waltham, USA
CD38	Alexa 700	90	ThermoFisher, Waltham, USA
IgD	Pacific Blue	11-26 c.2a	Biolegend, San Diego, USA
B220	BV510	RA3-6B2	Biolegend, San Diego, USA

Table 12. Staining panel for T follicular helper cells (T_{fh}) and memory T cells

Antibody	Color	Clone	Provider
ICOS	FITC	7E.17G9	ThermoFisher, Waltham, USA
***CXCR5-Bio**		L138D7	Biolegend, San Diego, USA
Streptavidin	PE		Biolegend, San Diego, USA
PD-1	PerCP-eFluor 710	J43	ThermoFisher, Waltham, USA
CD62L	APC	MEL14	Biolegend, San Diego, USA
CD3e	Alexa 700	eBio500A2 (500A2)	ThermoFisher, Waltham, USA
CD44	eFluor 450	IM7	ThermoFisher, Waltham, USA
CD4	BV510	RM4.5	Biolegend, San Diego, USA

*CXCR5-Bio+SA-PE: secondary antibody staining

Table 13. Staining panel for T helper cells (Th_1 and T_{fh})

Antibody	Color	Clone	Provider
****Bcl-6**	PerCP-eFluor 710	BCL-DWN	ThermoFisher, Waltham, USA
***IFN-g**	PE-Cy7	XMG1.2	Biolegend, San Diego, USA
***IL-21**	Alexa 647	K112-91	Biolegend, San Diego, USA
CD3e	Alexa 700	eBio500A2 (500A2)	ThermoFisher, Waltham, USA
CD4	Pacific Blue	RM4.5	Biolegend, San Diego, USA

*IFN-g and IL-21: Intracellular cytokine

**Bcl-6: Intranuclear staining

2.7.5 Histopathology

Spleen samples were fixed in 4% buffered formalin, embedded in paraffin, and cut into 2 µm thick sections. The staining was performed and evaluated by the Department of Veterinary Pathology, Freie Universitaet Berlin.

2.7.5.1 Hematoxylin and Eosin (HE) staining

Hematoxylin staining was performed by dedicated staining machine with following steps: tissues were immersed three times in xylene (Chemsolute Xylol Technisch, Th. Geyer, Germany) for 2 min, 2 min, and 3 min and then in a series of Ethanol solutions (Berkel, Germany) of decreasing concentrations (96%, 80%, and 70%) for 30 seconds. Samples were washed in water, stained with hematoxylin (Roth, Germany) for 8 mins, washes in water for 5 minutes and dyed in eosin (Waldeck, Germany) for 30 seconds. Spleens were immersed in increasing ethanol concentration (70%, 80%, 96%, and 100%) to eradicate the water. Finally, samples were absorbed four times in xylene immersions for 1 minute to replace the ethanol with xylene. Scanning of HE-stained slides was performed by Aperio CS2 slide Scanner (Leica Biosystems Imaging Ins., CA, USA).

2.7.5.2 Immunohistochemistry

Spleen samples were dewaxed in xylene (2 x 10 min), rehydrated in decreasing ethanol concentrations (2 x 100%, 2 x 96%, 1 x 70%; 3 min each) and the antigens were retrieved by heat (microwave 600 W) in 10 mM citric acid (pH 6.0). Tissue was incubated with anti-GL7 rat monoclonal antibody (Invitrogen, Carlsbad, USA) in 1:100 dilution, overnight at 4 °C. Negative controls were incubated with an irrelevant rat antibody (Biogenex rat, Innovative Diagnostic-Systeme, Germany) at the same dilution and conditions. Next, the samples were incubated with biotinylated goat anti-rat secondary antibody (Vector Laboratories, Germany), in 1:200 dilution, 30 min following incubation with HRP-coupled avidin-biotin complex (ABS-kit, Vector Laboratories, Germany) for 30 min. The color was developed by incubation with diaminobenzidine (DAB) for 8 min (ABS-kit, Vector Laboratories, Germany). The spleen samples were counterstained with hematoxylin (1 min) and dehydrated through graded ethanols

(70%, 2 x 96%, 2 x 100%, 3 min each) and immersed two times in xylene for 3 min before applying coverslip slides. The digital analysis of the samples was performed by Aperio nuclear v9 Algorithm (Leica Biosystems Imaging Ins., CA, USA).

2.8 Pigs as a large animal model

2.8.1 Immunization

Thirty healthy, six-week-old, 20 kg weight female pigs (German landrace *Sus domesticus*) purchased from BHZP (BHZP GmbH; An der Wassermühle 8; 21368 Dahlenburg-Ellringen) were kept at the animal facility of Friedrich-Loeffler-Institut, Bundesforschungsinstitut für Tiergesundheit Federal Research Institute for Animal Health Greifswald Insel Riems.

Pigs (n = 6) were immunized according to the schedule presented in Table 14 with following vaccines: (1) ST3-tetrasaccharide CRM197 conjugate, (2) ST3-tetrasaccharide Pneumolysin conjugate, (3) Prevnar13® as a positive control, (4) Alum only as a negative control. For the groups containing ST3-tetrasaccharide an amount of conjugate equal to 2.2 µg of synthetic oligosaccharide was used. Antigen was adsorbed onto 125 µg of Alum (Aluminium Hydroxide, Alhydrogel).

Table 14. Swine immunization regime

day	action
-1	Bleeding
0	1st immunization
10	Bleeding + 2nd immunization
15	Bleeding
20	Bleeding + final immunization
25	Bleeding
35/36	Bleeding + dissections

2.8.2 Antibody response

Blood was collected on day -1, 10, 15, 20, 25 by the *V. cava cranialis* punctuation and on day 35/36 by heart punctuation. Antibody titer and their subclasses were analyzed by glycan array and ELISA (as described previously in 2.4.3 and 2.4.4 respectively). Protective activity of the antibodies was evaluated by OPKA (described in 2.4.5). The neutralizing activity of anti-Ply antibodies was analyzed by RBC lysis assay (explained in 2.5.1.7)

2.8.3 FACS – T cells analysis

Blood after first (day 20) and second boost (day 35) was collected into Li-Heparin tubes. Peripheral blood mononuclear cells were isolated according to the following protocol. Blood samples were diluted twice in NaCl and carefully overlaid on 3 mL of Pancoll, without mixing phases. Samples were centrifuged at 800 x g for 20 minutes, room temperature and the lymphocytes, together with monocytes and platelets, were harvested from the white cells layer between the plasma samples and the Pancoll. The separated cells were washed twice with RPMI, diluted to the concentration of 12.5 x 10^6 cells per mL. Cells were stored overnight at 4°C.

Cells were seeded in the volume of 200 µL PBMC/well in triplicates and stimulated accordingly to the primary immunization either with CRM197 (40 µg/mL), native Pneumolysin (40 µg/mL), ST3-tetrasaccharide CRM197 conjugate (40 µg/mL) or ST3-tetrasaccharide Pneumolysin conjugate (40 µg/mL). After 2h, Brefeldin A was added in 1:1000 dilution and incubated for another 4h. The cell was stained accordingly to the panel described in Table 15 and analyzed by FACS CantoII.

Table 15. Staining panel for T-cell.

Antibody	Concentration	Color	Provider
DCE	1:500	506	ThermoFisher, Waltham, USA
Anti-CD14	1:50	Viogreen	Abcam, Cambridge, United Kingdom
Anti-CD3	1:200	PerCp-Cy5.5	Biolegend, San Diego, USA
Anti-CD8	1:100	FITC	Biolegend, San Diego, USA
Anti-CD4	1:100	Alexa 647	Biolegend, San Diego, USA
Anti-CD154	1:10	Pe-Vio770	Biolegend, San Diego, USA
IFNγ	1:400	PE	Biolegend, San Diego, USA,
TNFα	1:100	PB	Biolegend, San Diego, USA

3 Results and Discussion

3.1 The ST3-tetrasaccharide Glycoconjugate Adsorbs Significantly Better Onto Aluminium Hydroxide Particles.

Synthetic ST3-tetrasaccharide CRM197 conjugate was obtained by coupling the ST3-tetrasaccharide through the primary amine side chains of lysine residues and *N*-terminus of the Cross-Reactive-Material-197, called CRM197, using bis(4-nitrophenyl) adipate linker. CRM197 is a non-toxic mutant of a diphtheria toxin, where the single amino acid exchange of a glycine in position 52 to a glutamic acid reduces the protein toxicity (161), widely used as a carrier protein for polysaccharide conjugated vaccines. The resulting glycoconjugate was characterized by SDS-polyacrylamide gel electrophoresis shift assay (10% SDS-PAGE), and MALDI-TOF mass spectrometry to measure the average molecular size of the conjugate and the loading ratio (

Figure 18).

The proper conjugation and presentation of carbohydrate epitopes on a carrier protein regulate the glycoconjugate uptake and presentation that is crucial for the vaccine immunogenicity as it might influence the interaction of both carbohydrates – antigen-presenting cells and the MHCII – CD4 T-cell. CRM197 contains 39 lysines with a heterogeneous reactivity to conjugation reactions (e.g. steric accessibility or the individual pKa of the respective lysine residues). Reaction conditions and conjugate size might also influence the conjugation efficacy of individual sites (161). The average loading of the glycan epitopes on each CRM197 protein was calculated based on the average mass of the conjugate (64494 Da), a carrier protein (58000 Da) and ST3-tetrasaccharide (864.75 Da) (an exemplary calculation is shown in Figure 18a). Approximately 7.5 ST3-tetrasaccharide molecules were attached to CRM197 protein.

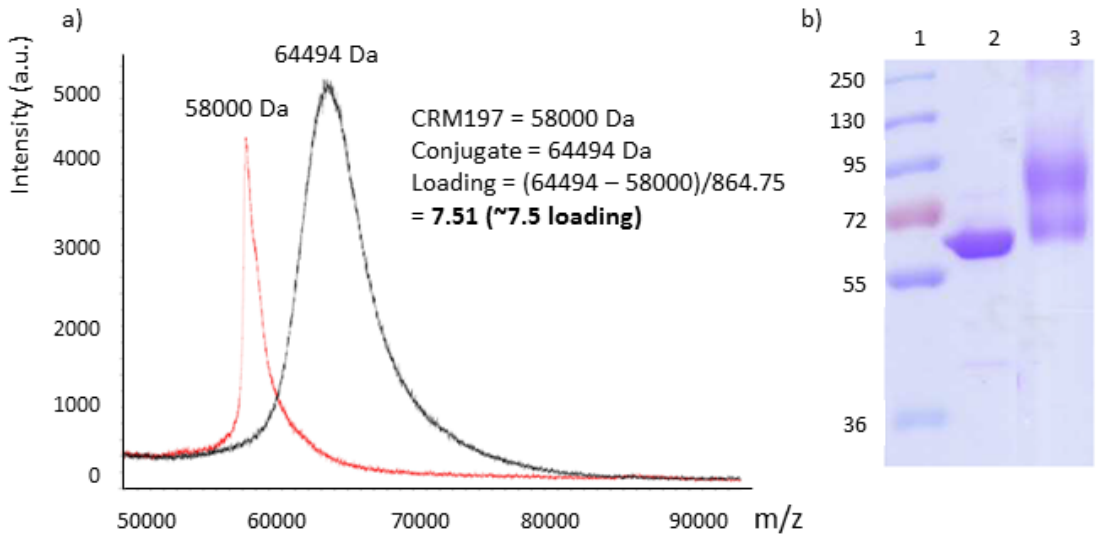


Figure 18. Characterization of the ST3-tetrasaccharide CRM197 conjugate by MALDI-TOF-MS and SDS-PAGE.
a) MALDI-TOF MS spectra of the glycoconjugate and CRM197 carrier protein. **b)** The 2 µg of CRM197 protein (line 2) and 2 µg of ST3-tetrasaccharide CRM197 glycoconjugate (line 3) were resolved on 10% SDS-PAGE and stained with PageBlue protein staining solution. The PageRuler Plus Prestained Protein Ladder was used as a molecular weight marker (line 1).

The choice of proper adjuvant is one of the most crucial steps in vaccine preparation. Aluminum-based adjuvants are common adjuvants used in marketed vaccines (e.g. Prevnar13®, Hepatitis A (Havrix) and B (Engerix-B) vaccine, Meningococcal group B MenB vaccine (Bexsero, Trumenba), etc.) The Alum-adjuvants are available in two different forms as aluminium hydroxide (Alhydrogel) and aluminium phosphate (Adju-Phos). During the formulation of the vaccine antigen adsorbed onto pre-formed $Al(OH)_3$ or $AlPO_4$ based on electrostatic attraction depending on the charge of both antigen and Alum-adjuvant type. Aluminum adjuvants have a large adsorptive capacity and usually, only a small dose is necessary to adsorb vaccine antigens. Nevertheless, sometimes a dose larger than required for maximal adsorption is necessary to achieve an optimal immune response (162, 163). The maximum amount of aluminum per dose in human vaccines in the United States is 0.85 mg Al^{3+} (162). To evaluate the best formulation of ST3-tetrasaccharide conjugated vaccine, different doses of glycoconjugate were used in combination with 0.125 mg of both aluminium hydroxide and aluminium phosphate. The chosen dose of adjuvant is equal to the amount of aluminium in commercial vaccine Prevnar13®.

The vaccines were prepared as described in Method 2.4.1. Samples were centrifuged and supernatants were transferred to new Eppendorf tubes. The amount of non-adsorbed conjugate was evaluated by the BCA assay (previously described in 2.4.2). The adsorption level of the glycoconjugate onto aluminium hydroxide is significantly higher than onto aluminium phosphate (percentage values varying depend on antigen amount, (Table 16)). The highest possible adsorption ratio is important for controlling the quality of the vaccine by the amount of antigen delivered and taken up by immune cells. Therefore, I want to ensure that the proper adjuvant choice results in the same adsorption level and no significant variation will be observed in vaccine success rate. The result of the experiment clearly indicated that aluminium hydroxide (Alhydrogel) is a better choice of adjuvant for the ST3-tetrasaccharide CRM197 conjugate vaccine.

Table 16. Adsorption level of ST3-tetrasaccharide CRM197 conjugate onto Alum particles in Aluminium-based adjuvants formulation with Al(OH)3 and AlPO4.
The adsorption percentage of the glycoconjugates were calculated as a recovery of protein in the non-adsorbed supernatants relative to controls without adjuvant. Glycoconjugate was incubated with adjuvant overnight (O/N) at 4°C while rotating. Protein concentration was measured by BCA assay. Data are shown as the average of free measurements.

Vaccine formulation (0.125 µg of Aluminium)	**Adsorption level (% of adsorption)**
Aluminium phosphate **+ 0.4ug** of glycan	50
Aluminium phosphate **+ 2.2ug** of glycan	~25
Aluminum phosphate **+ 4.4ug** of glycan	55-60
Aluminium hydroxide **+ 0.4ug** of glycan	85-90
Aluminium hydroxide **+ 2.2ug** of glycan	95
Aluminium hydroxide **+ 4.4ug** of glycan	82

3.2 Immunization With Different Doses Of Synthetic ST3-tetrasaccharide CRM197 Glycoconjugate Evokes the Production Of Anti-ST3 Capsular Polysaccharide Antibodies That Show *In-Vitro* Opsonophagocytic Activity

No general information regarding the optimal dosage of oligosaccharide antigen is available. However, to achieve the best immune response following vaccination, the proper dosage of antigen with respect to the purity of the product as well as an animal model, has to be established. Hence, six to eight-week-old C57BL/6 mice purchased from Charles River were divided into six groups (five animals per group) and immunized with an amount of ST3-tetrasaccharide CRM197 conjugate corresponding to a particular amount of ST3-tetrasaccharide antigen per injection (2.2 µg, 0.4 µg, 0.1 µg. 0.05 µg and 0.01 µg). Vaccines were formulated with aluminium hydroxide as described in 2.4.1. Immunization followed the standard prime-boost-boost-final boost regime (Table 17).

Table 17. Immunization regime for dose-finding study

Day	Action
0	Immunization; bleeding
10	Bleeding
21	1st boosting; bleeding
31	Bleeding
42	2nd boosting; bleeding
53	Bleeding
120	Bleeding
150	Bleeding
180	Final boost; bleeding
190	Bleeding

Serum collected at different time points was used to evaluate the *S.pneumoniae* serotype 3 capsular polysaccharide- and CRM197 carrier protein-specific antibody titer. ELISA assay shows that immunization with synthetic ST3-tetrasaccharide glycoconjugate elicits long term anti-carrier protein (CRM197-specific) antibody production on a similar level regardless of glycoconjugate dose. However, the higher doses of vaccine (2.2 µg and 0.4 µg) triggered a great antibody response already after the first immunization (day 10). The response to lower doses (0.1

µg, 0.05 µg, and 0.01µg) showed a slight delay (around 1 a.u. IgG titer after a first or second boost on day 31 or 35). The antibody level does not change significantly during the resting period (day 53 – 180) as well as after additional injections (final boost) of the vaccines (Figure 19). As protein antigens are in general stronger immunogens than glycans, especially in mice model the results are mostly used as an indication of successful immunization.

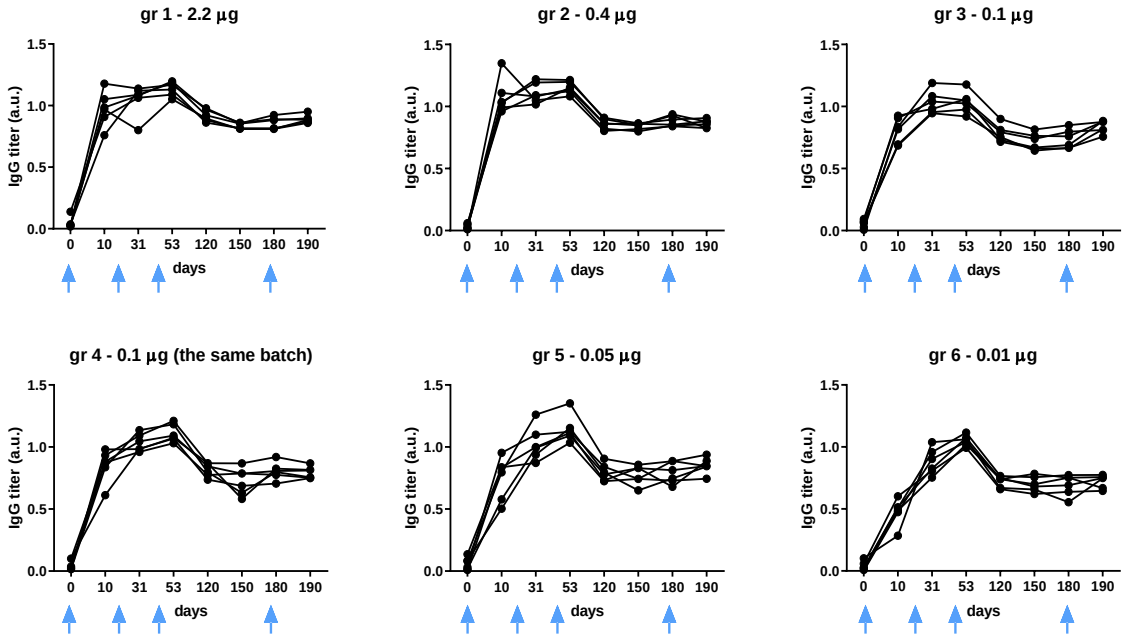


Figure 19. IgG antibody responses against CRM197 measured by ELISA.
Mice were s.c. immunized with glycoconjugate vaccines containing various doses of ST3-tetrasaccharide CRM197 conjugate corresponding to a 2.2 µg, 0.4 µg, 0.1 µg. 0.05 µg and 0.01 µg of ST3-tetrasaccharide. Blood was collected at different time points, and total serum IgG level was analyzed. The blue arrows indicate the immunization. Data are measurements of single animal antibodies titer on specified time points; n = 6 animal per group.

The aim of immunization with synthetic glycoconjugate is to trigger the production of antibodies against native capsular polysaccharide of *S.pneumonie* serotype 3. The anti-CPS antibody titer was monitored over the time of the study. In comparison to anti-protein response, the production of anti-CPS IgG was delayed in time (measurable response after the first boost on day 31). The response was unpredictable diverse both within the groups (not all animals produce anti-CPS antibodies) and between different vaccine doses. Anti-CPS antibody titer in a group 1 and group 2 immunized with the highest doses of synthetic tetrasaccharide (2.2 µg and 0.4 µg) was comparable to the group 6 injected with the lowest dose of 0.01 µg. Surprisingly, the group 5

(0.05 μg) shows the worst response. Only one out of five animals produce CPS-specific antibodies, while based on anti-CRM197 antibody level immunization by itself worked well (Figure 20 and Figure 21).

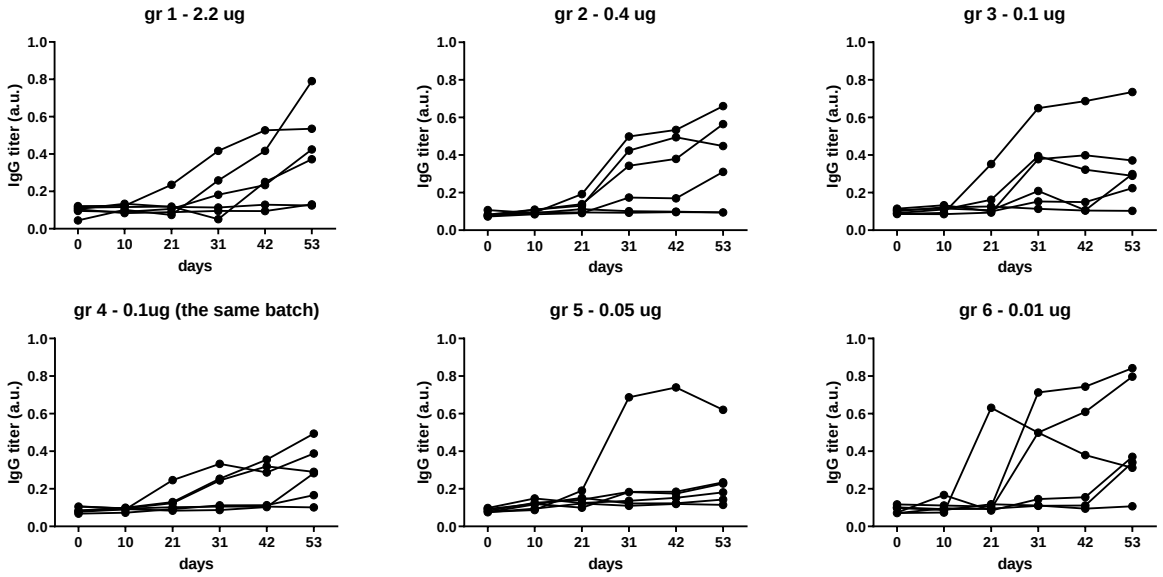


Figure 20. *S.pneumoniae* serotype 3 capsular polysaccharide specific antibody responses in groups immunized with different doses of ST3-tetrasaccharide measured by ELISA.
Mice were immunized with glycoconjugate vaccines containing various doses of ST3-tetrasaccharide. Blood was collected at different time points, and total IgG titer was analyzed in serum. The blue arrows indicate the immunization. Data are measurements of single animal antibody titers on specified time points; n = 6 animal per group.

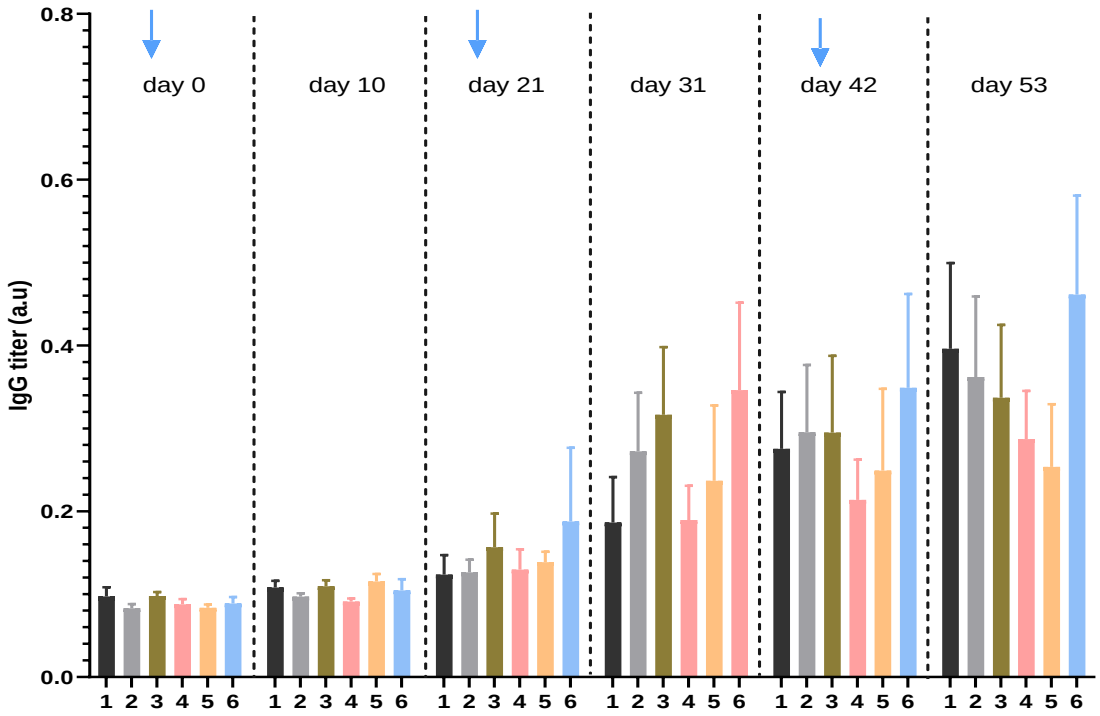


Figure 21. Comparison of the capsular polysaccharide of *S.pneumoniae* serotype 3 specific antibody titer between groups immunized with different doses of ST3-tetrasaccharide.
Mice were immunized with glycoconjugate vaccines containing various doses of ST3-tetrasaccharide; 2.2 µg (Group 1), 0.4 µg (Group 2), 0.1 µg (Group 3), 0.1 µg (different vaccine preparation, Group 4), 0.05 µg (Group 5) and 0.01 µg (Group 6). Blood was collected at different time points, and total IgG titer was analyzed in serum. The blue arrows indicate the immunization. Data are shown as mean ±SEM of each group at specified time points; n = 6 animal per group.

To evaluate the long term immune response, animals were rested for 18 weeks and received the final boost of ST3-tetrasaccharide CRM197 conjugated vaccine on day 180. No effect following the final immunization was observed (Figure 22). According to the regulation of the Office for Health and Social Affairs Berlin, the maximum allowed a length of the study was 209 days. We hypothesized that due to the time limitation, the relevant decrease in anti-CPS antibody titer could not be observed and the last boost did not cause an increase in the specific antibody level. Another assumption regarding the failure of final boosting might be caused by “overloaded” and unsensitized animals with the antigen, so it was not recognized as foreign anymore. The study conducted in infants says that previous or simultaneous exposure to an epitope or antigens may limit the immune response. The competition during antigen processing, presentation, or recognition of similar antigens can lead to lower immunogenicity for one or both antigens (164).

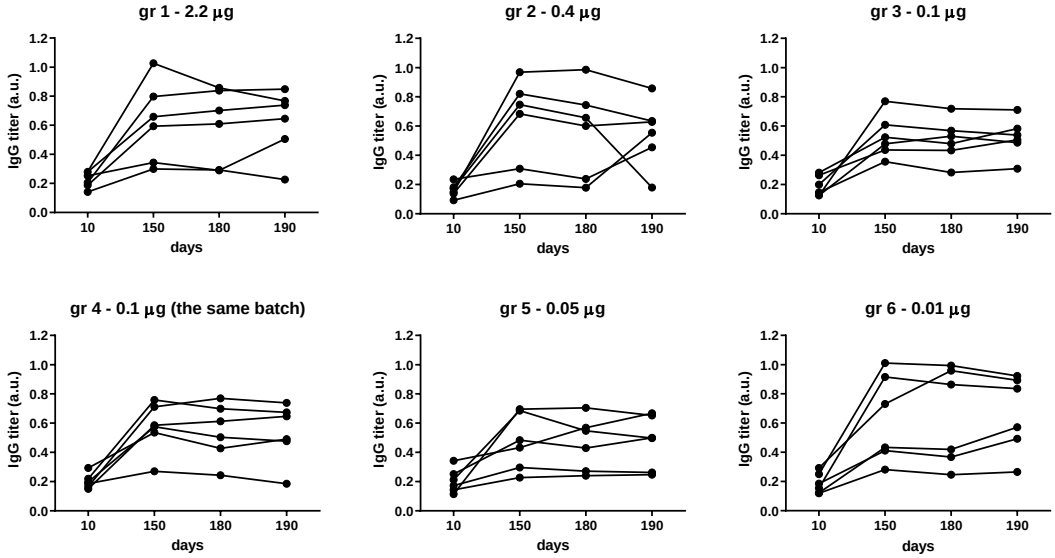


Figure 22. Long term IgG responses against native capsular polysaccharide of *S.pneumoniae* serotype 3 measured by ELISA.
Mice were immunized with glycoconjugate vaccines containing various doses of ST3-tetrasaccharide. Blood was collected at different time points, and total IgG titer was analyzed in serum. Late time points were plotted to show an immune response after final boosting in comparison to antibody level after priming Data are measurements of single animal antibodies titer on specified time points; n = 6 animal per group.

However, the study conducted by *Eskola et al*. has shown that lower antibody response is neither associated with impaired antibodies function nor with the induction of immune memory (165). Since antibodies to pneumococcal CPS protect the host against bacterial infection by opsonizing pneumococci and inducing phagocytosis, the opsonophagocytic killing assay (OPKA) has been widely accepted as the reference method for measuring the protective capacity of pneumococcal antibodies. The OPKA was performed to evaluate the variances in protective properties of serum from mice immunized with different doses of ST3-tetrasaccharide in glycoconjugate vaccines (as described in 2.4.5). Pooled sera of mice immunized with 2.2 µg (Group 1), 0.4 µg (Group 2), 0.1 µg (Group 3), 0.1 µg different vaccine preparation (Group 4), 0.05 µg (Group 5) and 0.01 µg (Group 6) of ST3-tetrasaccharide were used. Before the assay, sera were heat-inactivated to avoid any dissimilarities in the level of complement (24). The OPKA results confirm that the synthetic glycoconjugate vaccines elicit opsonic antibodies that can kill *S.pneumoniae* serotype 3. The dilutions of WHO 007sp typing serum necessary for 50% bacterial killing between 128 - 512 are considered biologically significant for successful vaccines and served as a control. The

serum dilution responsible for 50% of bacteria-killing varies from group to group (Figure 23). No significant differences were observed ($p > 0.05$), however, 0.4 µg of ST3-tetrasaccharide synthetic glycan conjugated to carrier protein CRM197 is the dose of the vaccine inducing antibodies with the best opsonophagocytic activity (the highest serum dilution responsible for the killing of 50% bacteria, IC_{50} = 4767). Interestingly, the second-best dose contains the lowest antigen amount (0.01 µg of synthetic glycan, IC_{50} = 3784,5). No correlation between antibody titer and opsonophagocytic protective activity was detected (p values > 0.05 and very low r^2) (Figure 24) so quantitative characteristics of antibodies (antibody level) do not contribute to OPA and the estimation of antibody activity.

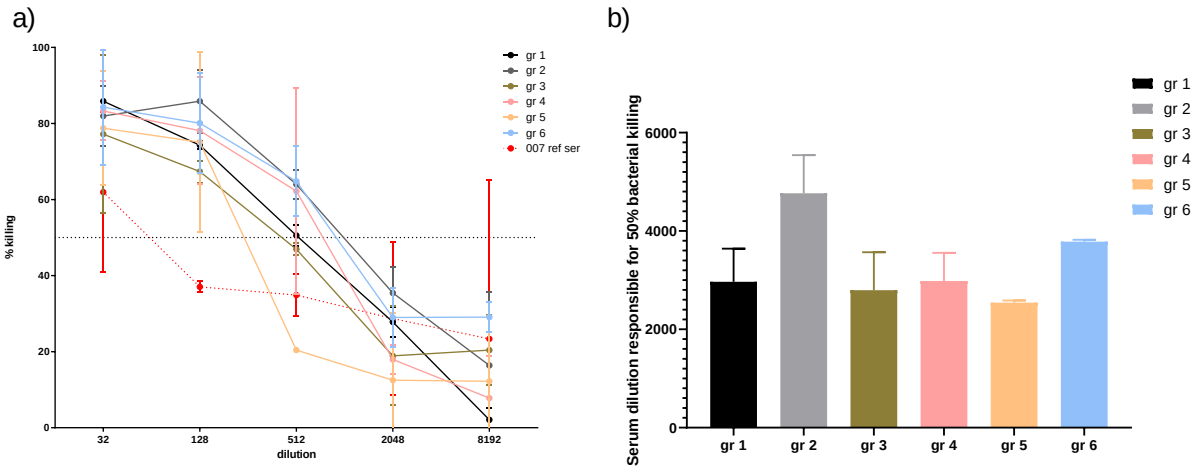


Figure 23**.** ***The in-vitro*** **opsonophagocytic activity of sera from mice immunized with glycoconjugate vaccine containing different doses of ST3-tetrasaccharide.**
a) A comparison of OPKA activity of pooled sera from mice immunized with 0.2 µg (Group 1), 0.4 µg (Group 2), 0.1 µg (Group 3), 0.1 µg (different vaccine preparation, Group 4), 0.05 µg (Group 5) and 0.01 µg (Group 6) doses of ST3-tetrasaccharide. Serum from blood collected on day 45 was used. Data are means ± SD of CFU reduction relative to negative control wells (samples lacking either antibody or complement) of three independent experiments. **(b)**. The serum dilution responsible for 50% killing of *S.pneumoniae* serotype 3 was estimated through non-linear interpolation of the dilution-killing OPKA data. Data are mean ± SD of 50% killing values from OPKA using blood collected on day 53 and 190. Results were analyzed by the two way ANOVA, and p-value of ≤ 0.05 was considered significant.

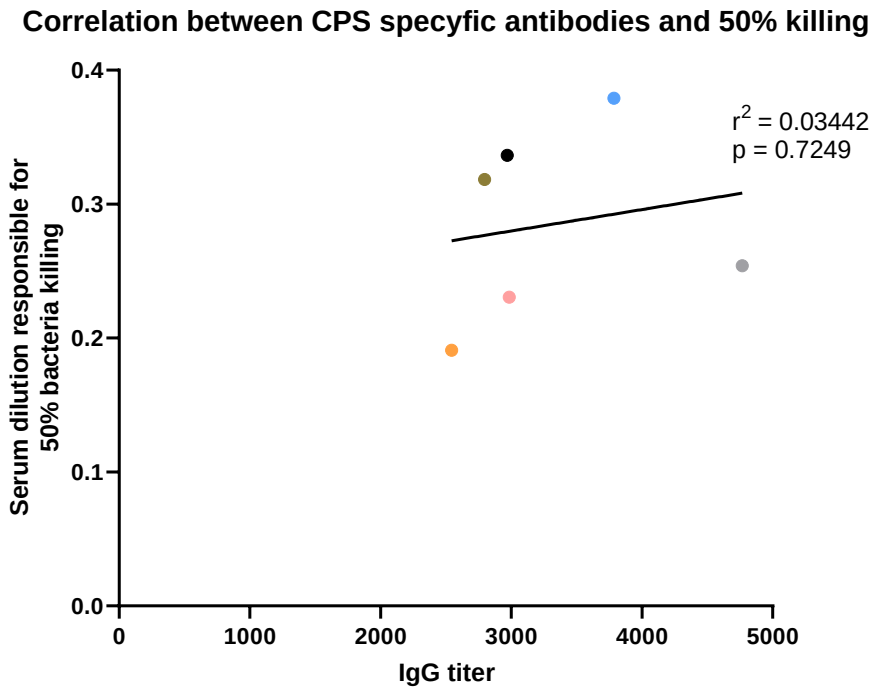


Figure 24. Correlation between specific antibody titer and serum dilution responsible for the killing of 50% bacteria.
Relationship between *S.pneumoniae* serotype 3 CPS specific antibody titer from group immunized with different amount of ST3-tetrasaccharide CRM197 conjugate corresponding to a particular amount of ST3-tetrasaccharide antigen (measured by ELISA) and serum dilution responsible for the killing of 50% bacteria (measured by OPKA). Plots show Pearson's correlations (r^2 value coefficient), a *P* value of ≤ 0.05 was considered significant.

In summary, the CPSs isolated from bacterial culture usually contain many impurities which influence the activity of vaccine and the overall antigen dose cannot be dramatically decreased. Chemical synthesis of the highly pure and active antigen is the biggest advantage of the ST3-tetrasaccharide semisynthetic conjugated vaccine. With this study, I proved that immunization with a significantly reduced amount of synthetic antigen does not decrease the protective activity of serum. Compared to the dose used in the commercial vaccine (2.2 μg), the lowest dose of 0.01 μg gives still better IgG titer responsible for 50% of the killing of bacteria.

As establishing the best dosage is a crucial step for vaccine development, for subsequent study, I decided to follow the results and use 0.4 μg of ST3-tetrasaccharide synthetic glycan conjugated to carrier protein CRM197, which gave the best protective effect. The data is relevant for the mouse model and has to be evaluated in clinical studies for human use.

3.3 Immunization With ST3-Pneumolysin Glycoconjugate Triggers Production of Protective Anti-Carbohydrate and Anti-Pneumolysin Antibodies

The bacterial capsule is the first target for the immune system and anti-CPS antibodies are highly protective. Therefore, commercial vaccines against *S.pneumoniae* contain capsular polysaccharides conjugated to the CRM197 carrier protein. However, each serotype of *S.pneumoniae* diverges based on differences in the polysaccharide structure and the replacement of serotypes included in vaccines with previously less prevalent pneumococcal serotypes occur. The serotype replacement problem has been a major reason for intensive research in the direction of developing a ‘universal’ broadly cross-protective pneumococcal vaccine. Targeting multiple membrane proteins based on their roles in bacterial pathogenicity and physiology seems to be a promising approach to achieve additive protection against pneumococci in mice. It has been well documented that protein carriers in glycoconjugate vaccines additionally induce an immune response against themselves, so-called "additional valency". Therefore, conjugation of synthetic oligosaccharides from the capsules of the most prevalent serotypes to conserved bacterial protein was the goal of this study.

Pneumolysin is a cholesterol-binding toxin and one of the major virulence factors of *S.pneumonie.* The detoxified version of the protein has been chosen as a proof of concept for broadening the protection of ST3-tetrasaccharide conjugated vaccine.

The plasmid expression vectors for the *S. pneumoniae* pneumolysin mutants (PlyW433E) were transformed into BL21 competent *E.coli,* and the recombinant pneumolysin proteins containing His-tagged were purified using the Ni-NTA column (Figure 25). The synthetic ST3-tetrasaccharide was conjugated to the PlyW433E by the bifunctional linker, and the ST3-tetrasaccharide pneumolysin conjugate (Ply-ST3-tetrasaccharide) was characterized by MALDI-TOF and SDS-PAGE gel electrophoresis (Figure 25) with the average loading ratio of seven synthetic tetrasaccharide molecules attached to a pneumolysin carrier protein.

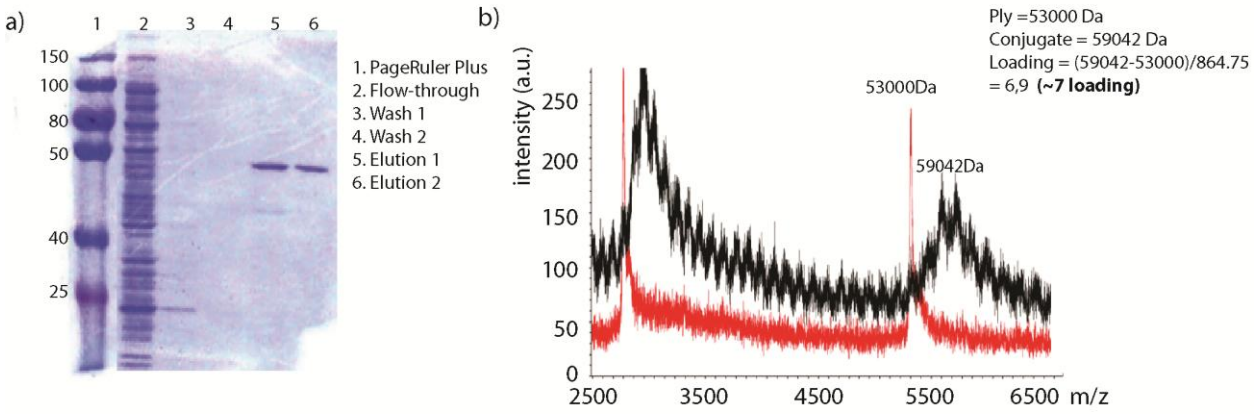


Figure 25. Characterization of purified pneumolysin PlyW433E by SDS-PAGE gel and ST3-tetrasaccharide pneumolysin conjugate by MALDI-TOF-MS.
a) Characterization of the fractions from the pneumolysin purification process. Flow-through (line 2), washing steps (line 3 and 4) as well as elutions (line 5 and 6) were separated by 10% SDS-PAGE electrophoresis and stained with PageBlue protein staining solution The PageRuler Prestained Protein Ladder was used as a molecular weight marker (line 1). **b)** Matrix-assisted laser desorption/ionization (MALDI) analysis was carried out to measure the average molecular size of the ST3 tetrasaccharide Pneumolysin conjugate and the Pneumolysin carrier protein as a standard.

Six to eight week old female C57Bl/6 mice were immunized s.c with the dose of 0.4 µg of ST3-tetrasaccharide conjugated to pneumolysin carrier protein adsorbed onto aluminium hydroxide following the schedule prime-boost-boost. Serum for analysis of the immune response to the vaccination was collected (Table 18).

Table 18**. Immunization regime for a vaccination with ST3-pneumolysin glycoconjugate.**

day	action
0	Immunization; bleeding
7	Bleeding
14	1st boosting; bleeding
21	Bleeding
28	2nd boosting; bleeding
35	Bleeding

The presence of the IgG antibodies against the synthetic ST3-tetrasaccharide on day 0, 21, and 35 were measured by glycan array (Figure 26a). Sera containing antibodies that are cross-

reactive with native CPS of *S.pneumoniae* serotype 3 and antibodies recognizing native pneumolysin was evaluated by ELISA (Figure 26b). The antibodies show protective activity against serotype 3 by *in vitro* opsonophagocytic killing assay (OPKA). No cross-protection with unrelated serotypes was proven, which leads to the conclusion that only glycan-specific antibodies can bind the complement and cause the killing of bacteria (Figure 26c). Nevertheless, the conjugation procedure does not influence the antigenic properties of the pneumolysin protein. This results support the use of pneumolysin as a carrier protein in order to broad pneumococcal vaccine coverage.

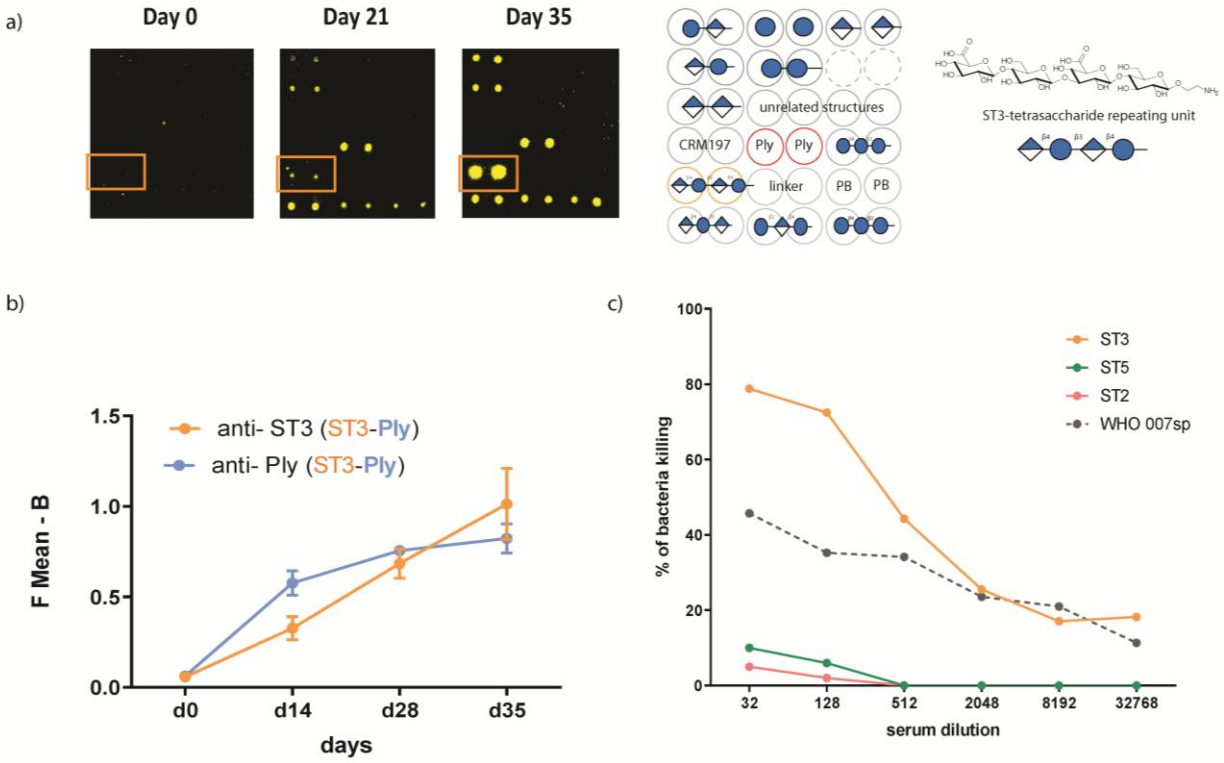


Figure 26. Mice immunized with ST3-tetrasaccharide pneumolysin conjugate vaccine produce protective ST3-tetrasaccharide and pneumolysin specific antibodies

a) Representative picture of glycan array analysis of serum from mice immunized with the ST3-tetrasaccharide pneumolysin conjugate. The dots marked with the frame showed the binding of the antibody to ST3-tetrasaccharide printed on a glass slide. The anti-mouse IgG Alexa594 in a dilution 1:400 was used as a secondary antibody. **b)** Evaluation of ST3-tetrasaccharide and pneumolysin specific antibody level by ELISA in mice sera collected on day 35. The plate was coated with both *S.pneumoniae* serotype 3 CPS (10 µg/mL) and native pneumolysin protein (10 µg/mL). **c)** The *in-vitro* opsonophagocytic activity of serum from mice immunized with ST3-tetrasaccharide pneumolysin conjugated. Blood was collected on day 35. Pooled sera from mice vaccinated with glycoconjugate vaccine as well as control WHO 007sp typing serum were used against *S.pneumoniae* serotype 2 (ST2), serotype 3 (ST3), and serotype 5 (ST5). Data are means of CFU reduction relative to negative control wells lacking either antibody or complement.

3.4 Anti-Pneumolysin Antibodies Inhibit the Hemolysis of Human Red Blood Cells by Whole Bacterial Cell Lysate and Prevent Alveolar Epithelial Cell Permeability *In-Vitro*

It has been shown in many studies that anti-pneumolysin antibodies reduce the cytotoxic effects of Pneumolysin and delay the onset of disease caused by *S.pneumoanie* (166, 167). As demonstrated by *Salha et al*., the anti-pneumolysin monoclonal antibody known to inhibit the hemolytic activity of the toxin *in vitro* was also able to confer protection *in vivo* by reducing tissue damage, proinflammatory IL-6 response, or neutrophil infiltration in lungs (166).

To prove that the anti-toxin antibody in serum collected from mice immunized with ST3-tetrasaccharide pneumolysin conjugated vaccine can reduce the cytotoxic effect of the protein, the *in vitro* human red blood cells hemolysis assay was performed. Briefly, a polyclonal mice sera (collected on day 35) containing anti-pneumolysin antibody was incubated with 25 ng of native pneumolysin in the presence of human red blood cells (hRBCs). The hemoglobin release measure at the absorbance $A_{540nm.}$indicates the damage of the red blood cell membrane by the toxin (erythrocytes lysis). Serum collected on day 35 shows strong toxin neutralizing activity. Anti-pneumolysin antibodies were able to completely inhibit the lysis of hRBCs compared to serum withdrawn on day 0. The neutralizing effect was visible up to 80 x serum dilution (Figure 27a,b). The assay was performed with pooled serum. Therefore, statistical analysis was not performed.

Pneumolysin is a conserved multi-functions membrane protein among all *S.pneumoniae* serotypes. To demonstrate that use of the detoxified pneumolysin as a carrier protein for synthetic carbohydrate vaccines can extend the cross-serotype protection by reducing the symptoms of the infection with a serotypes not included in a vaccine or during co-infection with non-related bacteria, hRBCs were incubated with the mice serum collected on day 35 and bacterial lysate of three different *S.pneumoniae* serotypes: serotype 2, 3 and 8. Significant reduction of hemoglobin released was observed independent on serotype used in the assay (Figure 27c) with probability values accordingly fo ST2 $p = 0.0065$, ST3 $p = 0.0008$ and ST8 $p = 0.0261$. As the bacteria cell lysate contains different toxins and lytic components; minor hemolysis was still noted. The most significant reduction in cytotoxicity was seen with lysate of

serotype 3 which might suggest the additional neutralizing effect of specific anti-glycan antibodies.

Epithelial cells are one of the targets for *S.pneumoniae.* They contribute to maintaining the barrier function in the lung as well as in the brain. The disruption of epithelial cell integrity of the lungs causes acute pneumonia and in a brain leads to meningitis. Hence, the effects of pneumolysin on epithelial barrier function was analyzed *in-vitro*. The human alveolar basal epithelial cell line A549 was exposed to cell lysate of *S.pneumoniae* serotype 2, 3 and 8 pre-incubated with and without the serum from mice immunized with the ST3-tetrasaccharide pneumolysin conjugated vaccine (blood was collected on day 35). Changes in transcellular electrical resistance of cell monolayers were analyzed by Electric Cell-substrate Impedance Sensing (ECIS). Serum containing anti-pneumolysin antibody significantly inhibit the effect of the toxin on the cells monolayer electrical resistance (Figure 27d), indicated as a lower decrease in A549 monolayer permeability compares to a cell with bacterial lysate only (p-value > 0.0001).

The above results indicate that mice immunized with the ST3-tetrasaccharide pneumolysin conjugated vaccine produce high levels of pneumolysin-neutralizing antibody, which may block acute lung injury in the early course of pneumococcal pneumonia and following respiratory failure. The antibodies against pneumolysin produce after immunization with the semi-synthetic ST3-tetrasaccharide pneumolysin conjugated vaccine, therefore have additional beneficial effects in the prevention and treatment of severe pneumococcal pneumonia.

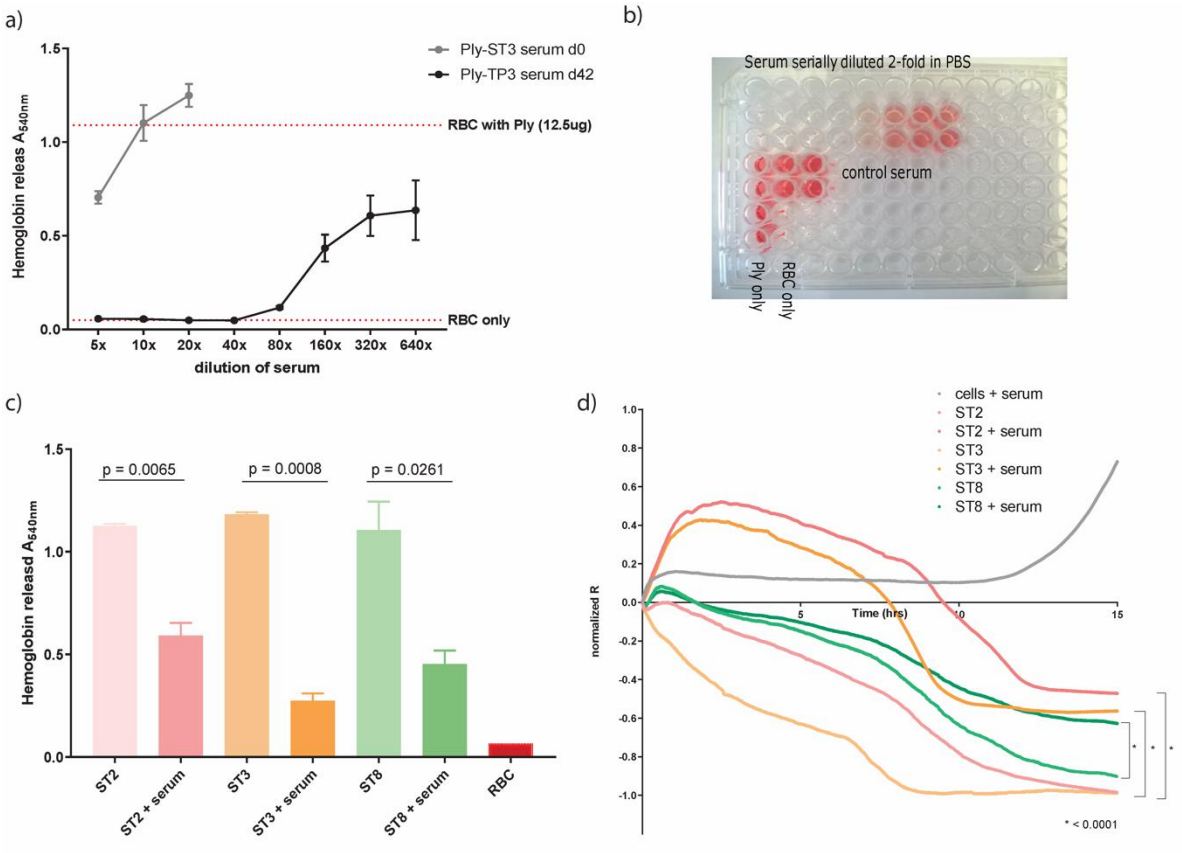


Figure 27. Inhibition of serotype independent pneumolysin induced lysis of human red blood cells by anti-pneumolysin antibodies from mice immunized with ST3-tetrasaccharide pneumolysin conjugated vaccine.

a) Inhibition of hemolytic activities of native pneumolysin by antibodies from mice immunized with ST3-tetrasaccharide pneumolysin conjugate. Hemoglobin release is expressed as the A_{540} of assay supernatants (mean ± SD). **b)** Representative picture of the results of hRBC hemolysis assay. **c)** The inhibition of hemolytic activities of the whole bacteria cells lysates of *S.pneumoniae* serotype 2, 3 and 8 by antibodies from mice immunized with ST3-tetrasaccharide pneumolysin conjugated vaccine. Hemoglobin release is expressed as the A_{540} of assay supernatants (mean ± SD). The statistical significance was calculated by paired t-test. **d)** Serum from mice immunized with ST3-tetrasaccharide pneumolysin conjugate protects pneumolysin-induced endothelial barrier disruption. A549 cells were treated with the whole cell lysate of *S.pneumoniae* serotype 2, 3 and 8 with and without serum from mice immunized with ST3-pneumolysin (blood collected on day 35). Transcellular electrical resistance was continuously monitored and normalized to baseline resistance. Statistical significance was calculated by unpaired t-tested between bacteria lysate with and without antibodies. * $p<0.0001$

3.5 Immunization With ST3-Tetrasaccharide Pneumolysin and CRM197 Conjugates Decreases the Bacterial Load in Blood and Lungs as well as Reduces Disease Severity in Mice Challenged With *S.Pneumoniae* Serotype 3 Bacteria

To determine whether ST3-tetrasaccharide pneumolysin and CRM197 conjugated vaccines are functional in a disease setting, mice were immunized with a dose of glycoconjugates corresponding to a 0.4 µg of ST3-tetrasaccharide antigen following the prime + boost + boost regime (Table 19) and intranasally infected with *S. pneumoniae* serotype 3 as described in 2.6.2. To evaluate the serotype-independent protection by anti-pneumolysin antibodies, an additional group of animals was challenged with *S. pneumoniae* serotype 2, (after the same immunization regime). Control mice received Prevnar13® and PBS. Clinical signs, body weight, and rectal temperature were monitored every 12 hours following a clinical score. The pulmonary bacterial outgrowth and blood bacterial load were examined after animals were sacrificed 36 and 48 hours post-infection. ST3-tetrasaccharide and carrier proteins specific antibody level was analyzed by glycan microarray and correlated with disease severity.

Table 19. Immunization and infection regime for *S.pneumoniae* serotype 2 and serotype 3 challenge study.

day	action
0	Immunization; bleeding
7	Bleeding
14	1st boosting; bleeding
21	Bleeding
28	2nd boosting; bleeding
35	Bleeding
42	Bleeding
49	Infection with *S.pneumoniae*
51-52	Dissection

Immunization with ST3-tetrasaccharide CRM197 conjugate and commercial vaccine Prevnar13® significantly alleviated the disease severity (no decrease in body weight and absence of hypothermia) when compared to the control PBS treated mice (Figure 28a and b). Clinical

manifestation of the diseases correlates with a bacterial burden in lungs and blood (Figure 28c and d) as well as specific antibody levels (Figure 29 and Figure 30). Immunization with ST3-tetrasaccharide pneumolysin conjugate also showed a substantial positive effect on mice infected with *S.pneumoniae* serotype 3 but lack of statistical significance was observed. Nevertheless, the ST3-tetrasaccharide pneumolysin conjugated showed promising results and required additional investigation.

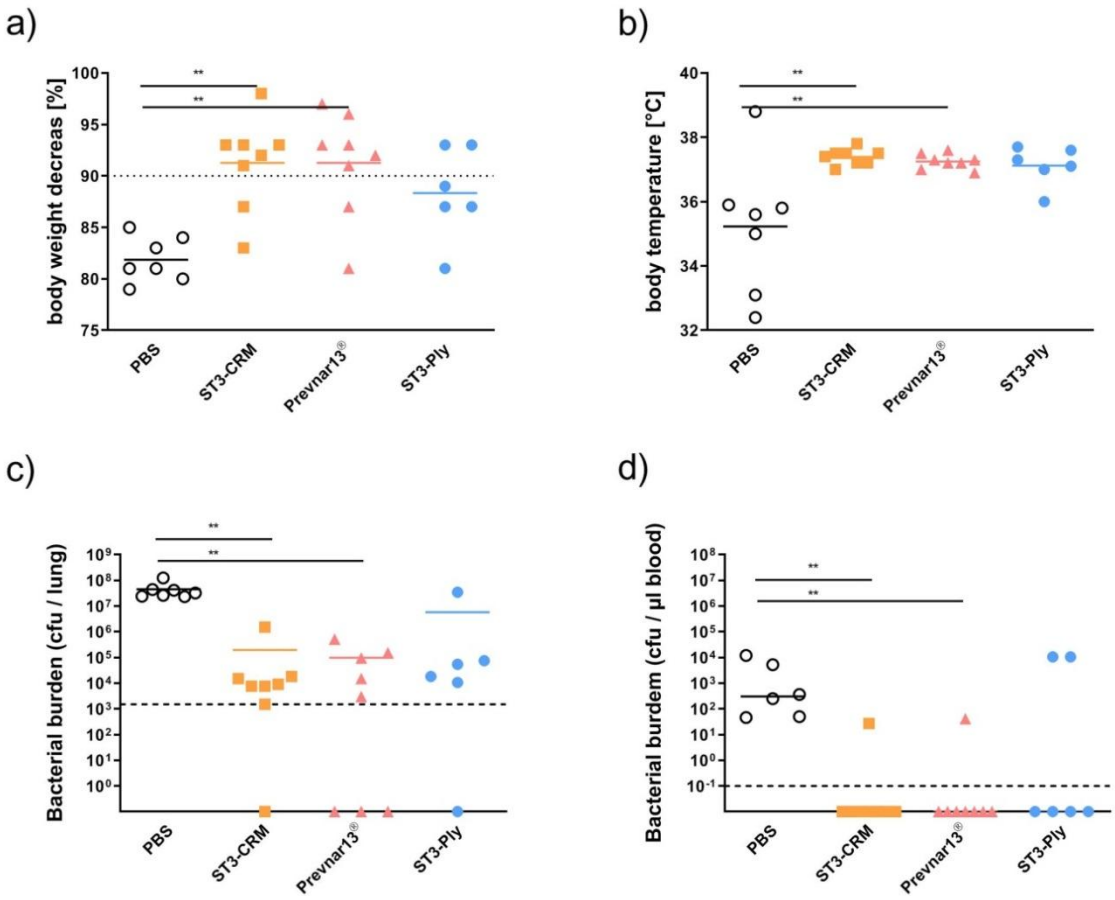


Figure 28. Evaluation of the protective effect of ST3-tetrasaccharide pneumolysin (ST3-Ply) conjugate, ST3-tetrasaccharide CRM197 (ST3-CRM) conjugate, and Prevnar13® vaccines in a mice challenge model of *S.pneumoniae* serotype 3.
a) The percentage decrease in body weight 48 h after transnasal infection with live *S.pneumoniae* serotype 3 in mice immunized with ST3-tetrasaccharide pneumolysin and CRM197 conjugates, as well as Prevnar13® vaccines. Control group received PBS only. **b)** Body temperature changes in mice receiving different vaccines 48h after challenge with bacteria. Healthy animals maintain the body temperature around 37°C and control PBS group shows hypothermia (below 36°C). Bacterial burden in lungs **(c)** and blood **(d)** collected 48h after infection with *S.pneumonie* serotype 3 after immunization with different vaccines candidates. Colony-forming units (CFU) were calculated for single animals, and values were transformed to a logarithm scale. Two animals from group vaccinated with ST3-tetrasaccharide pneumolysin conjugate were excluded as immunization did not work correctly (lack of specific antibodies). Data are individual values from n=8 mice (n=6 for ST3-tetrasaccharide pneumolysin conjugate group) with mean displayed. Statistical analysis was performed by the Kruskal-Wallis and Dunn's multiple comparison tests.

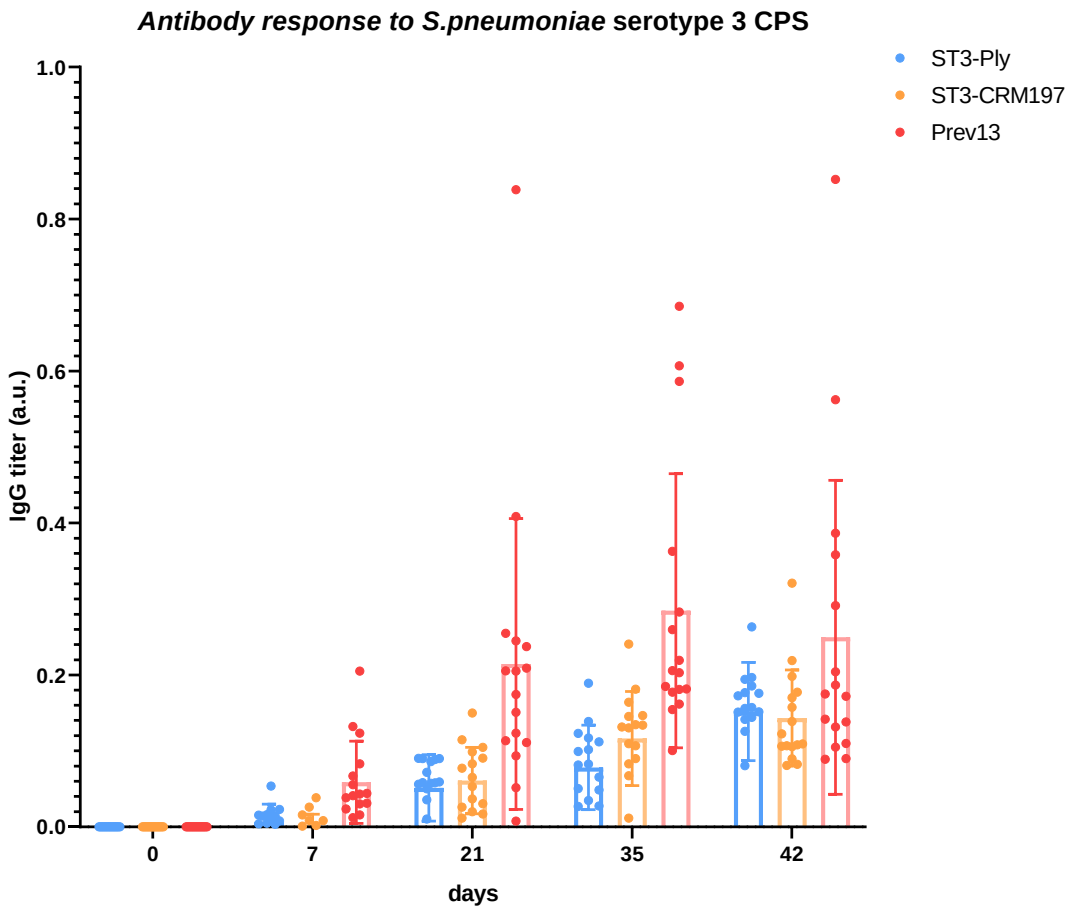


Figure 29. Evaluation of *S.pneumoniae* serotype 3 capsular polysaccharide specific antibody response in mice immunized with ST3-tetrasaccharide pneumolysin and CRM197conjugated vaccines, as well as Prevnar13®.
Antibodies from mice injected with semisynthetic ST3-pneumolysin (blue) and CRM197 (orange) conjugates recognize CPS of *S.pneumoniae* serotype 3. Animals immunized with Prevnar13® (red) were used as a control. Samples were analyzed by ELISA. Graphs show mean adsorption level (arbitrary units) ± SD value and individual points indicate a value for a single animal (n = 16).

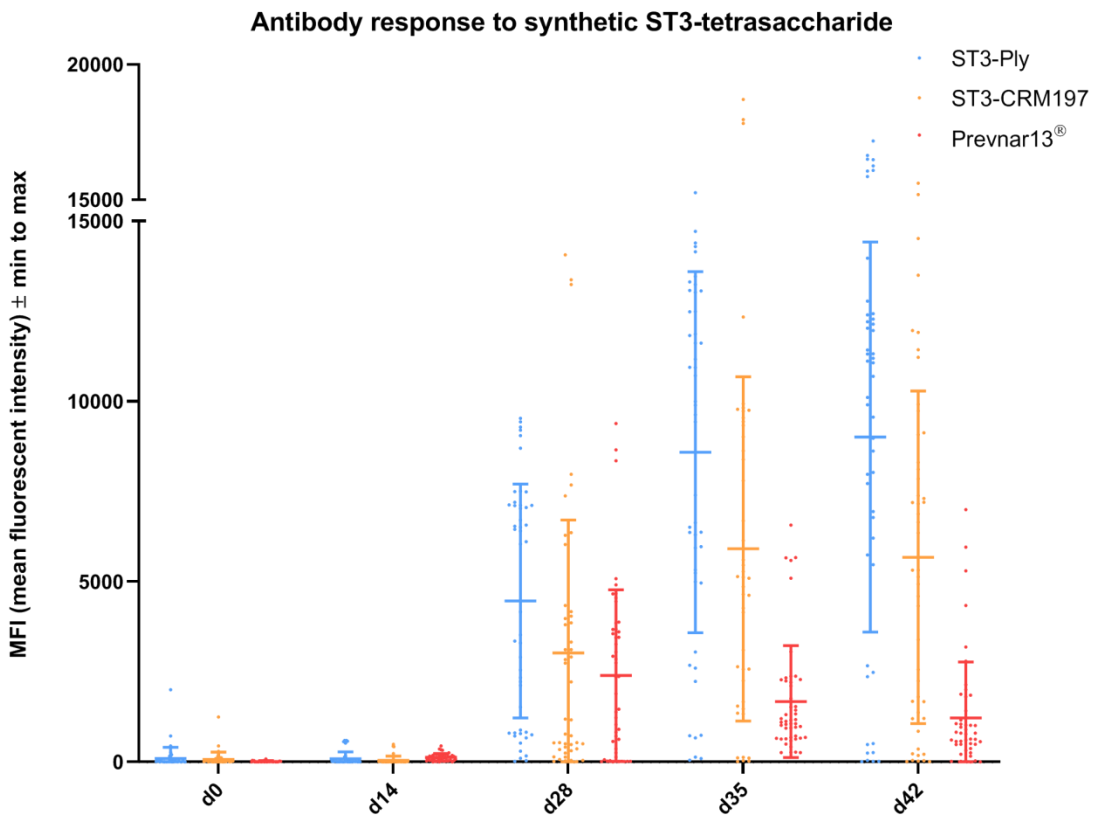


Figure 30. Evaluation of ST3-tetrasaccharide specific antibody response in mice immunized with ST3-tetrasaccharide pneumolysin and CRM197 conjugated vaccines, as well as Prevnar13®.
Recognition of ST3-tetrasaccharide by antibodies from mice immunized with ST3-tetrasaccharide pneumolysin conjugate (blue), ST3-tetrasaccharide CRM197 conjugate (orange) and commercial vaccine Prevnar13® (red). Samples were analyzed by glycan array. Graphs show mean fluorescence intensity ± SD value, and individual points indicate a fluorescence intensity for a single animal (n = 16) in triplicate.

To elucidate a potential systemic serotype independent effect of anti-pneumolysin antibodies in the *in vivo* model, mice were immunized with ST3-tetrasaccharide pneumolysin, and CRM197 conjugated vaccines, Prevnar13® as well as PBS control followed by intranasal infection with *S. pneumoniae* serotype 2. Clinical signs (body weight and temperature decrease), as well as bacteremia and pulmonary bacterial load, were evaluated (Figure 31). The data obtained from the experiment did not show any significant differences between treatment groups. Even control animals injected with PBS only did not demonstrate expected clinical symptoms. Therefore, we concluded that the challenge model was not efficient.

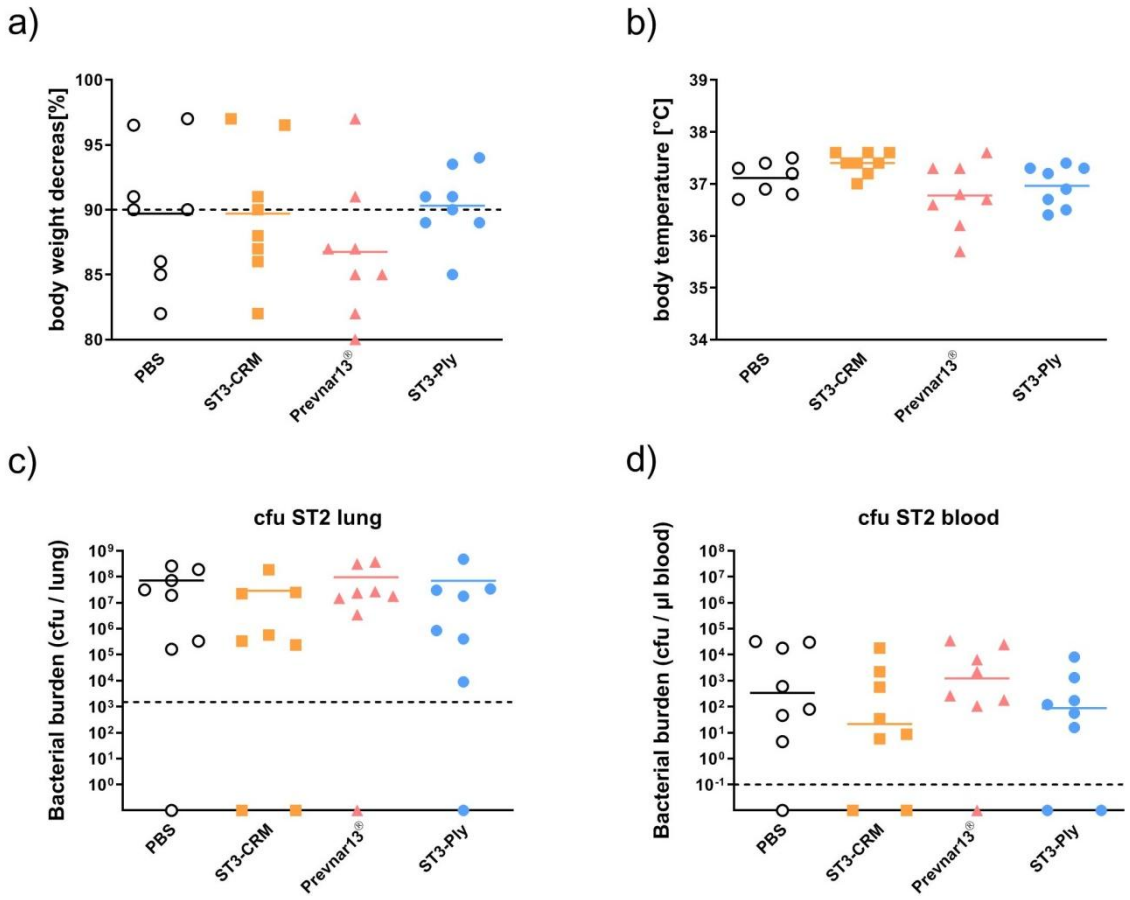


Figure 31. Assessment of the cross-serotype protective effect of ST3-tetrasaccharide pneumolysin, ST3-tetrasaccharide CRM197, and Prevnar13® vaccines in a mouse challenge model of *S.pneumoniae* serotype 2.

a) The percentage reduction of body weight in mice 36h after transnasal infection with live *S.pneumoniae* serotype 2 followed the immunization with ST3-tetrasaccharide pneumolysin and CRM197 conjugates, as well as Prevnar13® vaccines. Control group received PBS only. **b)** Body temperature changes in mice receiving different vaccines 36h after challenge with bacteria. Bacterial burden in lungs (**c)** and blood **(d)** collected 36h after infection with *S.pneumoniae* serotype 2 after immunization with different ST3 vaccine candidates and Prevnar13®. Colony-forming units (CFU) were calculated for single animals, and values were transformed to a logarithm scale. Data are individual values from n = 8 mice and the mean was presented. Statistical analysis was performed by the Kruskal-Wallis test and Dunn's multiple comparison test.

3.6 Nasal Immunization With ST3-Tetrasaccharide and ST2-Hexasaccharide Conjugated to Pneumolysin and PspA Inhibits Colonization of the Nasopharynx After Challenge With *S.pneumoniae* Serotype 2.

One of the most critical steps in the development of pneumococcal infections is nasopharyngeal colonization by the bacteria (168). It has been proved that *S.pneumoniae* virulence factors such as pneumolysin (Ply) and pneumococcal surface antigen A (PspA) play a crucial role in establishing the initial step and the subsequent spreading of the pathogen (32, 38, 169), therefore they are considered as a promising protein vaccine candidates. To evaluated the potential of pneumolysin and PspA as both carrier and an additional antigen for glycoconjugate vaccine, the proteins were conjugated to both synthetic ST3-tetrasaccharide and ST2-hexasaccharide. CRM197 carrier protein was used as a control. Glycoconjugates were characterized by SDS-PAGE (Figure 32) and MALDI-TOF-MS (Figure 33). The glycoconjugate conjugation ratios are shown in Table 20.

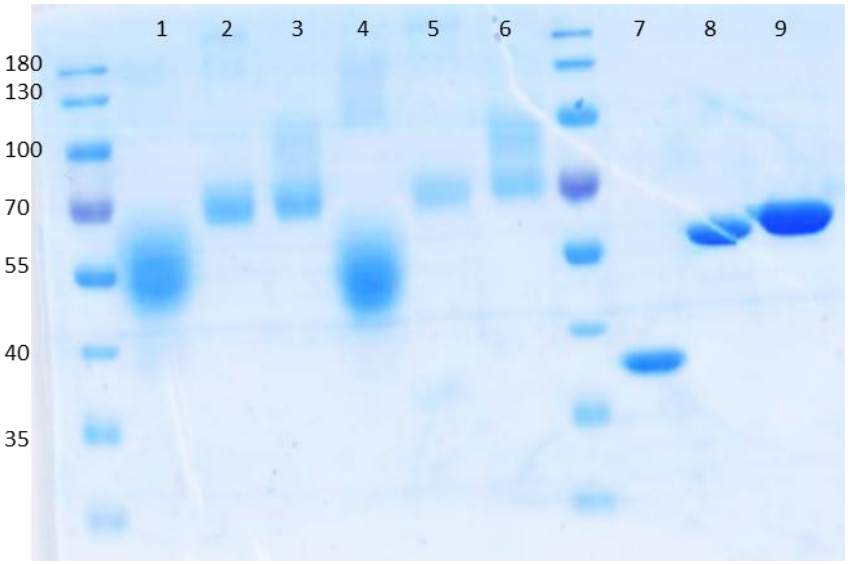


Figure 32. Characterization of ST3-tetrasaccharide and ST2-hexasaccharide conjugated to pneumolysin and PspA by SDS-PAGE electrophoresis.
Line 1: 1µg of ST3-tetrasaccharide PspA conjugate; Line 2: 1 µg of SP3-tetrasaccharide pneumolysin conjugate; Line 3: 1µg of SP3-tetrasaccharide CRM197 conjugate; Line 4: 1µg of SP2-hexasaccharide PspA conjugate; Line 5: 1µg of SP2- hexasaccharide pneumolysin conjugate; Line 6: 1µg of SP2- hexasaccharide CRM197 conjugate; Line 7: 1µg of PspA; Line 8: 1µg of pneumolysin; Line 9: 1µg of CRM197. PageRuler Plus Prestained Protein Ladder was used as a molecular weight standard.

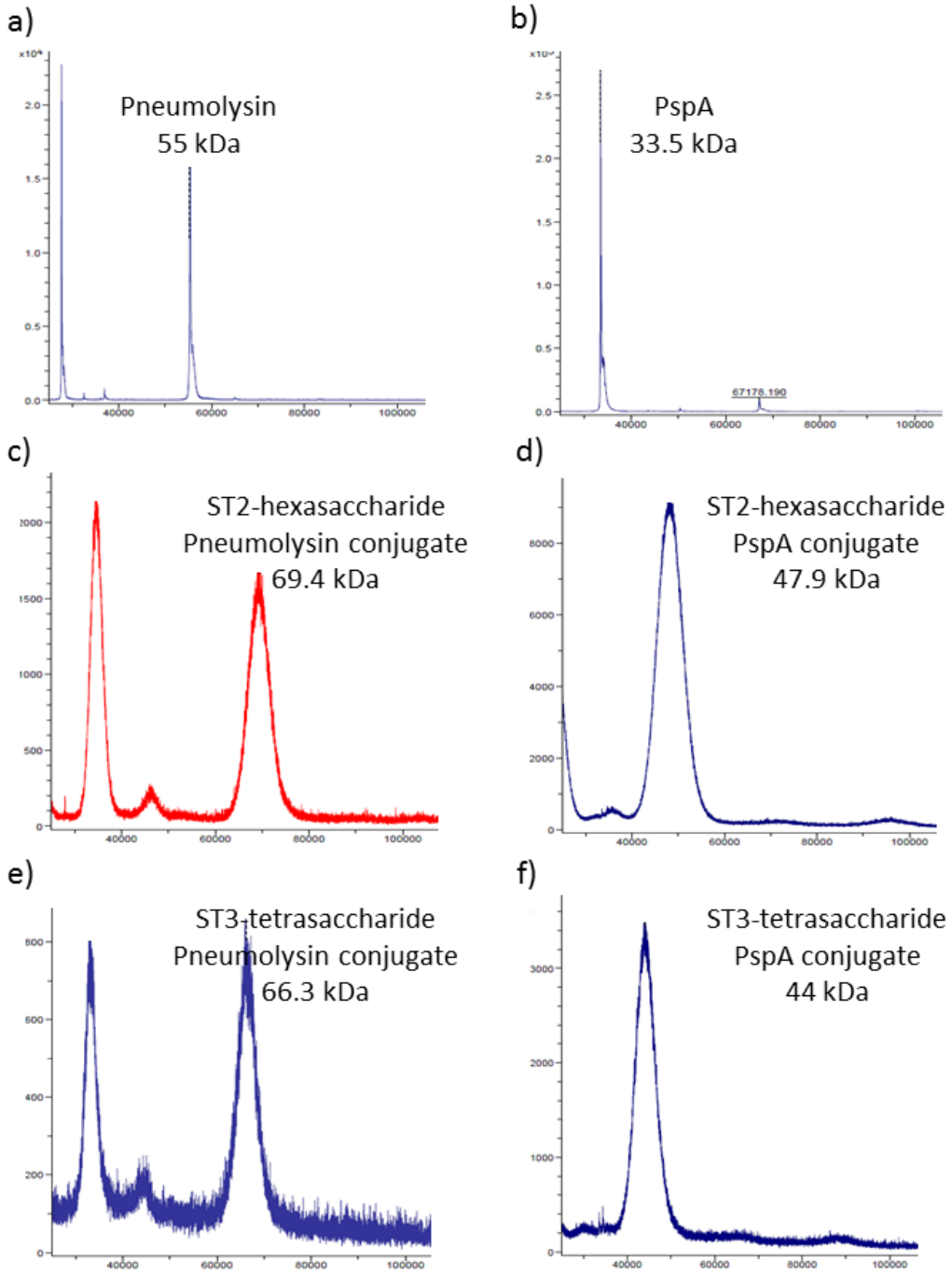


Figure 33. MALDI-TOF analysis of ST3-tetrasaccharide and ST2-hexasaccharide conjugated to pneumolysin and PspA carrier proteins.
The analysis was carried out to measure the average molecular size of conjugate and the loading ratio of the glycan on the carrier protein. Recombinant pneumolysin and PspA were used as standards.

Table 20. Glycoconjugates used for the nasal immunization of mice before challenge with *S.pneumoniae* serotype 2.

Construct name	Loading	Concentration (mg/ml)
SP2-hexasaccharide CRM197	9.7	2.26
SP2-hexasaccharide pneumolysin	12.2	2.34
SP2-hexasaccharide PspA	12.6	1.4 B
SP3-tetrasaccharide CRM197	11.0	2.56
SP3-tetrasaccharide pneumolysin	12.9	1.88
SP3-tetrasaccharide PspA	12.5	2.02

The pneumococcal colonization model is characterized by the bacteria present in the lower airways with an accompanying inflammatory cell response for at least 14 days post-infection without acute disease symptoms. *S.pneumoniae* D39 serotype 2 is one of the most suitable serotypes used for pneumococcal mouse colonization study (167) and was chosen as a bacteria challenge model.

Six to eight week old female inbred C57BL/6 mice were immunized intranasally three times in a two-week interval with ST3-tetrasaccharide and ST2-hexasaccharide conjugated to pneumolysin, PspA as well as a mixture of both glycoconjugates (Table 21). Animals received the dose of glycoconjugate equal to 2.5 μg of protein, corresponding to approximately 0.5 μg synthetic oligosaccharide antigen (the amount of glycan slightly varies based on a loading ratio). Vaccination dosage was chosen according to a published study. In work conducted by *Wu et.al*., PspA was used as an intranasal antigen in dosages varying from 150 ng up to 2.5 μg (170). In studies published by *Csordas et al.* and *Santamaria et. al*. mice were intraperitoneally injected with 2.5 μg to 5 μg of polysaccharide conjugated to the PspA which was sufficient to trigger an effective immune response (171, 172).

The glycoconjugates were formulated with Cholera Toxin subunit B (CTB) as an adjuvant in 10 μL total volume ST3-tetrasaccharide pneumolysin and PspA conjugates were used to prove the independent serotype protection against pathogen colonization. ST3-tetrasaccharide CRM197 conjugate serves as a negative control.

Table 21.Experimental groups for *S.pneumoniae* serotype 2 challenge study

Group	Vaccine	Animals number
1	ST2-PLY	12
2	ST2-PspA	12
3	ST2-PLY + ST2-PspA	12
4	ST3-PLY	12
5	ST3-PspA	12
6	ST3-PspA + ST3-PLY	12
7	ST3-CRM (negative control)	12
8	PBS (negative control)	12
		Σ = 96

Humoral immune responses following intranasal immunization were evaluated. Post-immune sera two weeks after the third immunization were collected, and total antigen-specific IgG titer was measured by ELISA. Intranasal immunization triggered a systemic immune response in all tested mice. Both pneumolysin, as well as PspA protein and *S.pneumoniae* serotype 3 capsular polysaccharide specific antibodies, were produced respectively to the vaccine received (Figure 34). However, mice injected with ST2-hexasaccharide glycoconjugate vaccine did not show any antibody response cross-reactive to the *S.pneumoniae* serotype 2 CPS.

The systemic antigen-specific IgG titer after intranasal immunization was comparable to the subcutaneous way of immunization and in conclusion, could be possibly exchanged in human as this non-invasive method is less painful and stressful especially for young children. Among many advantages, intranasal vaccination may trigger protection against infections at other mucosal sites, e.g., lungs, intestines or genital tract, and deliver cross-protection against different bacterial strains through mucosal antibody secretion (173).

In addition to preventing pathogen entry as one potential mechanism by which antibodies may confer protection (i.e., neutralization), antibodies can also control and help clear infections through non-neutralizing immune effector functions. Based on our previous results, the CPS-specific systemic serum antibodies correlate with the opsonophagocytic killing of pneumococci so the intranasal immunization can not only inhibit the local bacteria colonization but also provide systemic antibacterial protection.

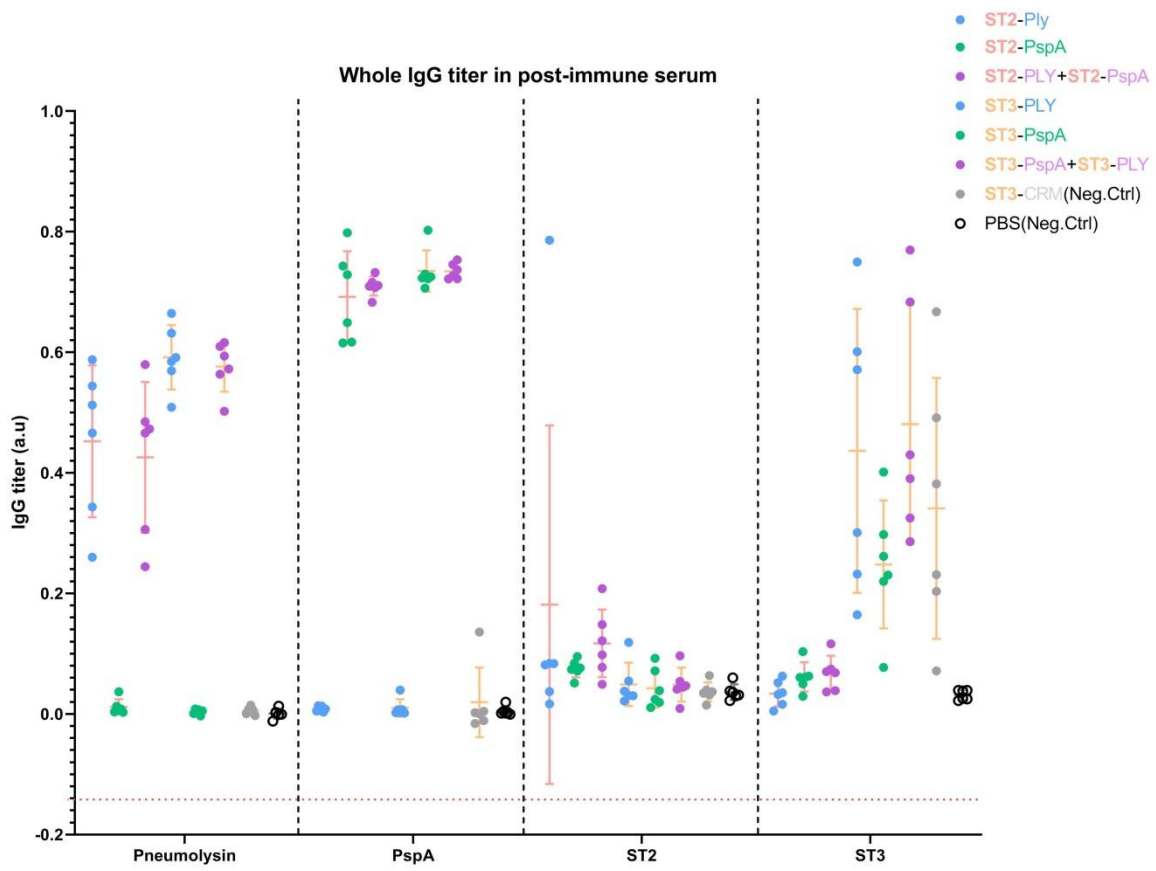
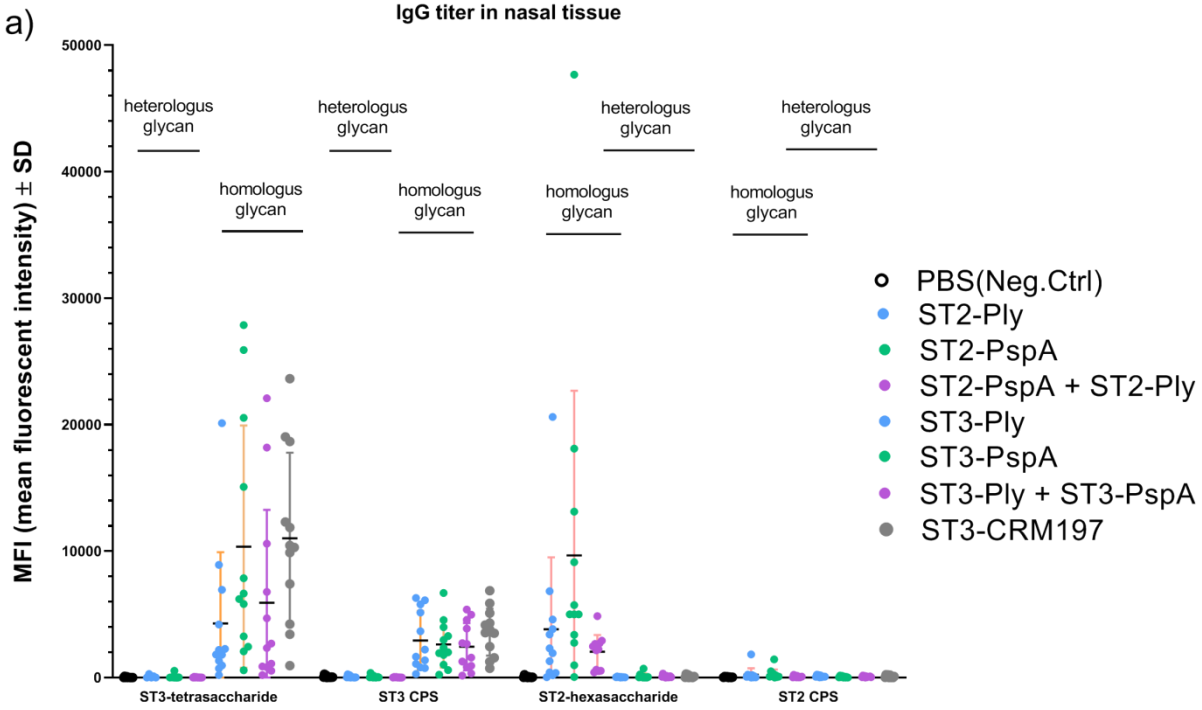


Figure 34. Pneumolysin, PspA, *S.pneumoniae* serotype 2 and serotype 3 capsular polysaccharide specific IgG titer in post-immune and final bleeding serum from mice intranasally immunized with ST3-tetrasaccharide, and ST2-hexasaccharide conjugated to Ply, PspA as well as a mixture of both.
Immune response to the ST2-hexasaccharide pneumolysin (ST2-Ply), ST2-hexasaccharide PspA (ST2-PspA), ST2-hexasaccharide pneumolysin + ST2-hexasaccharide PspA (ST2-Ply + ST2-PspA), ST3-tetrasaccharide pneumolysin (ST3-Ply), ST3-tetrasaccharide PspA (ST3-PspA), ST3-tetrasaccharide pneumolysin + ST3-tetrasaccharide PspA (ST3 Ply + PspA, ST3-tetrasaccharide CR197 (ST3-CRM) and PBS control vaccines in mice post- serum (n = 6 for post-immune, n=12 for final bleeding). Pneumolysin, PspA, *S.pneumoniae* serotype 2 (ST2-CPS) and serotype 3 (ST3-CPS) polysaccharide specific antibody levels were evaluated. Each dot represents an individual mouse immune response. Mean ± SD values were analyzed.

Three weeks after receiving the final dose of vaccine, mice were intranasally infected with a non-lethal dose of *S. pneumoniae* serotype 2. Three days later, animals were euthanized and live pneumococci were recovered from their nasal tissues. Mucosal immunity was evaluated. IgA and whole IgG both protein (pneumolysin and PspA) and glycan (CPS of *S.pneumoniae* serotype 2,

CPS of *S.pneumoniae* serotype 3, ST2-hexasaccharide and ST3-tetrasaccharide) specific antibody levels were measured using glycan arrays (Figure 35 and Figure 36).



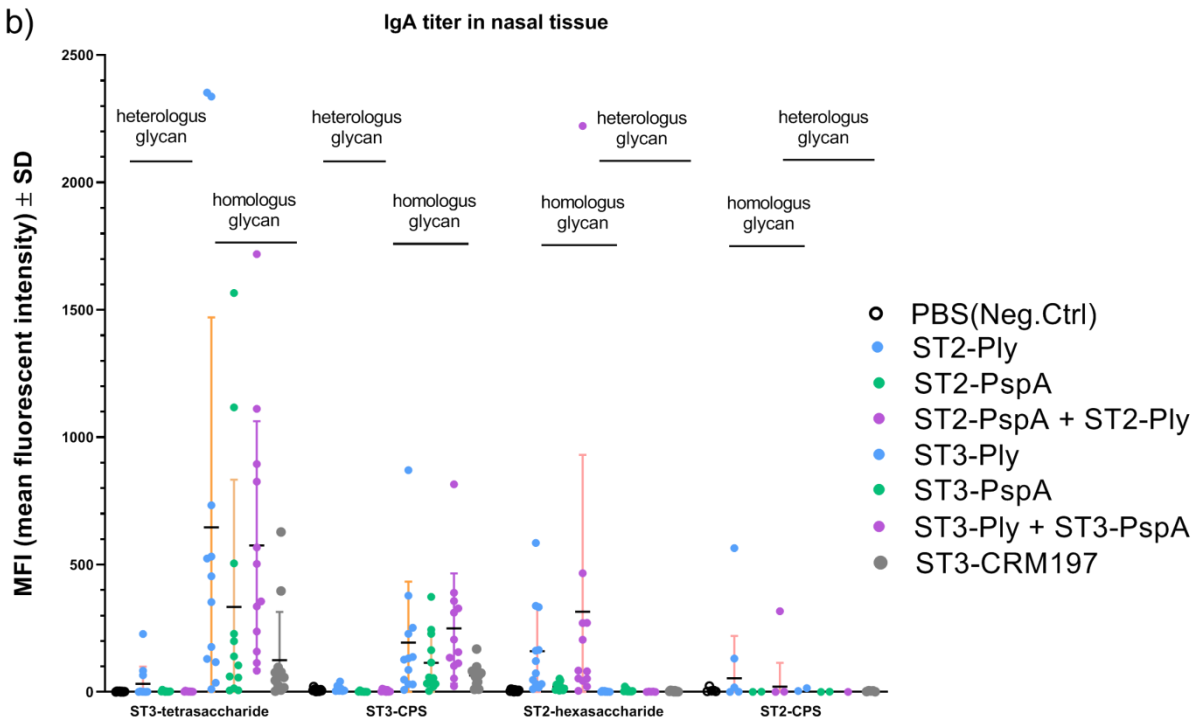
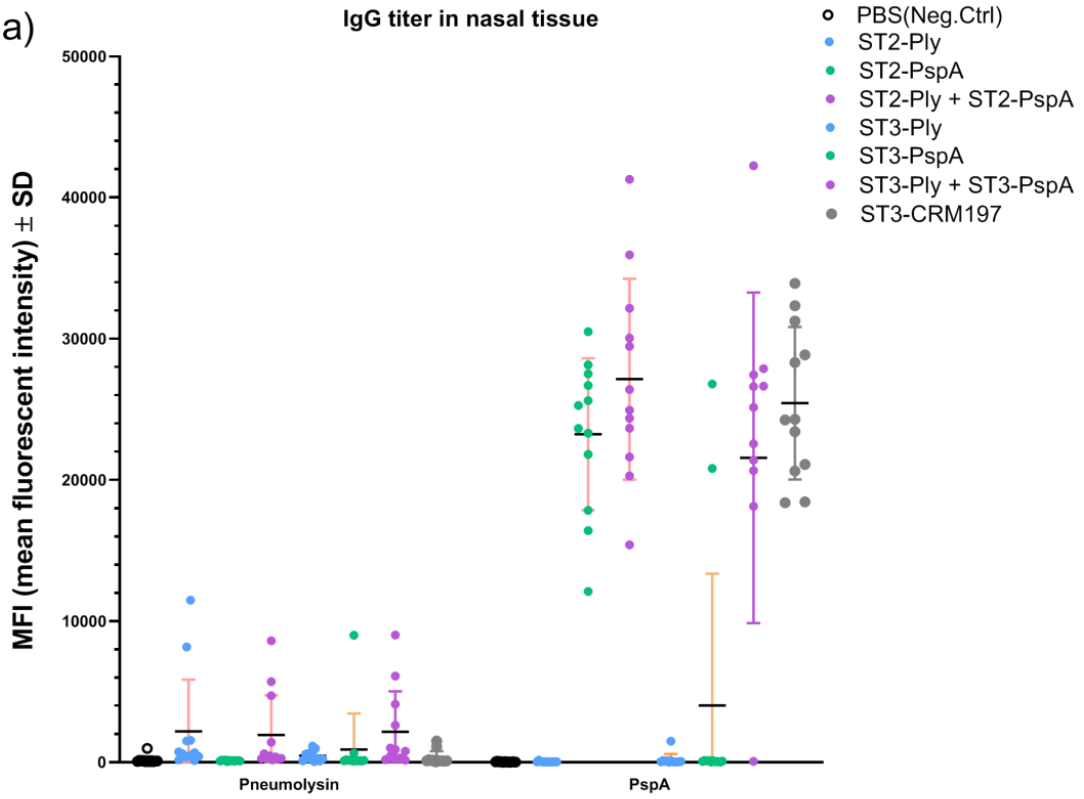


Figure 35. Evaluation of mucosal IgG and IgA response to CPS of *S.pneumoniae* serotype 3, CPS of *S.pneumoniae* serotype 2, ST3-tetrasaccharide and ST2-hexasaccharide in nasal tissue of mice intranasally immunized with ST3-tetrasaccharide, and ST2-hexasaccharide conjugated to Ply, PspA as well as a mixture of both.
IgG and IgA immune response to the ST2-hexasaccharide Pneumolysin (ST2-Ply), ST2-hexasaccharide PspA (ST2-PspA), ST2-hexasaccharide Pneumolysin + ST2-hexasaccharide PspA (ST2-Ply + ST2-PspA), ST3-tetrasaccharide Pneumolysin (ST3-Ply), ST3-tetrasaccharide PspA (ST3-PspA), ST3-tetrasaccharide Pneumolysin + ST3-tetrasaccharide PspA (ST3 Ply + PspA, ST3-tetrasaccharide CR197 (ST3-CRM) and PBS control vaccines. Antibody level was evaluated in homogenized nasal tissue collected from mice. CPS of *S.pneumoniae* serotype 3, ST2-hexasaccharide and ST3-tetrasaccharide specific antibody levels were evaluated by glycan array. Each dot represents an individual mouse immune response (n = 12). Mean ± SD values were analyzed.

Mice intranasally immunized with the above-mentioned vaccines show a relatively hight local immune response (both IgA and IgG) to CPS of *S.pneumoniae* serotype 3, synthetic ST3-tetrasaccharide and synthetic ST2-hexasaccharide However, mice injected with ST2-hexasaccharide glycoconjugate vaccine did not show a local antibody response cross-reactive to the *S.pneumoniae* serotype 2 CPS. Both pneumolysin, as well as PspA protein and *S.pneumoniae* serotype 3 capsular polysaccharide specific mucosal IgA and IgG, were also produced respectively to the vaccine received (Figure 34). This proved that the vaccine is immunogenic and able to trigger the local mucosal immune response essential for inhibition of colonization.

a)


IgG titer in nasal tissue
MFI (mean fluorescent intensity) ± SD
50000
40000
30000
20000
10000
0
Pneumolysin
PspA
PBS(Neg.Ctrl)
ST2-Ply
ST2-PspA
ST2-Ply + ST2-PspA
ST3-Ply
ST3-PspA
ST3-Ply + ST3-PspA
ST3-CRM197

b)

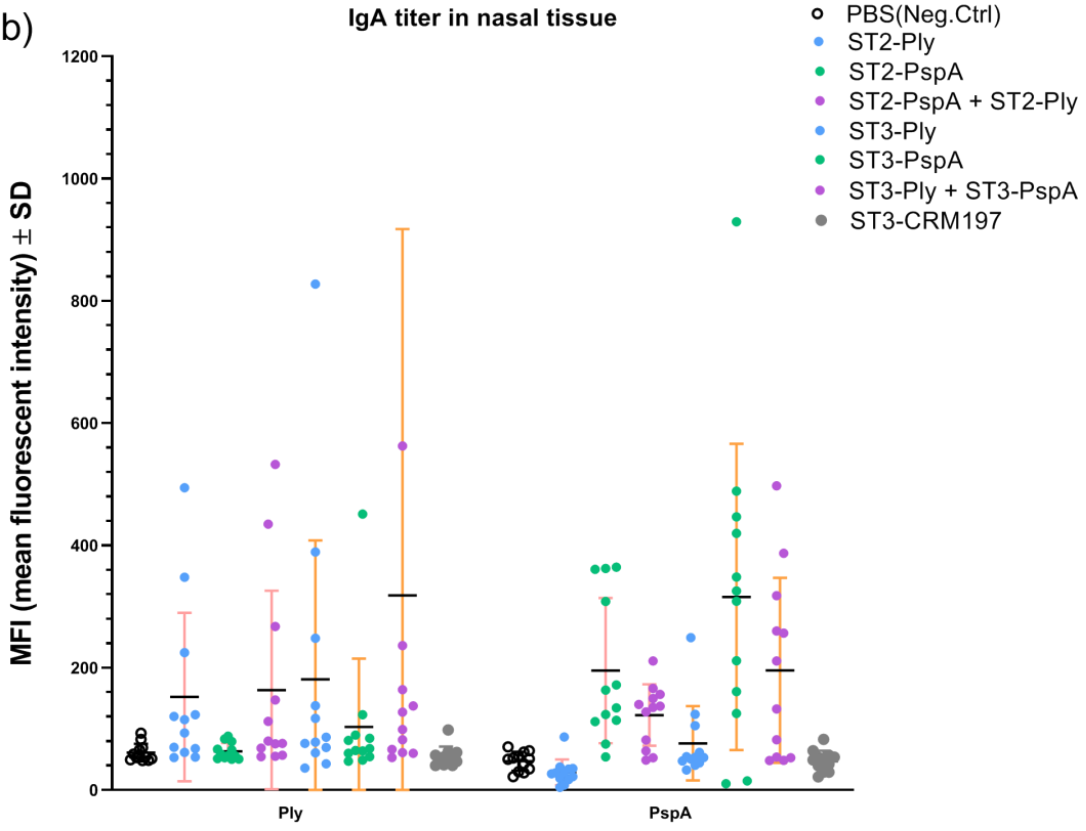

IgA titer in nasal tissue
MFI (mean fluorescent intensity) ± SD
1200
1000
800
600
400
200
0
Ply
PspA
PBS(Neg.Ctrl)
ST2-Ply
ST2-PspA
ST2-PspA + ST2-Ply
ST3-Ply
ST3-PspA
ST3-Ply + ST3-PspA
ST3-CRM197

Figure 36. Evaluation of mucosal IgG and IgA response to Pneumolysin and PspA carrier proteins in nasal tissue of mice intranasally immunized with ST3-tetrasaccharide, and ST2-hexasaccharide conjugated to Ply, PspA as well as a mixture of both
IgG and IgA immune response to the ST2-hexasaccharide Pneumolysin (ST2-Ply), ST2-hexasaccharide PspA (ST2-PspA), ST2-hexasaccharide Pneumolysin + ST2-hexasaccharide PspA (ST2-Ply + ST2-PspA), ST3-tetrasaccharide Pneumolysin (ST3-Ply), ST3-tetrasaccharide PspA (ST3-PspA), ST3-tetrasaccharide Pneumolysin + ST3-tetrasaccharide PspA (ST3 Ply + PspA, ST3-tetrasaccharide CR197 (ST3-CRM) and PBS control vaccines. Antibody level was evaluated in homogenized nasal tissue collected from mice. CPS of *S.pneumoniae* serotype 3, ST2-hexasaccharide and ST3-tetrasaccharide specific antibody levels were evaluated by glycan array. Each dot represents an individual mouse immune response (n = 12). Mean ± SD values were analyzed.

The mucosal immune response (both IgG and IgA antibodies) is responsible for inhibition of bacteria in the nasopharynx. Indeed, the intranasal vaccination with ST2-hexasaccharide pneumolysin conjugate, ST2-hexasaccharide PspA conjugate, ST2-hexasaccharide pneumolysin + ST2-hexasaccharide PspA conjugates mixture, ST3-tetrasaccharide pneumolysin conjugate, ST3-tetrasaccharide PspA conjugate, and ST3-tetrasaccharide pneumolysin + ST3-tetrasaccharide PspA conjugates mixture induced a significant reduction of bacterial load in the nasal cavity compared to the bacterial load in mice injected with PBS only (Figure 37).

The carrier proteins used in vaccines play the most important role in the reduction of pneumococci colonization in the nasopharynx within three days post-infection regardless the use of either homologous (ST2-hexasaccharide) or heterologous (ST3-tetrasaccharide) glycan. Previous studies have already proven that a highly conserved pneumolysin and PsaA, provides cross-protection in a mouse model of colonization following intranasal immunization. My study adds additional value, as the proteins could be combined with synthetic oligosaccharide antigens that are protective epitopes in an *S.pneumoniae* infection model (decrease the bacteria load in blood and lungs as well as reduce the disease severity in mice challenged with *S.pneumoniae* serotype 3 bacteria, see 3.5).

As mentioned before, the carrier proteins originate from the same pathogen, induce an additional response, so-called “additional valency”. Conserved among all *S.pneumoniae* serotypes pneumolysin and PspA were chosen as a proof of concept to broaden the spectrum of protection of the pneumococcal vaccine. I was able to prove for the first time that conjugation of the synthetic oligosaccharide to conservative carrier proteins pneumolysin and PspA gives both local

protection against bacterial colonization, the first step for pneumococcal infection but also a systemic defense by antibody-mediated opsonophagocytic clearance of the bacteria, clearing of the bacteria during the infection.

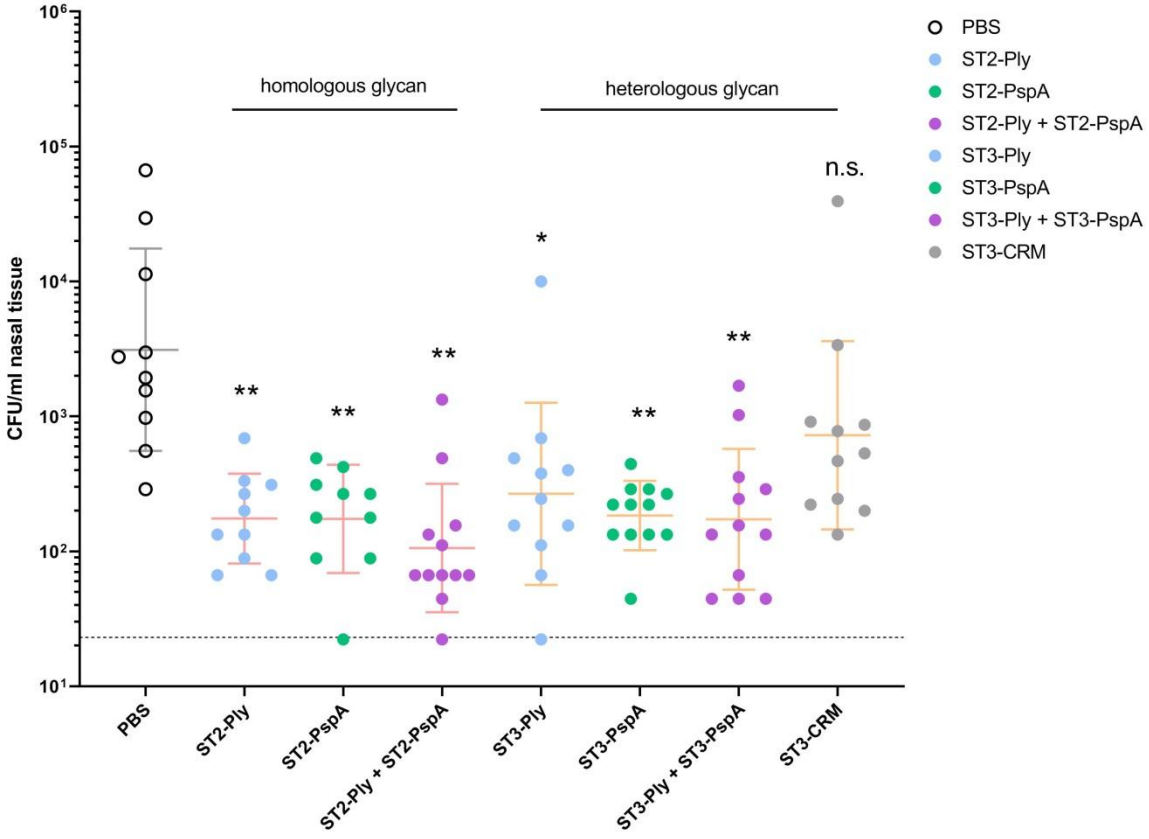


Figure 37. Intranasal vaccinations with the ST2-hexasaccharide (homologous glycan) and ST3-tetrasaccharide (heterologous glycan) Pneumolysin or PspA conjugated vaccines reduce pneumococcal colonization in mice.
Bacterial recovery of *S. pneumoniae* serotype 2 (D39) from nasal tissue 3 days after the intranasal challenge of C57BL/6 mice (n = 12) with 3.5×10^6 CFU. Each dots represent single animal and bars show the group median ±SD. The dotted line indicates the lower limit of the infection model. Data were analyzed using a one-way ANOVA multi comparison test; *p < 0.001, **p<0.0001.

3.7 Formulation of ST3-tetrasaccharide CRM197 Conjugate Vaccine Depends on Adjuvant Properties

The adjuvant is a crucial portion of a vaccine and, as has been shown in many studies, significantly changes the immune response induced by the vaccination (129). Alum-based adjuvants are currently the most popular type of adjuvants used in commercial vaccines. Unfortunately, very often Alum adjuvants do not allow to reveal the maximum capability of the antigen as the mechanism, by which they improve the immune response, is still not fully understood. The importance of adjuvant formulation has been realized, and the number of approved adjuvants is gradually increasing.

Given that carbohydrate antigens are weaker immunogens than proteins, the proper choice of the adjuvant formulation is the major challenge during the carbohydrate vaccine development process. Developing adjuvant formulations with improved potency and safety will be of great value. To maximize the immunogenic and protective potential of the ST3-tetrasaccharide CRM197 conjugate, we formulate the vaccines with various adjuvants (Table 21).

The ST3-tetrasaccharide was conjugated to the CRM197 carrier protein by a bifunctional linker. The conjugate was characterized by MALDI-TOF and by a gel shift assay (Figure 38). The average loading ratio of four glycans per protein was obtained. The loading ration can vary from batch to batch depending on, e.g., the amount of glycan used in the particular reaction. Nevertheless, based on previous studies from the Seeberger group, it was known that the loading ratio between three to twelve glycans is sufficient for the protection (174). The conjugation procedure conducted in a small batch for the research purpose varies a lot and has to be optimized for the large scale production according to guidelines that are related to good manufacturing practices (GMP).

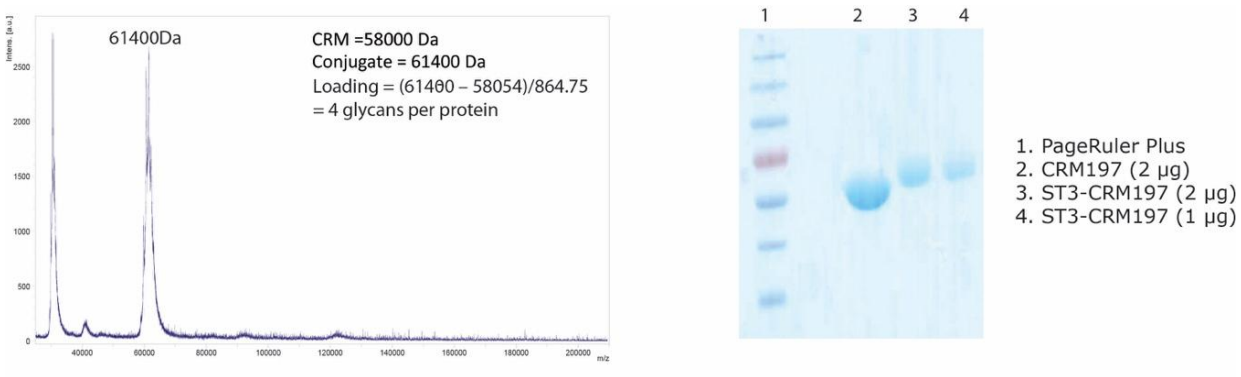


Figure 38. Characterization of ST3-tetrasaccharide CRM197conjugat.
a) MALDI-TOF analysis was carried out to measure the average molecular size of conjugate and the loading ratio of the glycan on the carrier protein. The recombinant CRM197 was used as standard. **b)** The conjugate visualization by gel shift assay (10% SDS-PAGE)

Given the long-term success of "gold standard" aluminum adjuvant, all new adjuvants formulations have to be compared to Alum (162). As investigated before (see 3.1) the adsorption of the ST3-tetrasaccharide CRM197 conjugate onto Aluminum hydroxide particles is significantly better than onto aluminium phosphate. Therefore, the ST3-tetrasaccharide CRM197 conjugated formulated with aluminium hydroxide was the first group used in the study. The antigen was adsorbed onto 125 µg Alum ($Al(OH)_3$) as an aluminum source (Alhydrogel, Brenntag, Denmark).

The particular vaccine formulation was decided according to the chemical and biological properties of the adjuvant (Table 23). All groups contain the same dose of antigen equal 0.4 µg of synthetic ST3-tetrasaccharide conjugated to the CRM197 and amount of adjuvant decided base on commercially available vaccine formulation or published data.

Emulsions have been used as adjuvants for both human and animal vaccines for many years. Traditional oil-based emulsion adjuvants, such as complete or incomplete Freund's adjuvant, have been reported with many side post-immunization reactions (175). MF59^{TM} is a squalene-based oil-in-water emulsion, currently licensed as part of an influenza vaccine by Novartis, mostly acting by enhancing recruitment of antigen-presenting cells. Therefore, the second group to be formulated with the ST3-tetrasaccharide CRM197 conjugate formulated was MF59^{TM}. The ST3-tetrasaccharide CRM197 conjugate was adjuvanted with MF59^{TM} and an emulsion was prepared by "two-syringes" method in 1:1 volume ration of antigen and adjuvant.

It has been well known that the majority of microbial components, referred to as pathogen-associated molecular patterns (PAMPs) stimulate the innate immune response through pattern recognition receptors such as Toll-like receptors (TLRs) (176). Vaccines containing TLR ligand-based adjuvants activate and trigger the innate immune response and enhance the protection induced by vaccines. A derivative of lipopolysaccharide, monophosphoryl lipid A (MPLA) already approved in many commercial vaccines (177), a powerful immunostimulator for the treatment of cutaneous cancers in various clinical trials Resiquimod (R-848) (178) and one of the oldest but very potent components of attenuated vaccines – bacterial RNA (179), were chosen as adjuvants for the synthetic ST3-tetrasaccharide CRM197 conjugated vaccine. The way of vaccine formulation depends on the chemical properties of the adjuvant. As all of the above-mentioned adjuvants can not be chemically attached to the antigen, nanoparticles were selected as a delivery system. Many studies showed that using nanostructures functionalized with antigen and adjuvant might augment the immunogenicity of the vaccine by enhancing the activation of antigen-presenting cells (158, 180). Polylactic acid (PLA) particles made of biodegradable approved for human-used polyester are broadly described as a co-delivery system of vaccine components (181). The use of PLA particles in the formulation of ST3-tetrasaccharide – CRM197 conjugate with TLRs ligands (MPLA, Resiquimod, and RNA) offers the possibility to delivery the antigen and immunostimulatory molecules to the immune cells in more natural spherical structures and help to overcome the problem the adjuvant formulation. Particles were prepared by a water/oil/water (W1/O/W2) double emulsion solvent evaporation technique (2.7.2). One of the greatest benefits of particulate antigen delivery systems is their ability to co-deliver antigens and immunostimulatory molecules simultaneously to the same APCs (137). MPLA and Resiquimod (TLR4 and TLR7/8 activators) were chosen as a prove of synergistic effects that may result in a more effective or longer-lasting immune response (162). For groups 3 to 6, the ST3-tetrasaccharide CRM197 conjugate together with adjuvant were encapsulated into biodegradable PLGA particles (as described in Particle preparation 2.7.2.). In addition, the dummy control particles were prepared without the antigen or adjuvant.

Table 22. The experimental group used in the evaluation of adjuvant formulation on the effect of ST3-tetrasaccharide CRM197 conjugated vaccine.

	Group name	Adjuvant	Adjuvant dose	Adjuvant provider	Formulation
1	SP3-AlH	Aluminium hydroxide	0.125 mg of Aluminium/dose	10% Alhydrogel (Brenntag, Denmark)	adsorption
2	SP3-MF59	MF59TM	1:1 (v/v)	AddaVax™ (InvivoGen, San Diego, USA)	emulsion
3	SP3-MPLA	Monophosporyl lipid A (MPLA, PHAD®)	10 µg/dose	Avanti Polar Lipids (Sigma Aldrich, St. Louis, USA)	PLGA particles
4	SP3-R848	Resiquimod (R848)	10 µg/dose	InvivoGen, San Diego, USA	PLGA particles
5	SP3-MPLA + R848	Monophosporyl lipid A (MPLA, PHAD®) + Resiquimod (R848)	10 µg + 10 µg/ dose	Avanti Polar Lipids (Sigma Aldrich, St. Louis, USA) InvivoGen, San Diego, USA	PLGA particles
6	SP3-RNA	Isolated bacterial RNA	5 µg/dose	Sander Group (Charité – Universitätsmedizin Berlin)	PLGA particles
7	Prevnar®13	Aluminum hydroxide	1/5 of the human dose	Pfizer, New York, USA	solution
8	Empty particles	-	-	-	PLGA particles

Particle characterization is a crucial step before further development for applications. The presence of the adjuvant and antigen, size and distribution of the particles were determined. The flow-through solutions after washing steps were monitored by Nano-Drop and BCA assay for the presence of the non-encapsulated conjugate. The ST3-tetrasaccharide CRM197 conjugate was not detected at any point, so the encapsulation efficiency was assumed to be complete.

The encapsulation and localization of the antigen on the particles were confirmed by flow cytometry and confocal microscopy analysis. ST3-tetrasaccharide CRM197 conjugate encapsulated particles were incubated with the ST3 and CRM197 specific monoclonal antibodies followed by detection using a FITC conjugated secondary antibody. Confocal microscopy analysis revealed the specific surface localization of ST3-tetrasaccharide CRM197 on the particles (Figure 39a). The changes in fluorescence intensity according to the control with

secondary antibodies only were measured by flow cytometry. The shift of the signal to the right indicates the presence of both carbohydrate and protein components on the particles (Figure 39b). Surface localization of the ST3-tetrasaccharide antigen is an important feature considering that polysaccharide specific immune responses are primarily mediated by cross-linking of the B cell receptor.

The parameters for nano- (<500 nm) and micro- (2-8 μm) particle fabrication are well established. The dimension of particles loaded with antigen and adjuvant was analyzed by dynamic light scattering (DLS) measurement and scanning electron microscopy (SEM). The size distribution by intensity and number shows the average magnitude of the ST3-tetrasaccharide CRM197 and adjuvant encapsulated particles between 300 – 500 nm depending on the formulation (Figure 39c and d). Inconsistent results provide no definite answer as to which PLGA particles size is the most effective for vaccine delivery. Some studies have shown that particles less than 450 – 600 nm induced the most robust immune response after s.c. injection (activation of CD8+ cells) (182). In regards to IgG production, the 200 nm and 500 nm particles elicited similar antibody titer (183). Microparticles of 0.5 – 5 μm are embrace via phagocytosis or macropinocytosis, mostly induce humoral responses (155, 184, 185).

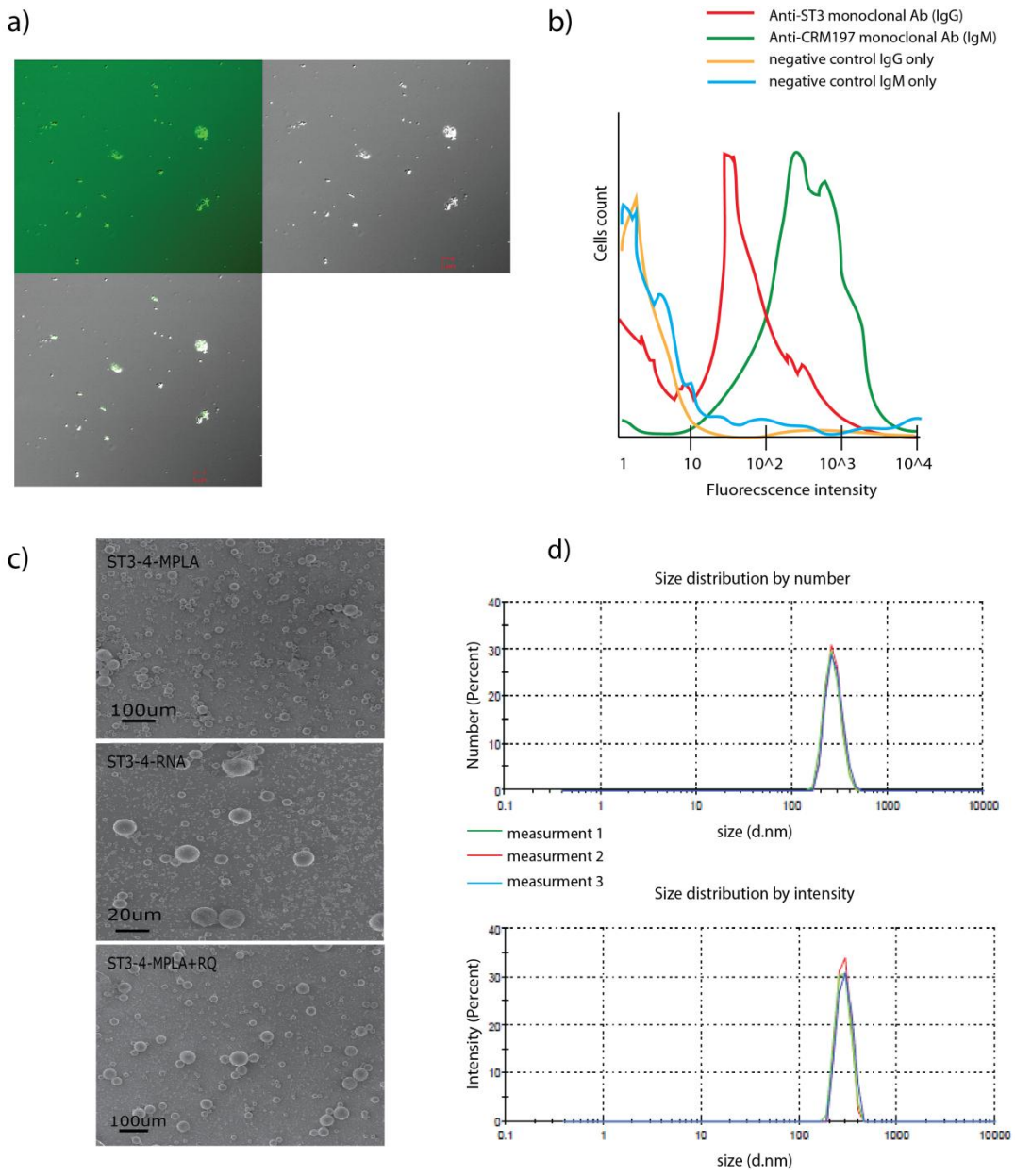


Figure 39. Exemplary characterization of PLA particles.
a) The picture of ST3-tetrasaccharide CRM197 conjugate and RQ loaded particles observed under the Transmission Electron Microscope (TEM). Scale bars indicated in the pictures. **b)** Encapsulation of ST3-tetrasaccharide CRM197 conjugate into PLA particles analyzed by Flow Cytometry. The shift in the intensity of the fluorescence signal (x-axis) indicates the presence of the glycan (monoclonal IgG antibodies against ST3-tetrasaccharide in green) and protein (monoclonal IgM antibodies against CRM197 in red) part of the conjugate compare to the control of secondary antibodies only (anti-IgG-FITC and anti-IgM-FITC). The same number of events was recorded for all measurements. **c)** Particle size and polydispersity measured by Dynamic Light Scattering (DLS). Representative pictures of ST3-tetrasaccharide CRM197 conjugate and RQ loaded particles. Graphs show the particle size by number and intensity. Measurement was repeated three times.

3.8 The Immune Response Following Immunization with ST3-Tetrasaccharide CRM197 Conjugate Depends on the Choice of Adjuvant and Vaccine Formulation

Six to eight-week-old C57BL/6 mice were s.c immunized with an amount of ST3-tetrasaccharide CRM197 conjugate corresponding to a dose of 0.4 µg of ST3-tetrasaccharide antigen per injection in different vaccine formulations. Animals were vaccinated according to the long-term immunization regime prime + boost + boost + final boost (Table 23). Blood was collected, and the antibody response was analyzed.

Table 23. Immunization regime for adjuvant formulation study

days	procedure
d0	Immunization, bleeding
7	Bleeding
14	1st boost
21	Bleeding
28	2nd boost
35	Bleeding
42	Bleeding
49-140	Resting period
140	Bleeding
161	Final boost
168	Bleeding
175	Dissection

Based on broad knowledge about T-cell differentiation and a role of pathogen-associated molecular patterns (PAMPs) in the induction of immune responses, in theory, it is possible to selectively induce cell-mediated or humoral and T-helper 1 or T-helper 2 (T_h1/T_h2) responses by adjustment of vaccine adjuvant formulations. In a mouse model, the IgG subclasses induced after immunization are an indirect measurement of Th_2-type versus Th_1-type immune response. The production of IgG_1-type antibodies is principally induced by T_h2-type cytokines, whereas the IgG_2 and IgG_3 reflect the involvement of T_h1-type cytokines (186). A specific IgG subclass can contribute to the clearance of encapsulated bacteria by particular mechanisms. Murine IgG_3 is

highly protective, while the IgG_2a, IgG_2b, and IgG_1 isotypes are poorly or non-protective and have a significantly lower affinity. Mice IgG_2 display the strongest binding to Fc receptors and together with IgG_3 can activate the complement better than IgG_1 (IgG_3>IgG_2b>IgG_2a>IgG_1) (187, 188). Therefore, an immune response with a broad subclass distribution may be beneficial against encapsulated bacteria. In human IgG_3 and IgG_1 are the best complement activators (189).

The effect of adjuvant formulation on the T_h1 and T_h2 profile of humoral immune responses was analyzed by determining the level of IgG_1, IgG_2, and IgG_3 antibodies recognizing a carrier protein as well as synthetic ST3-tetrasaccharide and native CPS of *S.pneumoniae* serotype 3. The blood from a single animal was screen by glycan arrays using IgG_1, IgG_2 and IgG_3 secondary antibodies conjugated to different fluorescent dyes.

The influence of the adjuvant formulation on the magnitude within a time course and functional activity of the antibody response upon vaccination with ST3-tetrasaccharide CRM197 conjugate vaccine was also tested. It was presented that the use of different adjuvants correlates with antibody response kinetic as they differ in mode of action. MF59^{TM} promptly stimulate an early immune response while Alum entraps the antigen and slows down antibody production two weeks after priming (129). The analysis revealed that the kinetic of the humoral response induced by ST3-tetrasaccharide CRM197 conjugate strongly depends on the adjuvant formulation in the mouse model (Figure 40). However, neither special pattern nor correlation was perceived.

Immune memory is one of the critical features of a successful vaccine. Effector memory for short-incubation diseases and central memory for long-incubation diseases must be formed after boosting immunization. Animals immunized with ST3-tetrasaccharide CRM197 conjugate in combination with T_h1 adjuvants (MPLA, RQ-848, bacterial RNA as well as a combination of MPLA and RQ-848) showed high anti-ST3-tetrasaccharide IgG_2 and IgG_3 titer. The same groups demonstrate an excellent IgG_2 response just after the final boost. The quick and specific recognition of the previously encountered antigen and a corresponding high immune response is indirect evidence for the presence of oligosaccharide specific memory B-cells. Using the TLR ligands as adjuvants (MPLA, RQ-848, RNA) substantially shifted the immune response toward a T_h1 phenotype.

The profile of *S.pneumoniae* serotype 3 CPS-specific IgG subclasses (IgG_1, IgG_2, and IgG_3) differs from the ST3-tetrasaccharide-specific IgG subclass antibody response. The vaccine formulated with Alum and MF59^{TM} (o/w squalene) evoked a prevalence of the IgG_1 subclass, (indicative of a T_h2-biased response), while the MPLA, RQ-848, and RNA strongly indicated the T_h1 cell profile by induction of IgG_2 and IgG_3 subclasses. Improved production of IgG_2 antibodies was similarly observed for the formulation of ST3-tetrasaccharide CRM197 conjugate with MF59^{TM} even though the adjuvant is considered more T_h2. Nonetheless, the *in vitro* results have to be interpreted very carefully as the immune system is very complex, and the response in the murine model differs. In summary, the choice of adjuvant plays a significant role in the level of involvement of the T_h1 and T_h2 responses in mice immunized with ST3-tetrasaccharide CRM197 conjugate.

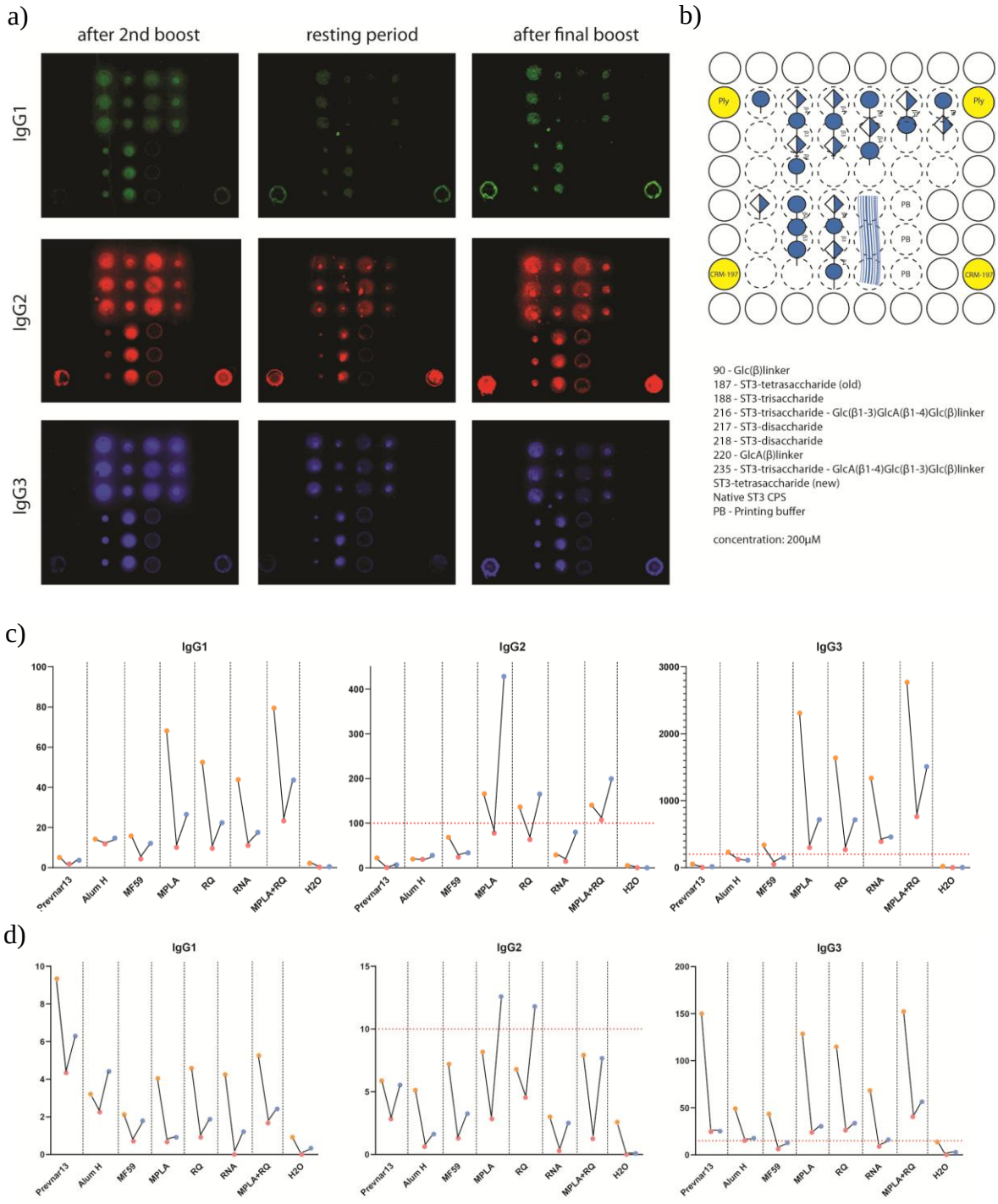


Figure 40. Immune response analysis of mice immunized with the ST3-tetrasaccharide CRM197 conjugate in the different adjuvant formulations.

a) Representative picture of glycan array analysis of serum from mice immunized with the ST3-tetrasaccharide CRM197 conjugate and RQ encapsulated PLA particles. Serum was collected seven days after the second boost (day 35), during the resting period (day 140) and seven days

after final bleeding (day 168). The IgG1, IgG2, and IgG3 were labeled with different fluorescent dyes, and intensity was measured at 300 PMT. **b)** The printing pattern of glycan array used for screening of serum samples. All glycans were structures spotted onto a glass slide in the concentration of 200 μM and were part of the MPS glycan library. **c)** Kinetic of ST3-tetrasaccharide specific antibody response in mice immunized with different formulations of ST3-tetrasaccharide CRM197 conjugate seven days after the second boost, during the resting period and seven days after the final boost evaluated by glycan array analysis. **d)** Cross-reactivity and kinetics of antibody response against native CPS of *S.pneumoniae* serotype 3 from mice vaccinated with ST3-tetrasaccharide CRM197 conjugate in various adjuvant formulations. Mean fluorescence intensity (MFI) of eight animals measured in triplicates were plotted.

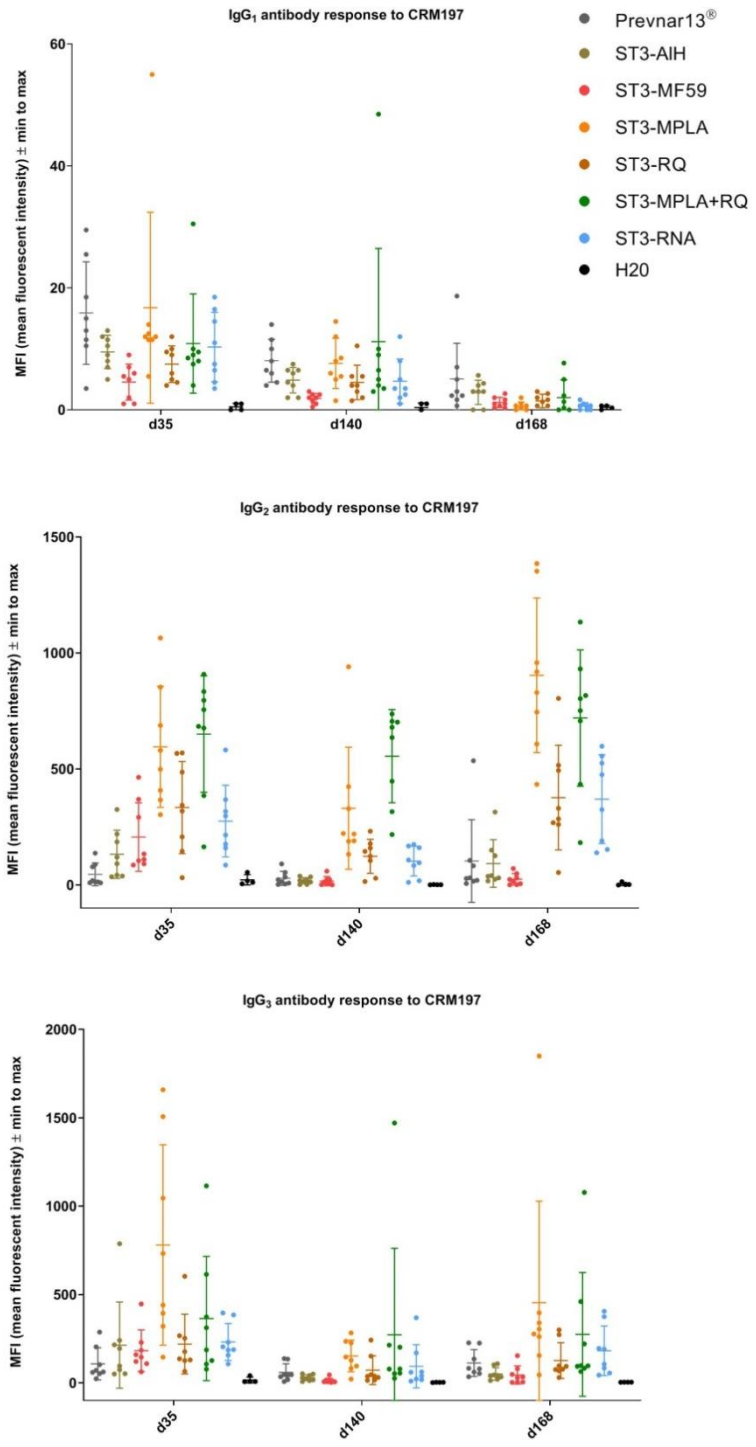


Figure 41. Glycan array evaluation of IgG_1, IgG_2, and IgG_3 antibody response to CRM197 in mice immunized with ST3-tetrasaccharide CRM197 conjugate vaccine using different adjuvant formulations.

Data are measurements of single animal antibodies titer on day 35 (after 2nd boost), day 148 (during the resting period) and day 168 (after final boost). Graphs show mean fluorescence intensity ± SD, n = 8.

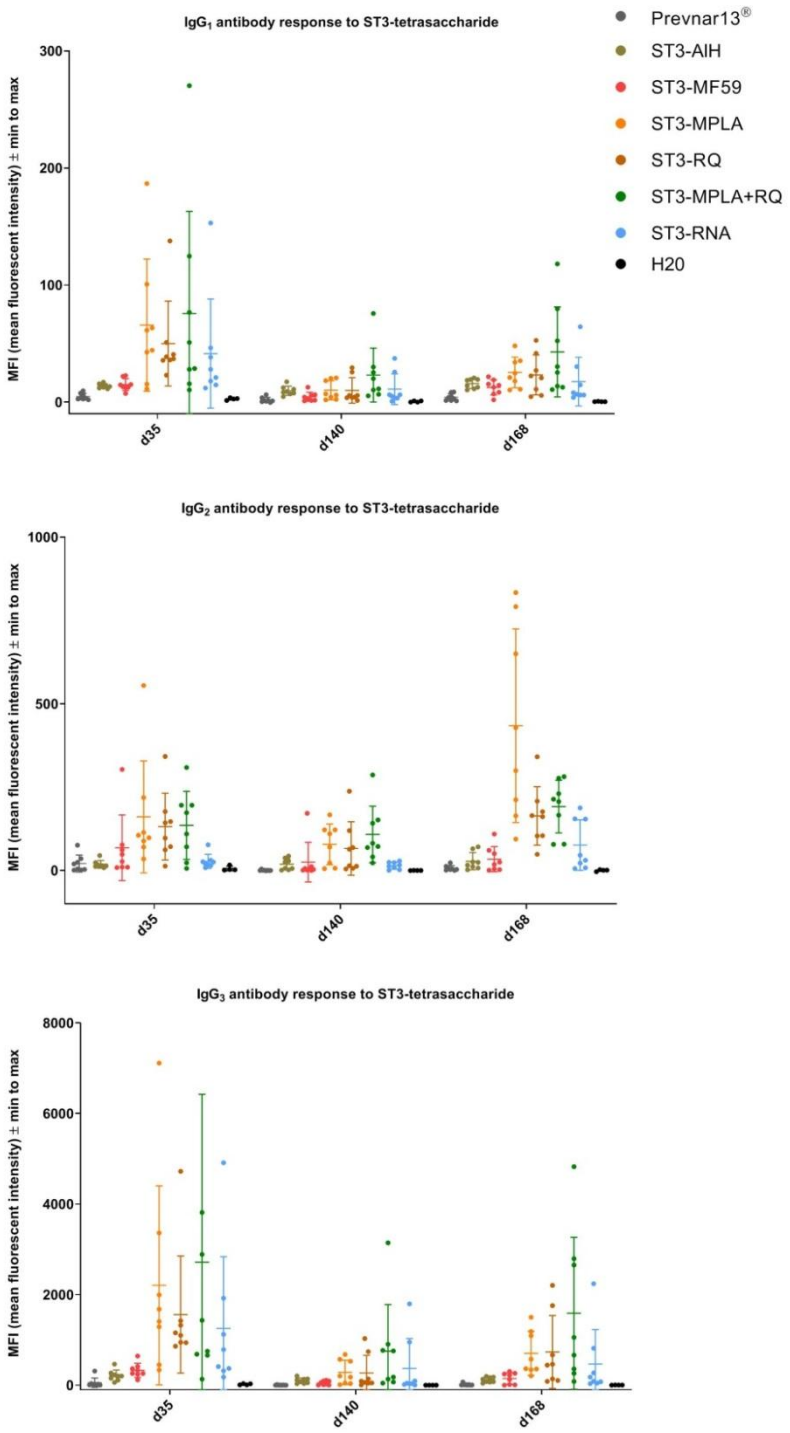


Figure 42. Glycan arrays evaluation of IgG_1, IgG_2, and IgG_3 antibody response to ST3-tetrasaccharide in mice immunized with ST3-tetrasaccharide CRM197 conjugate using different adjuvant formulations.

Data are measurements of single animal antibodies titer on day 35 (after 2nd boost), day 148 (during the resting period) and day 168 (after final boost). Graphs show mean fluorescence intensity ± SD, n = 8.

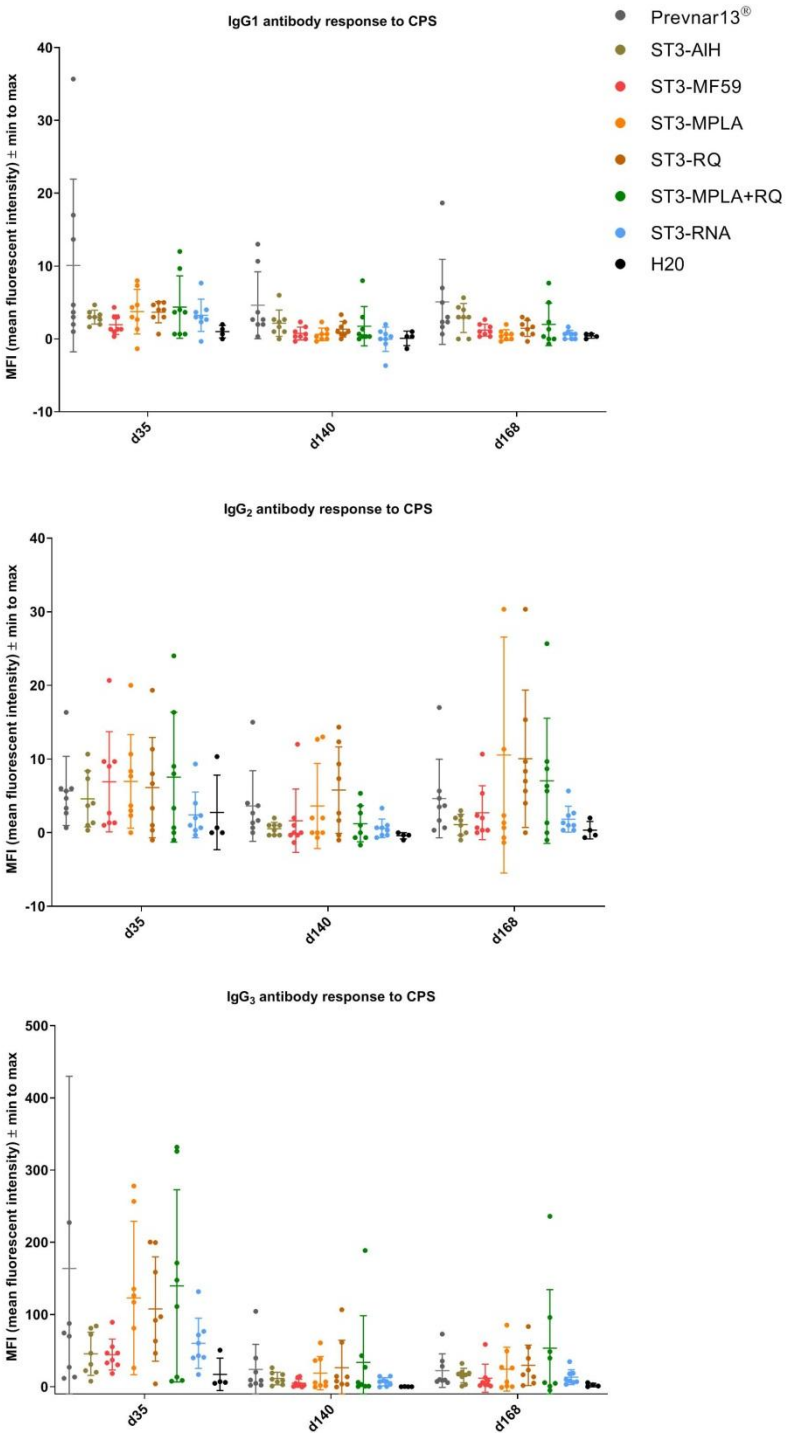


Figure 43. Glycan array evaluation of IgG_1, IgG_2, and IgG_3 antibody response to native CPS of *S.pneumoanie* serotype 3 in mice immunized with ST3-tetrasaccharide CRM197 conjugate using different adjuvant formulations.

Data are measurements of single animal antibodies titer on day 35 (after the second boost), day 148 (during the resting period) and day 168 (after final boost). Graphs show mean fluorescence intensity ± SD, n = 8.

Polysaccharide specific antibodies are the primary protective mechanism against pneumococcal bacteria. To determine antibody-mediated bacterial killing *in-vitro,* an opsonophagocytic killing assay (OPKA) was performed. This very well-established method evaluates the vaccine-induced immunoprotection by measuring the reduction of viable bacteria in the presence of phagocytes, antibodies, and complement (160, 190).

The OPKA results indicated that the RQ-848 and MF59^{TM} and are the best candidates for the formulation of the ST3-tetrasaccharide CRM197 conjugate vaccine with the highest serum dilutions responsible for 50% killing of bacteria (IC_{50}), respectively 1236 and 998 (Figure 44). The IC_{50} for a control Prevnar13® vaccinated group equaled 357. The group containing aluminium hydroxide showed IC_{50} = 360; MPLA IC_{50} = 341; the combination of MPLA and RQ-848 IC_{50} =; 260 and RNA IC_{50} = 203. No statistical significance was calculated ($p > 0.05$).

It has been claimed that quantitative characteristics of antibodies (= antibody levels) contribute to OPA and the estimation of immunity, those correlations between the measured concentrations of IgG and opsonophagocytic activity have been required (191, 192). Unfortunately, no correlation between antibody titer and opsonophagocytic protective activity in groups immunized with ST3-tetrasaccharide CRM197 conjugate and the different adjuvant formulation was observed (p values > 0.05 and very low r^2 value) (Figure 45).

However, it is a controversial question of whether the quality or quantity of antibodies is mostly responsible for vaccine efficacy. An antibody may be highly correlated with protection or synergistic with other functions. Antigen-binding antibodies mediate effector functions through interactions between their Fc domains and Fc receptors (FcR) found either on all innate immune cells or components of the complement system. The outcome of Fc/FcR interaction might be phagocytosis, induction of cell lysis or degranulation. Antibodies can also recruit complement to directly kill pathogens or use additional innate immune clearing activities via complement receptors (193).

Based on the results, I strongly believe that functional activity plays a crucial role in protection, not the number of antibodies induced by vaccination (OPK activity). It has been described that modifications of the Fc domain, as well as changes in Ab isotype/subclass and glycosylation, may have a profound impact on antigen-antibody complex delivery and can improve affinity for particular Fc receptors (194). A detailed investigation of the antibody generated after

immunization with ST3-tetrasaccharide CRM197 conjugate vaccine with different adjuvant formulations has to be performed. Knowing the avidity and character of immunocomplexes helps to fully understand the vaccine formulation effect.

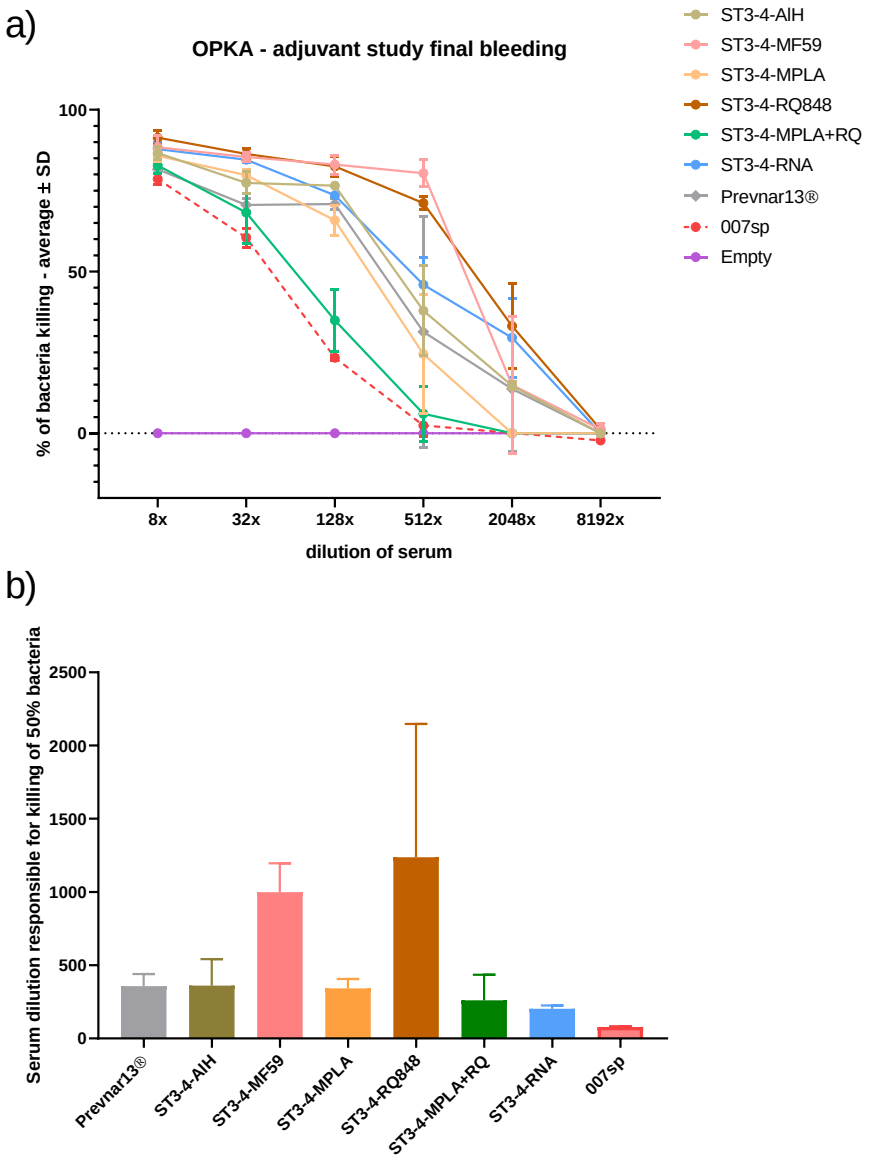


Figure 44. Comparison of the *in-vitro* opsonophagocytic activity of sera from mice immunized with ST3-tetrasaccharide CRM197 conjugate using different adjuvant formulations

a) A comparison of OPKA activity of pooled sera from mice immunized with ST3-tetrasaccharide CRM197 conjugate in different adjuvants formulation. The commercial vaccine Prevnar13® and WHO human reference serum 007sp were used as controls. Data are means ± SD of CFU reduction relative to negative control wells (samples lacking either antibody or

complement) of two independent experiments. **b)** Evaluation of serum dilution responsible for the killing of 50% bacteria by the *in-vitro* opsonophagocytic killing assay. Graph shows mean ±SD of results from two independent experiment estimated through non-linear interpolation of the dilution-killing OPKA data. Data were analyzed by the two way ANOVA, and p-value of $\leq$ 0.05 was considered significant. Human reference serum 007sp was used as a control.

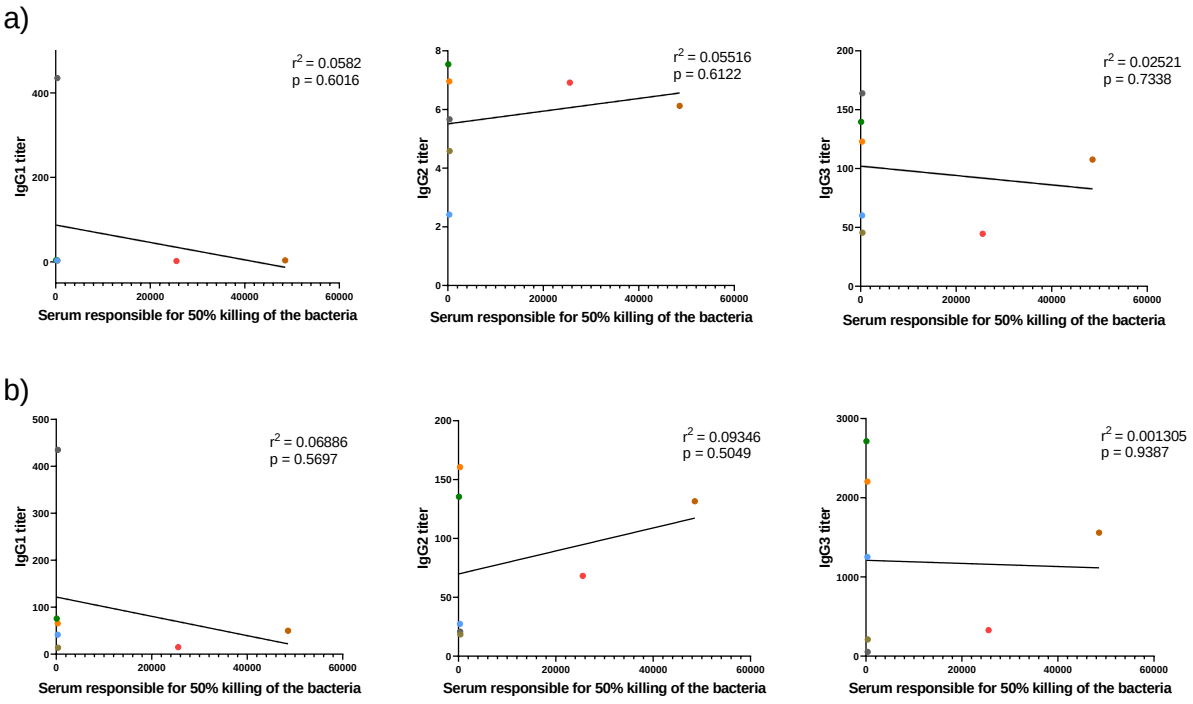


Figure 45. Correlation between specific antibody titer and serum dilution responsible for the killing of 50% bacteria.

Relationship between **(a)** *S.pneumoniae* serotype 3 CPS and **(b)** ST3-tetrasaccharide specific IgG subclasses titer from group immunized with ST3-tetrasaccharide CRM197 conjugate in the different adjuvant formulation (measured by ELISA) and serum dilution responsible for the killing of 50% bacteria (measured by OPKA). Plots show Pearson's correlations (r^2 value coefficient), a *P* value of ≤ 0.05 was considered significant.

To evaluate the composition of the immune cells from animals immunized with various formulations of ST3-tetrasaccharide CRM197 conjugate vaccine, flow cytometric analysis of spleen cells and bone marrow was conducted. The experiment provides detailed information about the numbers and phenotypes of the immune cells.

The spleen cells obtained from immunized animals were first differentiated based on B220 epitope (one of the epitopes of CD45 protein, which is expressed on all murine B cells) and presence of IgD which allows identification of different stages of B cells. Double negative (IgD^- $B220^-$) cell subsets were further analyzed by the presence of CD138 (a marker of plasma cells) and CD19 (expressed in all B lineage cells, except for plasma cells). $IgD^-B220^-CD138^+CD19^-$ cell population was identified as long-lived plasma cells, mainly responsible for maintaining the level of serum antibodies. Follicular B (FO B cells) cells live in the well-established lymphoid follicles of spleen and lymph nodes and present T-dependent antigen to activated T cells. FO B cells were characterized by the presence of IgD, high level of B220 as well as CD19 and CD38 (expressed on follicular B cells but down-regulated on germinal centers) ($B220^+IgD^+D19^+CD38^+$). Memory B cells are other subpopulations of B cells. They are responsible for a quick and robust antibody-mediated immune response in the case of re-infection. Memory B cells were distinguished from $B220^+IgD^-CD19^+$ subpopulation by $CD38^+FAS^-$ staining. The Fas receptor responsible for maintaining homeostasis in the peripheral lymphoid organs (195), was used as a marker for germinal center B cell (IgD^- $B220^+$ $CD19^+CD38^-$ FAS^+GL7^+). GL7 serves as a marker for germinal centers in the immunized spleen. Activated $GL7^+$ B cells have a very high functional activity for producing antibodies and presenting antigens (196) (Figure 46). No significant differences were observed between specific B cell subsets in groups immunized with ST3-tetrasaccharide CRM197 conjugate vaccine in the different adjuvant formulation (Figure 47). A similar analysis was performed for bone marrow immune cells (Figure 48). The level of B cell subset does not differ depending on the adjuvant used. A lack of variance might be due to the analysis of the whole B cells population and not ST3-tetrasaccharide specific cells. The population of carbohydrate-specific B cells is very low and difficult to detect.

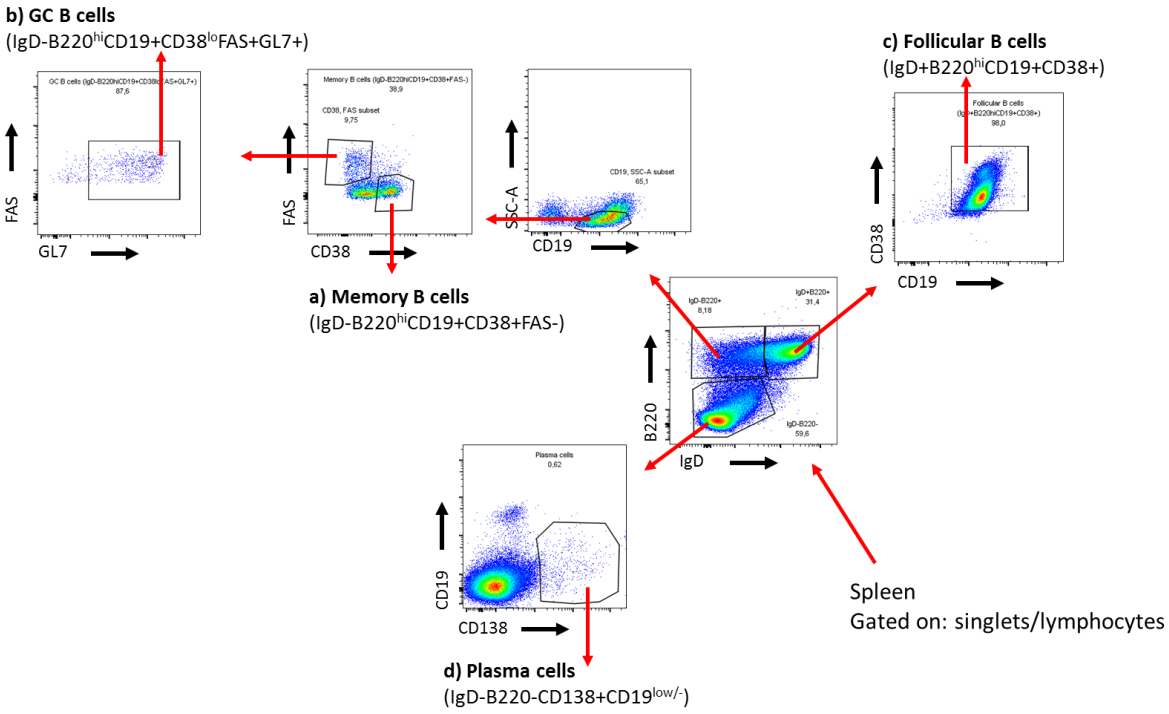


Figure 46. Gating strategy for flow cytometry assay of B-cells from the spleen.
Spleen cells were divided into different B-cells subclasses base on B220 and IgD staining. Aggregates were excluded by FSC-H vs. FSC-A gating. **a)** Memory B-cells were defined as $B220^{+}CD19^{+}CD38^{+}$ and IgD^{-} FAS^{-} cells among the whole spleen cells lymphocytes. **b)** Germinal center B-cells subpopulation were differentiated from memory B-cell subpopulation base on FAS^{+} and $GL7^{+}$ staining. **c)** Follicular B-cells were distinguished among the whole spleen cells lymphocytes by $B220^{+}IgD^{+}$ and $CD19^{+}CD38^{+}$ gating. **d)** Plasma cells were defined as $CD138^{+}CD19^{low/-}$ follow $IgD^{-}B220^{-}$ gating.

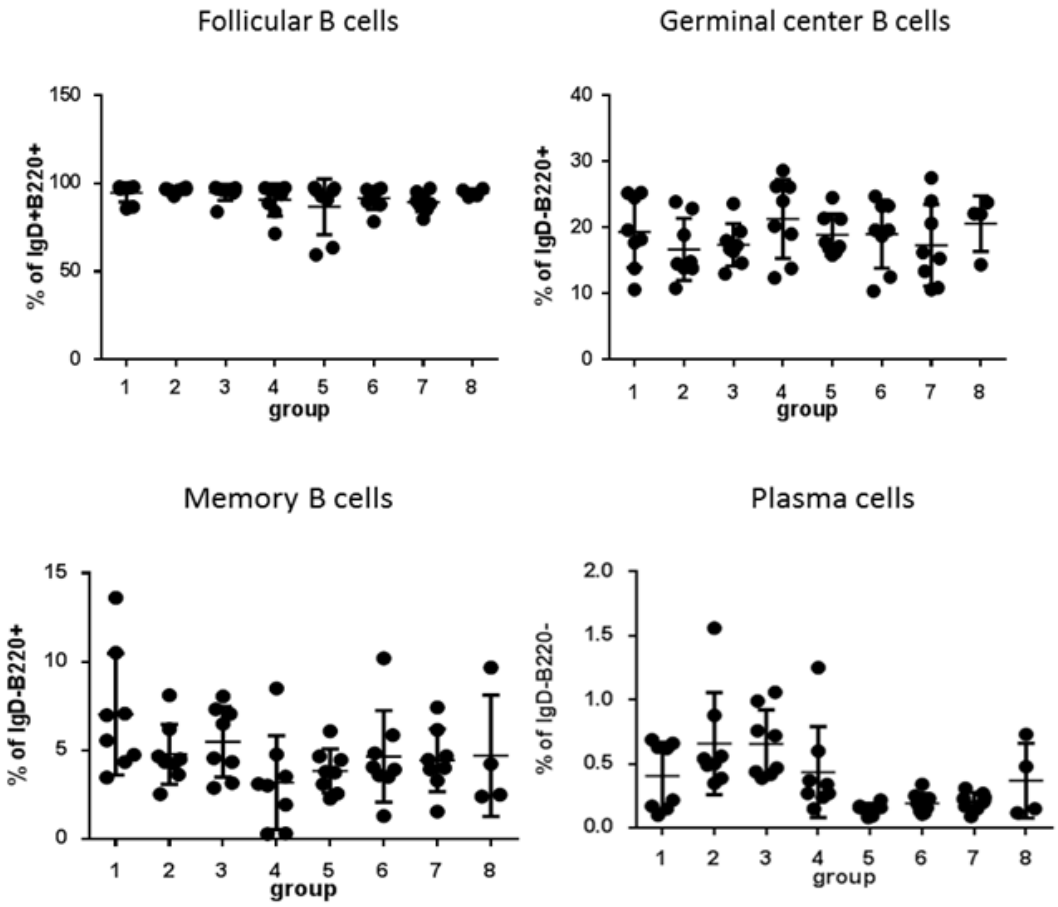


Figure 47. Flow cytometry analysis of B-cells in the spleen from mice immunized with ST3-tetrasaccharide-CRM197 conjugate using different adjuvant formulations.
The percentage of different B cell subsets from spleens, such as follicular B cells, germinal center B cells, memory B cells, and plasma cells were analyzed by FACS staining. Mice were immunized with: (1) Prevnar13®, ST3-tetrasaccharide-CRM197 conjugate adjuvanted with (2) aluminum hydroxide, (3) MF59, (4) MPLA, (5) Resiquimod (RQ), (6) MPLA + RQ, (7) bacterial RNA and (8) negative control, empty particles. The values for a single animal per each group were plotted and the mean of n = 8 (and n = 4 for the control group) was calculated.

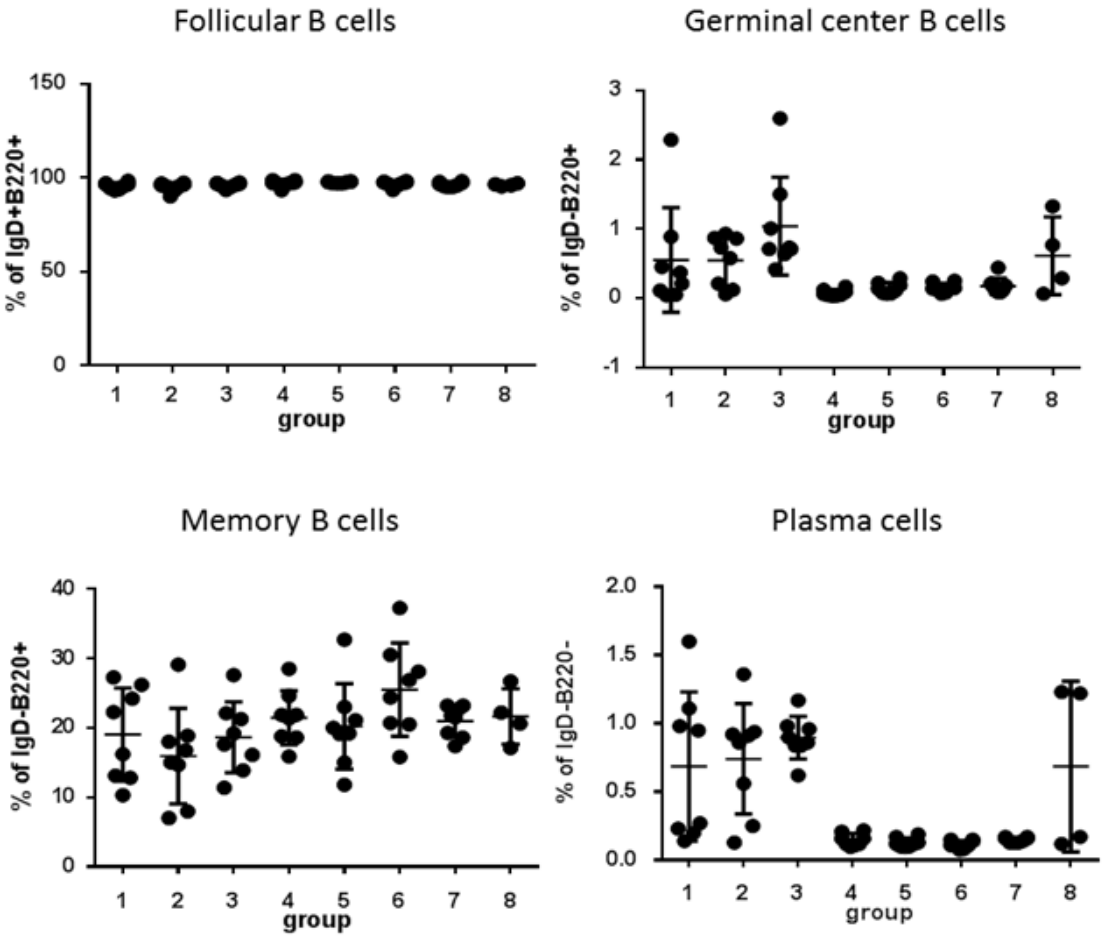


Figure 48. Flow cytometry analysis of B-cells in bone marrow from mice immunized with ST3-tetrasaccharide-CRM197 conjugate using different adjuvant formulations.
The percentage of follicular B cells, germinal center B cells, memory B cells, and plasma cells in bone marrow were analyzed by FACS staining. Mice were immunized with: (1) Prevnar13®, ST3-tetrasaccharide-CRM197 conjugate adjuvanted with (2) aluminum hydroxide, (3) MF59, (4) MPLA, (5) Resiquimod (RQ), (6) MPLA + RQ, (7) bacterial RNA and (8) negative control, H_2O Particles only. The values for a single animal per each group were plotted, and the mean of n = 8 (and n = 4 for the control group) was calculated.

Carbohydrates conjugated to the carrier protein are considered T-cell dependent antigens. The T cell subpopulation, such as naïve T cells, T follicular helper cells, and central memory T cells from spleen and bone marrow, were analyzed by flow cytometry. CD3 antigen appears at all stages of T cell development, which makes it an ideal T cell marker and CD4 molecule help to recognize T helper (197). T cells can be further divided into naïve and memory cells based on

CD44 and CD62L adhesion molecules. Naïve T cells express high levels of CD62L and low CD44 ($CD3^+CD4^+CD62L^+CD44^-$), while memory T cells are recognized by high CD44 and low CD62L expression ($CD3^+CD4^+CD62L^+CD44^+$). T follicular helper cells were gated as $CD3^+CD4^+CD62L^-CD44^-$ and additionally, the high level of the programmed cell death-1 (PD-1) and distinctive for them CXCR5 marker (Figure 49). Similar to B cells, no significant differences were observed for both spleen and bone marrow cells (Figure 50) as the whole T cells, not ST3-tetrasaccharide T cells, were analyzed. The presence of T carbs has still been doubted as their identification is very challenging.

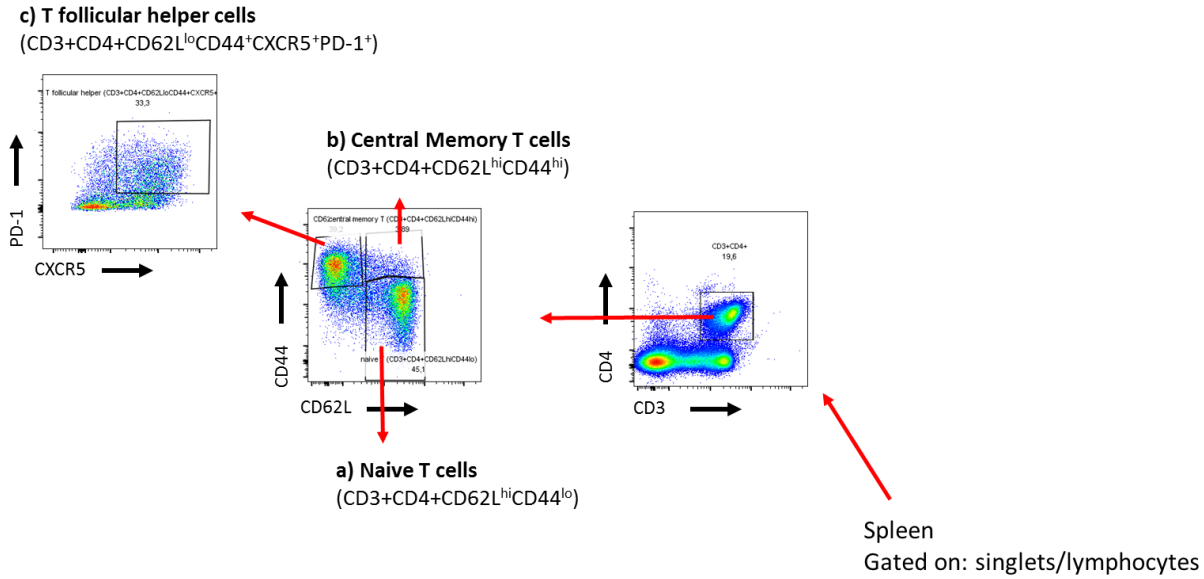


Figure 49. Gating strategy for flow cytometry assay of T-cells from the spleen.
Spleen cells were divided into different T-cells subsets base on CD3 and CD4 staining. Aggregates were excluded by FSC-H vs. FSC-A gating. **a)** Naïve T cells were described as CD3+CD4+CD62L+ CD44low/- population among whole spleen T-cells. **b)** Central Memory T-cells subclass were CD62L+CD44+ among CD3+CD4+ lymphocytes. **c)** T-follicular Helper cells were gated on PD-1+ and CXCR5+ among CD44+CD62Llow/-.

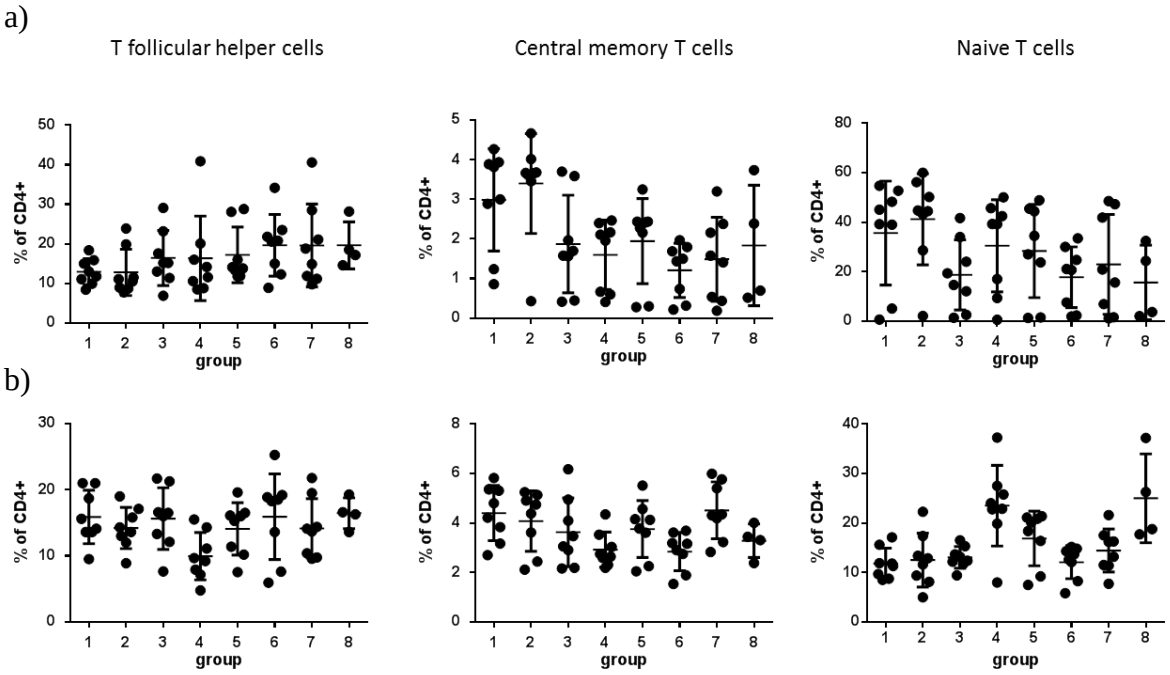


Figure 50. Flow cytometry analysis of T-cells in spleen and bone marrow from mice immunized with ST3-tetrasaccharide-CRM197 conjugate in the different adjuvant formulations.
The percentage of T follicular helper cells, central memory T cells, and naïve T cells in **(a)** spleen and **(b)** bone marrow were analyzed by FACS staining. Mice were immunized with: (1) Prevnar13®, ST3-tetrasaccharide-CRM197 conjugate adjuvanted with (2) Aluminum Hydroxide, (3) MF59, (4) MPLA, (5) Resiquimod (RQ), (6) MPLA + RQ, (7) bacterial RNA and (8) negative control, H_2O Particles only. The values for a single animal per each group were plotted, and the mean of n = 8 (and n = 4 for the control group) was calculated.

Due to the presence of B and T lymphocytes, the spleen is one of the recommended organs to evaluate for enhanced histopathology of the immune system. Functionally, two major zones of the organ can be distinguished: hematogenous red pulp and the lymphoid white pulp composed of the periarteriolar lymphoid sheath (T-cell area), the adjacent follicles (B-cell area), and marginal zone (B-cell area) (198). The lymphoid follicles are very rich in naïve B cells and serve as a filter for antigen, allowing B cells to make contact with the antigen (an activation of B cells and germinal center reaction). Histological analysis of spleen provides a deeper insight into the immune response to antigenic stimuli by observation of area and size of follicles with prominent germinal centers (Figure 51). Activated B cells express the GL7 epitope serving as a marker for germinal centers in the immunized spleen (Figure 52and Figure 53). Unfortunately, no

significant difference in overall histopathology of spleen from mice immunized with the various formulation of ST3-tetrasaccharide CRM197 conjugated vaccine was observed (Figure 54).

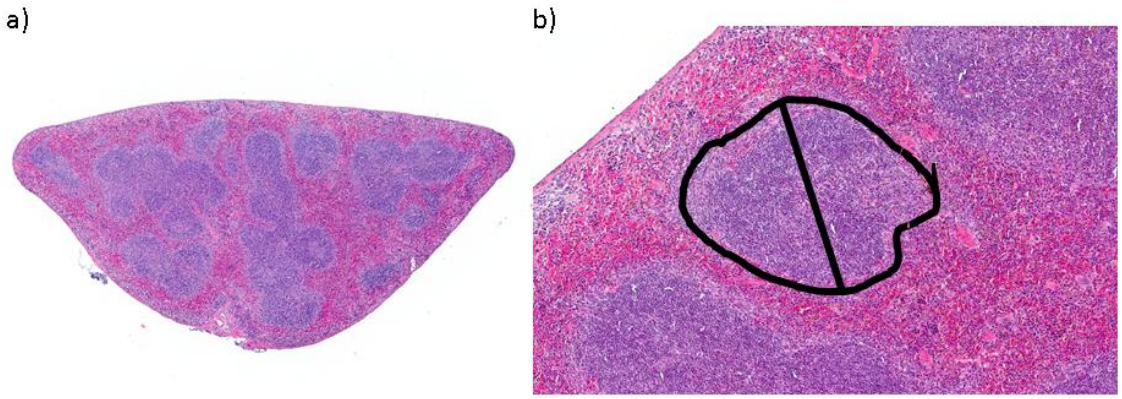


Figure 51. Representative picture of hematoxylin and eosin (HE) staining of a spleen.
Spleen was cut into 2 μm thick sections, and HE-stained. **a)** The whole section of a spleen **b)** Follicle area and follicle diameter.

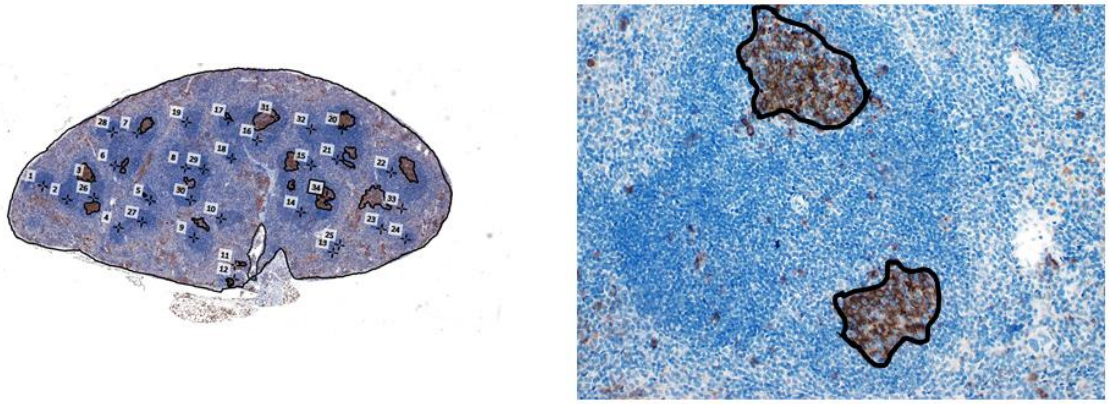


Figure 52. Exemplary picture of immunohistochemistry staining of GL7+ follicle centers in a spleen.
A number of lymphoid follicles per mm^2, a number of GL7+ follicles centers per mm^2, mean area of GL7+ follicle centers (mm^2) and percentage area of GL7+ follicle centers/ spleen area were evaluated.

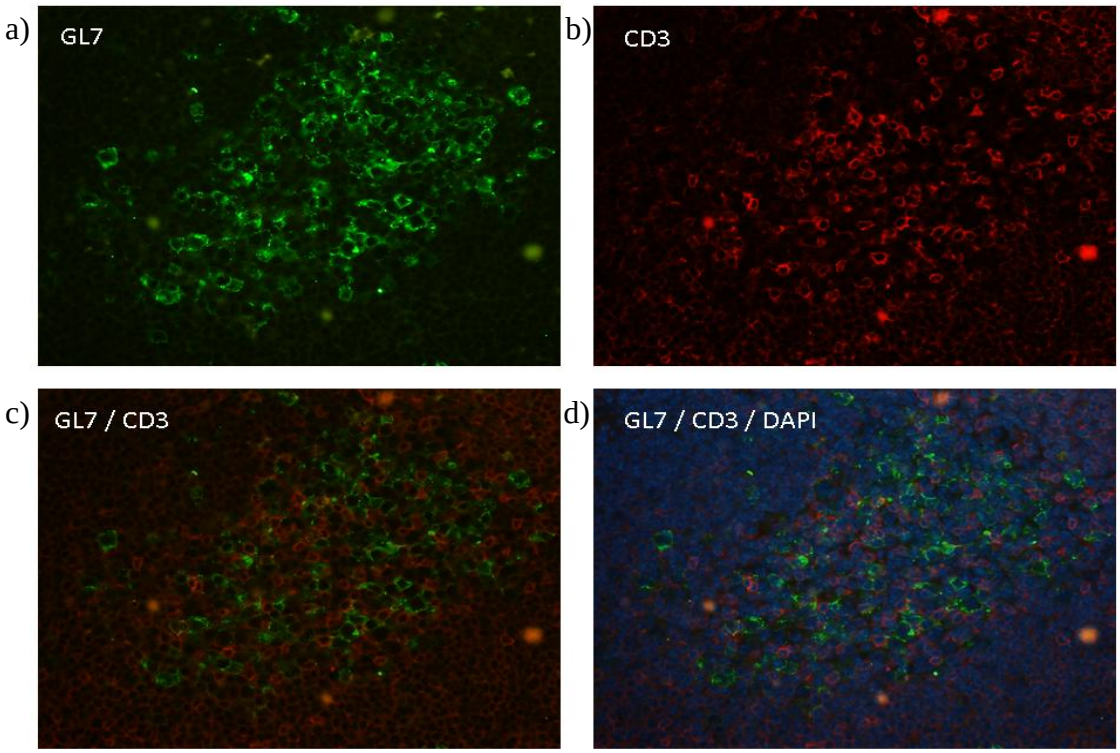


Figure 53. Representative image of spleen immunofluorescent staining for germinal centers.
Spleen was stained with **(a)** anti-GL7 antibodies, **(b)** anti-CD3. The merged image of **(c)** GL7/CD3 positive cells and **(d)** GL7/CD3/DAPI.

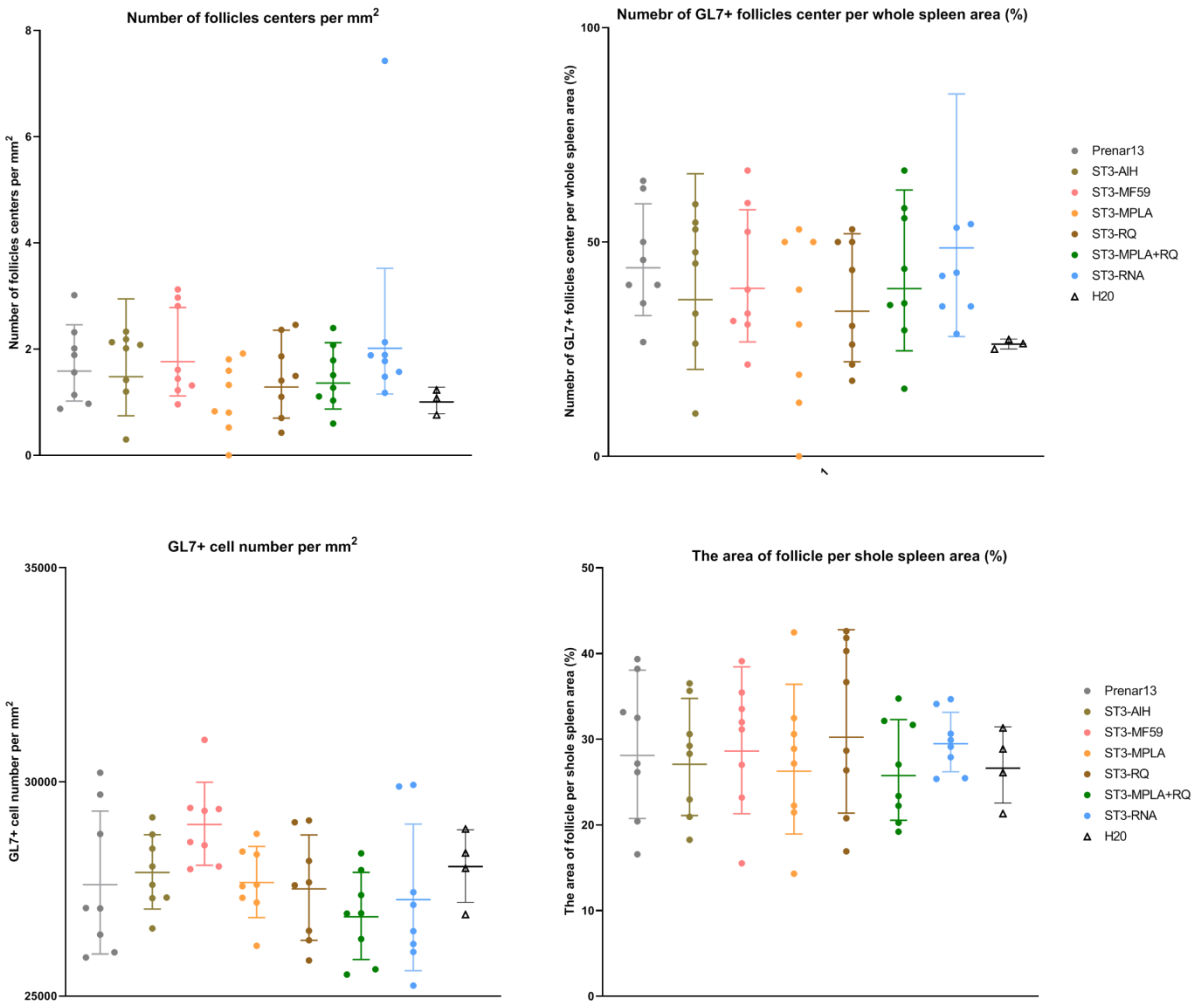


Figure 54. Histopathological analysis of spleens from mice immunized with the various formulation of ST3-tetrasaccharide CRM197 conjugated vaccine.
A number of follicles center per mm^2 **(a)** and percentage of follicles area per whole spleen area **(b)** calculated based on HE staining. A number of GL7+ cells per mm^2 **(c)** and a number of GL7+ follicles center per whole spleen area **(d)** were analyzed after immunohistochemistry staining.

3.9 ST3-tetrasaccharide CRM197 And Pneumolysin Conjugated Vaccines Are Immunogenic In The Swine Model

Despite many essential differences in terms of immunology and physiology between mice and humans, the murine model still has been the gold standard for early pre-clinical studies (199). In many respects, mouse models reflect human diseases very poorly. The complexity of the human disease, the inherent nature of the mouse model, sterile housing conditions, significant differences in cellular composition between mouse and human tissues, differences in the molecular as well as immune response make mouse models unsuitable (200). Domestic pigs are very similar to humans in terms of anatomy, immunology, and physiology. Moreover, porcine immune responses are similar to human responses with >80% of studied parameters, whereas in mice the response has <10% of overlap (201). Additionally, several pathogens such as Gram-positive *Staphylococcus aureus* (202) and Gram-negative *Bordetella pertussis (203)*; viruses like influenza virus (204), infect both humans and pigs, the latter has been described as a very suitable model for the studying human immunology and response to infectious diseases (205). The size of the animal also allows for repeated measurements in time without killing it (206, 207). Therefore, the swine model is considered a highly relevant model for vaccine development.

Herein, six-week-old healthy, female domestic pigs (German landrace *Sus scrofa* were s.c. or intramuscular (i.m) immunized with 2.2 µg of ST3-tetrasaccharide conjugated to CRM197 or pneumolysin and formulated with 0.125 mg of aluminium hydroxide. The control groups were Prevnar13® (positive control) and Alum only (negative control). Conjugates were prepared and characterized as previously described in section 2.3.1 and 2.3.2. An immunization was conducted according to the prime + boost + boost regime (Table 24). Blood was collected on day 0, 10, 20, and final bleeding on day 35/36 and use for antibody response analysis.

Table 24. Immunization regime for pigs

days	Procedure
0	Immunization, bleeding
10	1st boost, bleeding
20	2nd boost, bleeding
35-36	Dissection and bleeding

Since the immune response to protein antigens is much stronger than to carbohydrate antigens, the anti-carrier protein antibody response was measured to estimate whether the immunization by itself was successful and the vaccine was immunogenic in a pig model. A high level of pneumolysin-specific antibodies was measured in the group immunized with ST3-tetrasaccharide pneumolysin conjugate. Animals injected with vaccines containing CRM197 as a carrier protein (ST3-tetrasaccharide CRM197 conjugate and Prevnar13®) show significantly elevated titer of anti-CRM197 IgG (Figure 55). The expected anti-protein response was observed in the immunized group, therefore semi-synthetic ST3-tetrasaccharide CRM197 and pneumolysin conjugated vaccines were immunogenic in the swine model. However, a detectable level of a specific protein response was observed in unrelated groups due to nonsterile housing and breeding of the animals. Those conditions are in fact preferable, as they reflect the human situation such as differences in genetic background, diverse microbiota from food and environment or contact with pathogens.

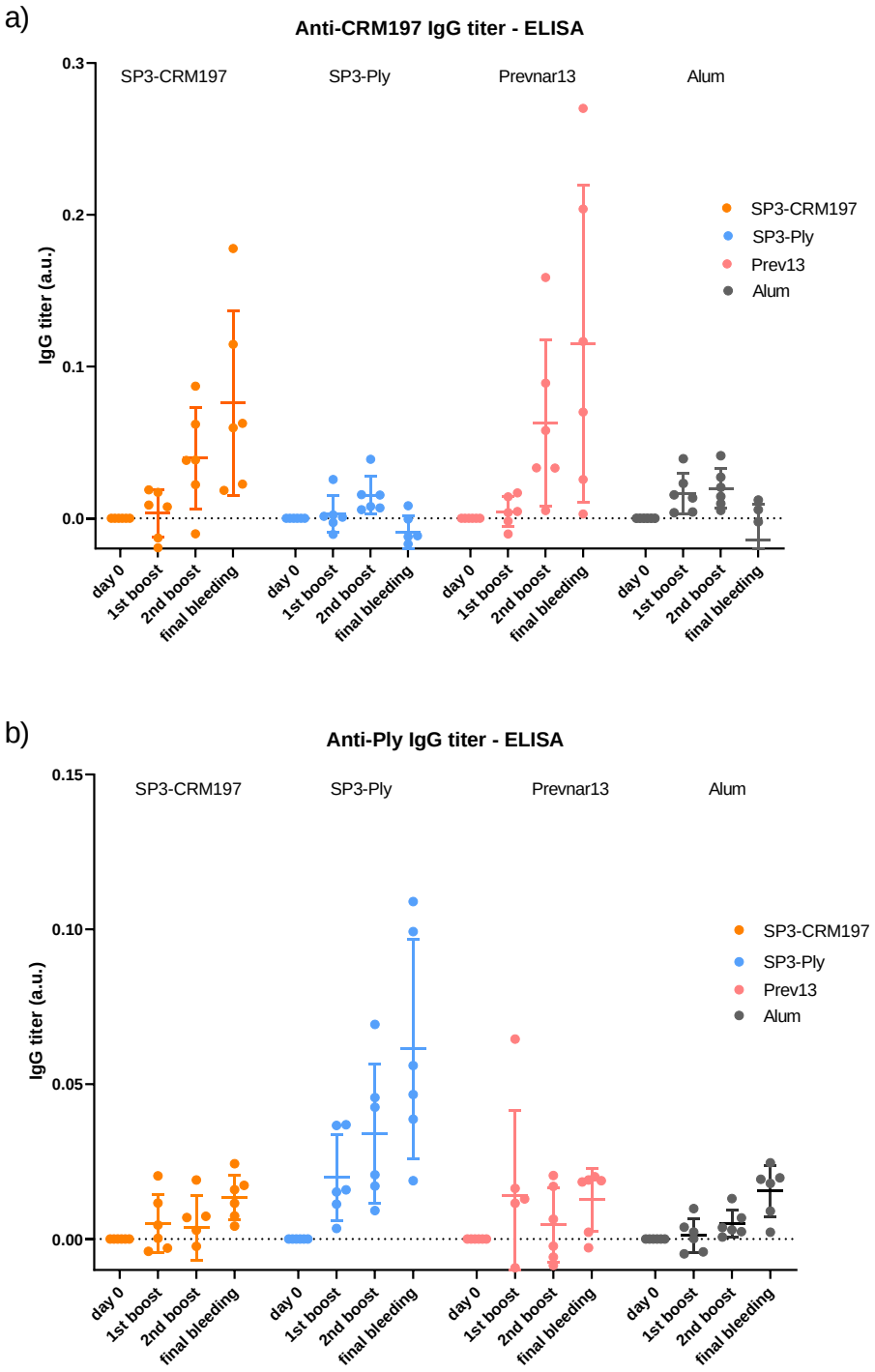


Figure 55. The evaluation of IgG response to (a) CRM197 and (b) Pneumolysin carrier proteins measured by ELISA.

Carrier protein-specific antibody levels were analyzed at day 0, day 10 (first boost), day 20 (second boost), and day 35-36 (final bleeding). Data are measurements of single animal antibodies titer on specified time points; n = 6 animal per group.

The presence of ST3-tetrasaccharide and *S.pneumoniae* serotype 3 CPS cross-specific IgG_1 and IgG_2 were examined by glycan arrays. The arrays contain synthetic and native structures spotted on the glass slides (printing pattern shown in Figure 40b). Antibody isotype distribution of anti-carrier protein antibodies was checked as well. Both CRM197 and Ply specific IgG1, and IgG2 were identified in groups immunized respectively with Prevnar13® and ST3-tetrasaccharide CRM197 glycoconjugate as well as ST3-tetrasaccharide pneumolysin conjugate. For groups immunized with semisynthetic ST3-tetrasaccharide CRM197 and pneumolysin conjugated carbohydrate vaccines, ST3-tetrasaccharide specific IgG_1 titer is significantly higher than IgG_2 (IgG_1: IgG_2 ratio 2.8 and 2.7 respectively). The vaccines were adjuvanted with aluminium hydroxide that has Th_2 properties (mostly IgG_1 production) (Figure 56a). *S.pneumoniae* serotype 3 CPS-cross reactive antibody level was significantly elevated in a group immunized with Prevnar13® as the vaccine contain isolated CPS. Groups immunized with semi-synthetic vaccine show 3.5 x increase in CPS-specific IgG_1 titer and twice higher IgG_2 levels compared to a negative control immunized with Alum only. The study provides the first evidence for the immunogenicity of the ST3-tetrasaccharide CRM197 and pneumolysin conjugated glycoconjugate vaccine in a swine model. The positive results of the semi-synthetic glycoconjugate vaccine candidate in two different animal models might considerably help in advancing the vaccine to clinical trials.

Furthermore, the first success of semi-synthetic glycoconjugates in pigs might open new opportunities in the field of synthetic carbohydrate vaccines for veterinary use. Piglets can develop severe diseases such as sepsis, meningitis, pneumonia, endocarditis as well as arthritis upon infection with *Streptococcus suis* (208). Infected animals are treated individually with a mixture of antibiotics which are very expensive and difficult to control. This leads to a high level of antimicrobial resistance (99.61% of multidrug resistance bacteria were found in pigs) (209). Infection caused by this bacteria can be also transmitted from animal to humans (outbreak in 2005, Sichuan Province in China – 640 pigs and 39 people died from *S. suis* infection) *(210)*. Therefore, a lot of effort has been invested in developing the *S. suis* vaccine for pigs.

To confirm the protective activity of ST3-tetrasaccharide specific antibodies, a standard OPKA was performed using *S. pneumoniae* serotype 3 and HL-60 cells. Human reference serum WHO 007sp served as standardized control to prove that assay was performed within an acceptable range. The results demonstrated that serum from animals immunized with ST3-tetrasaccharide

CRM197 (serum dilution responsible for 50% killing of bacteria, IC_{50} = 528) and pneumolysin (IC_{50} = 240) conjugated vaccines have significantly higher antibody titer responsible for 50% killing of the bacteria than group injected with Prevnar13® (IC_{50} = 122) (Figure 57). *In-vitro* opsonophagocytic killing assay, the gold standard to measure the efficacy of pneumococcal vaccines proved that antibody raised in pigs immunized with semi-synthetic vaccines have protective opsonophagocytic properties.

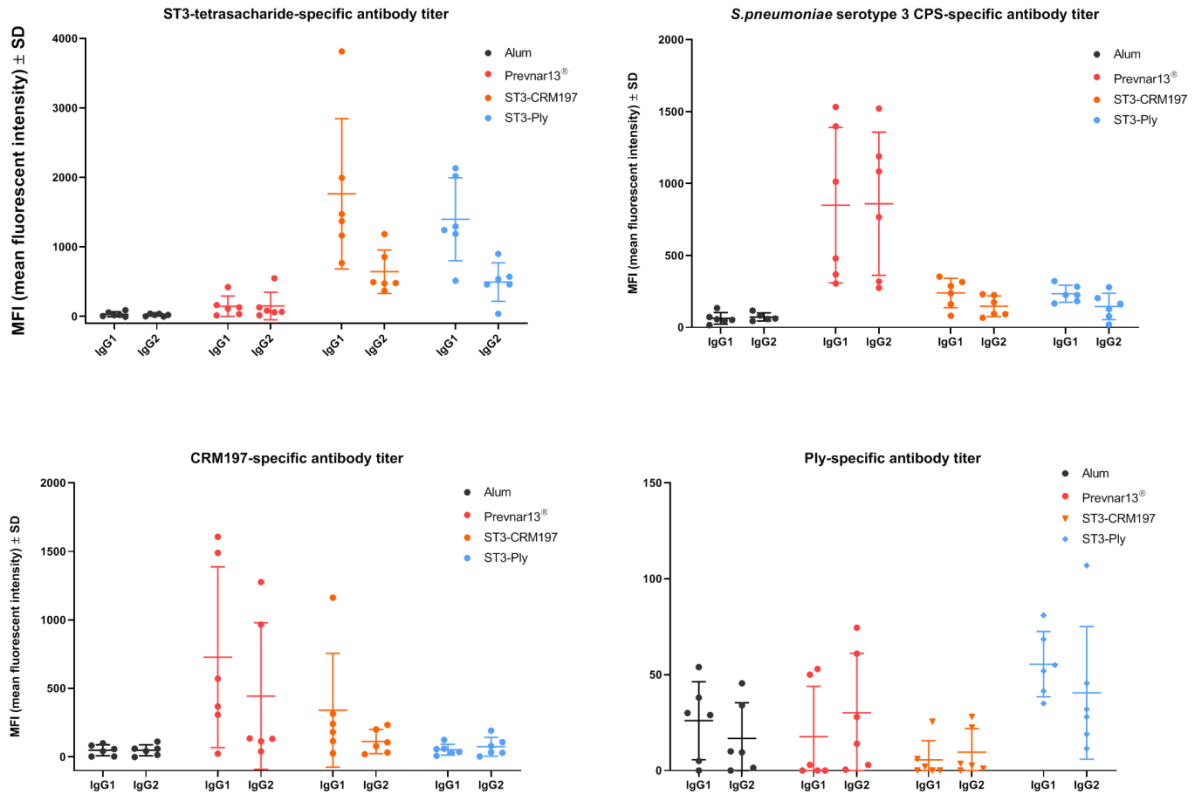


Figure 56. Evaluation of IgG immune response of pigs immunized with ST3-tetrasaccharide CRM197 and pneumolysin conjugates in blood collected at the final time point.

Antibody response against **(a)** synthetic ST3-tetrasaccharide, **(b)** native CPS of *S.pneumoniae* serotype 3, **(c)** CRM197 and **(d)** native pneumolysin, measured in serum obtained from mice vaccinated with Alum only, Prevnar13®, ST3-tetrasaccharide CRM197 conjugate, and ST3-tetrasaccharide Plneumolysin conjugate. Mean fluorescence intensity (MFI) of six animals measured in triplicates were plotted. Data were analyzed by t-test, *$p < 0.05$.

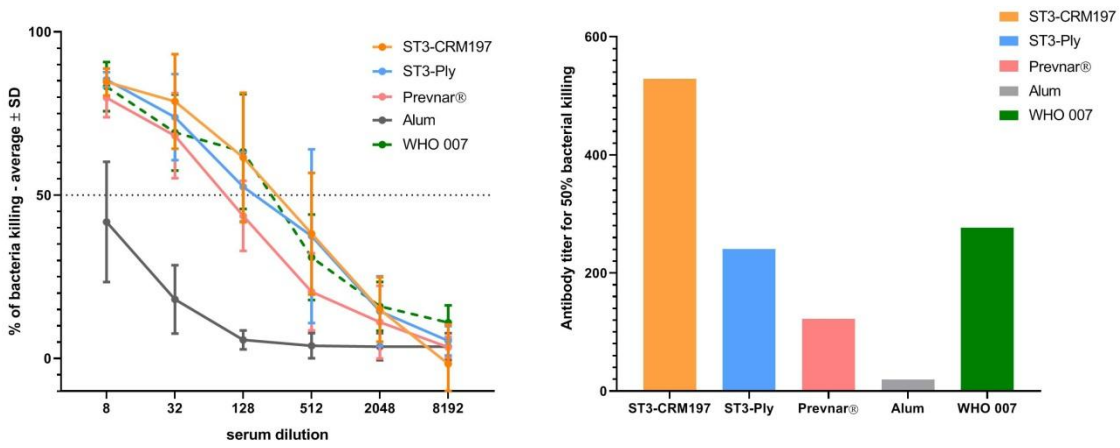
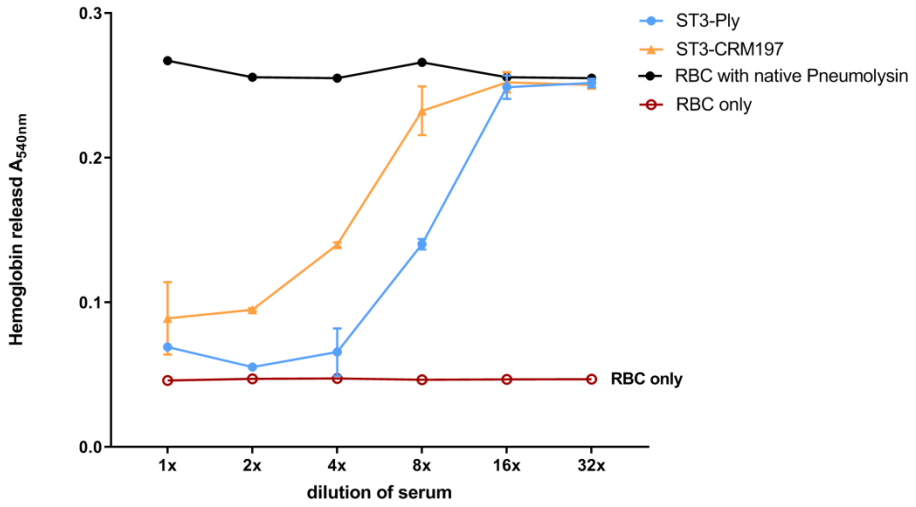


Figure 57. *In-vitro* opsonophagocytic killing activity of antibodies from pigs immunized with ST3-tetrasaccharide CRM197 and pneumolysin conjugates.
a) A comparison of OPKA activity of pooled sera from pigs immunized with ST3-tetrasaccharide CRM197 and ST3-tetrasaccharide pneumolysin conjugates. The commercial vaccine Prevnar13® and Alum only were used as a positive and negative control, respectively. Data are means ± SD of CFU reduction relative to negative control wells (samples lacking either antibody or complement) of two independent experiments. **b)** Evaluation of antibody titer of pooled serum responsible for 50% killing of bacteria in the opsonophagocytic killing assay. IgG titers are expressed as the reciprocal serum dilution mediating 50% bacterial killing, estimated through non-linear interpolation of the dilution-killing OPKA data. Human reference serum 007sp was used as a control.

Antibodies from group immunized with ST3-tetrasaccharide pneumolysin conjugated vaccine showed toxin neutralization activity. These antibodies are able to inhibit the hemolysis of red blood cells by Pneumolysin *in-vitro* (Figure 58). As animals are kept in non-sterile conditions, some of them acquired neutralizing antibodies cross-reactive with pneumolysin. Nevertheless, the group that was immunized with ST3-tetrasaccharide pneumolysin conjugate showed a statistically significant decrease of hemoglobin release compared to Prevnar13® vaccinated animals ($p = 0.0185$) and a substantial reduction with regards to ST3-tetrasaccharide CRM197 ($p = 0.15$) and Alum injected groups ($p =. 0.29$)

Piglets are the natural host for *Streptococcus suis* which share many similarities in pathogenesis with *S. pneumoniae* infection in humans. Piglets could develop severe disease upon infection with *S. suis* like sepsis, meningitis, pneumonia, endocarditis as well as arthritis (208). Therefore, establishing the infection model of *S. pneumoniae* and exploring the protective ability of the ST3-tetrasaccharide pneumolysin conjugated vaccine is planned in the future.

a)



b)

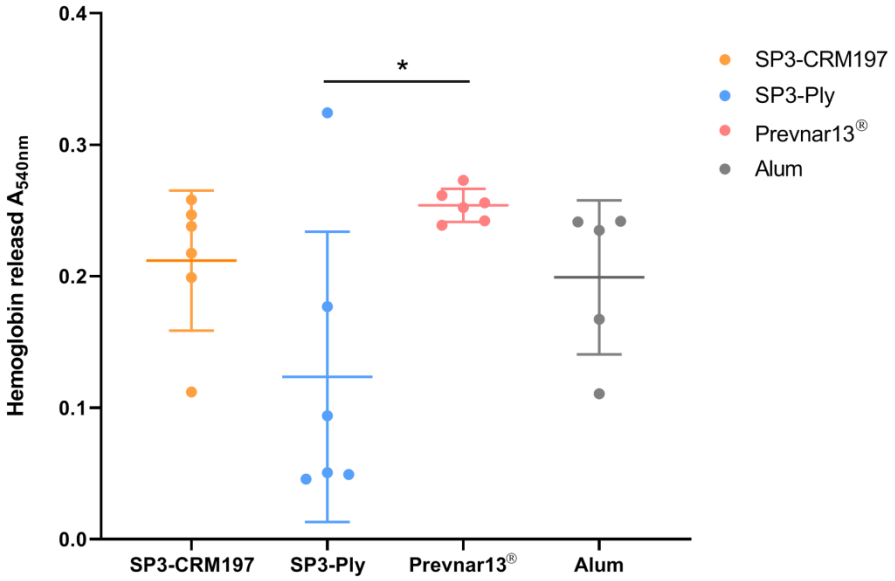


Figure 58. Inhibition of pneumolysin induced lysis of human red blood cells by anti-pneumolysin antibodies from the blood of pigs immunized with ST3-tetrasaccharide Pneumolysin glycoconjugate collected during final bleeding.
a) Inhibition of hemolytic activities of native pneumolysin by antibodies from pigs immunized with ST3-tetrasaccharide Pneumolysin conjugate. Blood was collected during final bleeding on day 35-36. Hemoglobin release is expressed as the A_{540} of assay supernatants (mean ± SD). **b)** Comparison of the toxin neutralization activities of antibodies from mice immunized with ST3-tetrasaccharide CRM197 and ST3-tetrasaccharide pneumolysin conjugates, as well as Prevnar13® and Alum control at the fivefold serum dilution. Data points are shown for single animals in each group (n = 6). Hemoglobin release is expressed as the A_{540} of assay supernatants. The statistical significance was calculated by one-way ANOVA; * $p<0.05$.

The magnitude of an immune response to a glycoconjugate vaccine depends not only on antibodies producing B cells but also on a relatively small population of antigen-specific T lymphocytes which helps B cells as well as control the inflammation and protection in response to the pathogen. Therefore, understanding the role of antigen-specific T cells in the host immune response is essential for developing effective T cell-dependent vaccines (211). A population of antigen-specific T cells in the absence of infection is very tiny (around 1 in 10^{-6} within a naïve and 1–100 in 10^{-5} within the memory of conventional T cells) what makes the analysis of the cell population very difficult (207). The abundance of carbohydrate specific T-cell (T_{carbs}) is even smaller and more challenging to visualize; thus, this subclass is often neglected. It was shown that the expression of the marker CD154 (expressed by conventional CD4 T cells after TCR stimulation), on pig $CD4^+$ T cells occurs within around four hours after antigen restimulation and $CD4^+CD154^+$ cells coexpress cytokines such TNFα (207).

The CD154 expression on swine $CD4^+$ T cells (from immunized animals) restimulated either with a carrier protein included in the semisynthetic ST3-tetrasaccharide glycoconjugate vaccine (CRM197 or pneumolysin) and commercial Prevnar13® (CRM197) or entire glycoconjugates were analyzed. The frequencies of TNFα producing CD154+CD4+ T cells were evaluated according to gating strategy (Figure 59). PBMCs stimulated with medium alone served as a control. The swine study was performed to understand if antigen-specific CD4+ T cells can be monitored at different time-points and detected across all immunized animals.

By restimulation of PBMC obtained from animals immunized with semi-synthetic carbohydrate vaccines, both ST3-tetrasaccharide pneumolysin and ST3-tetrasaccharide CRM197 conjugates, with carrier protein pneumolysin and CRM197 accordingly, I was able to prove the presence of vaccine-specific CD4+T cells in the blood of animals after the third boost. The same PBMC samples re-stimulated with ST3-tetrasaccharide pneumolysin and ST3-tetrasaccharide CRM197 conjugate instead of carriers protein only, showed even higher frequencies of cytokine-producing specific T cells, statistically significant compared to unstimulated PBMC ($p < 0.001$ for ST3-tetrasaccharide CRM197 conjugate and $p < 0.01$ for ST3-tetrasaccharide pneumolysin conjugate) as well as to CRM197 restimulation only ($p < 0.01$) (Figure 60). This observation might suggest the involvement of carbohydrate specific T cells (T_{carbs}). However, it needs further investigation to fully understand the contribution of T_{carbs} in response to semi-synthetic carbohydrate conjugated vaccine.

The above results strongly prove the involvement of T cells in response to the semi-synthetic carbohydrate conjugated vaccine (without the infection present) used in an outbred animal model from conventional husbandry.

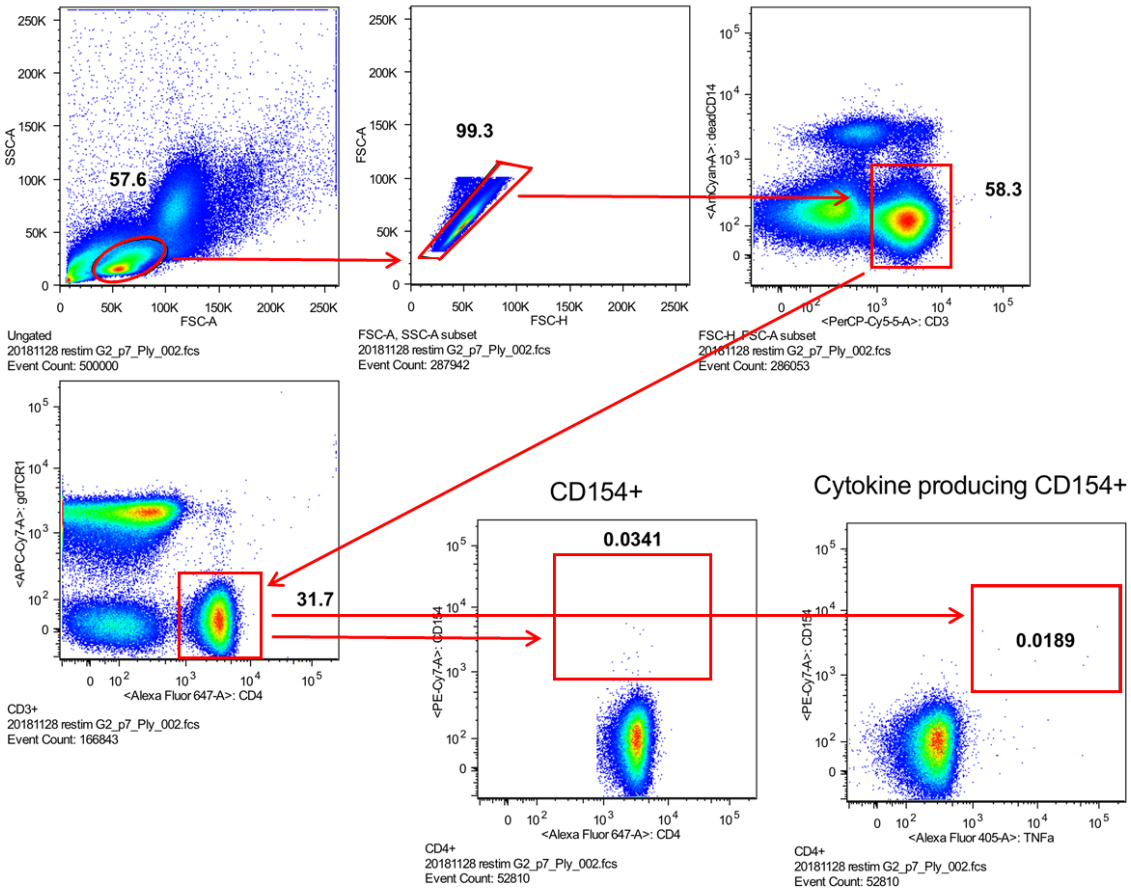
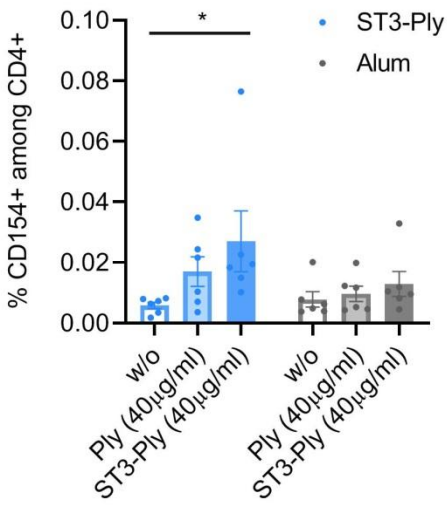
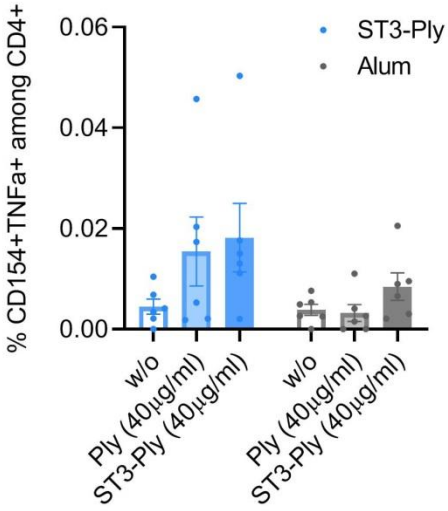
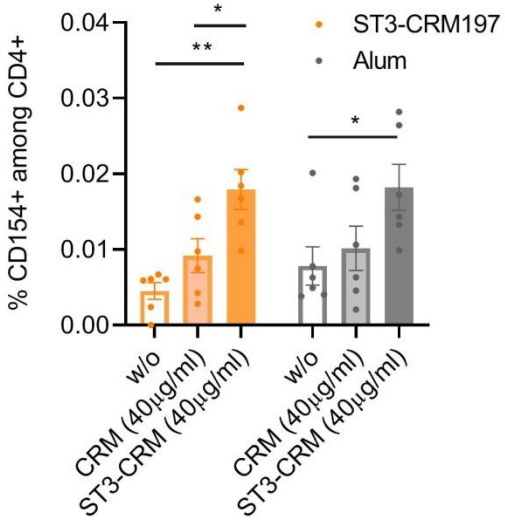


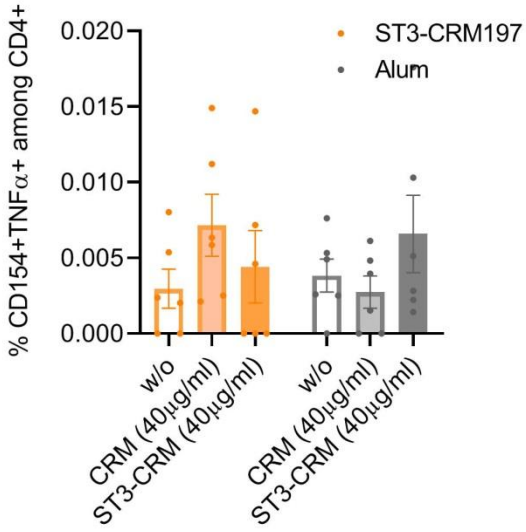
Figure 59. Gating strategy for the CD154+ and TNFα+CD154+ cells from *ex-vivo* PBMC gated on CD4$^+$ T cells that were either unstimulated (w/o) or stimulated with carrier proteins used for vaccination.

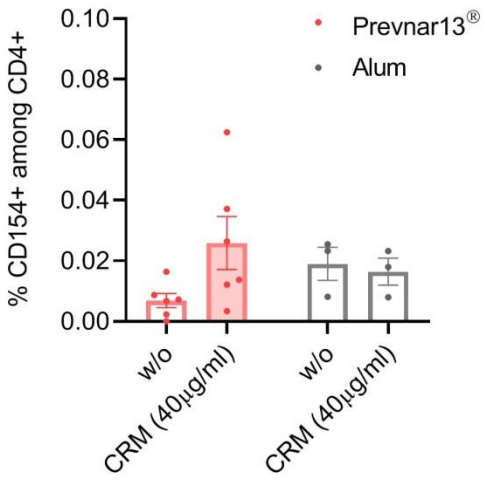
Spleen cells were divided into different T-cells subsets base on CD3+ and CD4+ staining. Aggregates were excluded by FSC-H vs. FSC-A gating. **a)** Naïve T cells were described as CD3+CD4+CD62L+ CD44low/- population among whole spleen T-cells. **b)** Central Memory T-cells subclass were CD62L+CD44+ among CD3+CD4+ lymphocytes. **c)** T- follicular Helper cells were gated on PD-1+ and CXCR5+ among CD44+CD62Llow/-.


% CD154+ among CD4+
0.10
0.08
0.06
0.04
0.02
0.00
*
ST3-Ply
Alum
w/o
Ply (40µg/ml)
ST3-Ply (40µg/ml)
w/o
Ply (40µg/ml)
ST3-Ply (40µg/ml)


% CD154+TNFa+ among CD4+
0.06
0.04
0.02
0.00
ST3-Ply
Alum
w/o
Ply (40µg/ml)
ST3-Ply (40µg/ml)
w/o
Ply (40µg/ml)
ST3-Ply (40µg/ml)


% CD154+ among CD4+
0.04
0.03
0.02
0.01
0.00
*
**
*
ST3-CRM197
Alum
w/o
CRM (40µg/ml)
ST3-CRM (40µg/ml)
w/o
CRM (40µg/ml)
ST3-CRM (40µg/ml)


% CD154+TNFα+ among CD4+
0.020
0.015
0.010
0.005
0.000
ST3-CRM197
Alum
w/o
CRM (40µg/ml)
ST3-CRM (40µg/ml)
w/o
CRM (40µg/ml)
ST3-CRM (40µg/ml)


% CD154+ among CD4+
0.10
0.08
0.06
0.04
0.02
0.00
Prevnar13®
Alum
w/o
CRM (40µg/ml)
w/o
CRM (40µg/ml)

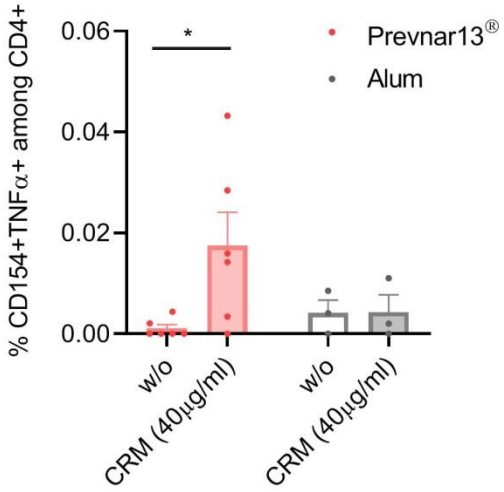

% CD154+TNFα+ among CD4+
0.06
0.04
0.02
0.00
*
Prevnar13®
Alum
w/o
CRM (40µg/ml)
w/o
CRM (40µg/ml)

Figure 60. Restimulation of swine PBMCs accordingly to the primary immunization with carrier protein CRM197 or pneumolysin and glycoconjugates ST3-tetrasaccharide CRM197 or ST3-tetrasaccharide pneumolysin shows increased levels of CD154+ and TNFα+CD154+ antigen-specific CD4+ cells.

To detect antigen-reactive T cells, PBMCs collected during final bleeding (day 35-36) were *ex vivo* restimulated with carrier proteins or glycoconjugates and unstimulated cells. The percentage of CD154+ and TNFα+CD154+ antigen-specific cells among the CD4+ population was analyzed by FACS. Comparison of antigen-specific CD154+ and TNFα+CD154+ cells after restimulation of PBMC from animals immunized with **(a)** ST3-tetrasaccharide Pneumolysin glycoconjugate vaccine and Alum control group with Pneumolysin (40 μg/mL) and ST3-tetrasaccharide Pneumolysin conjugated (40 μg/mL) as well as unstimulated group; **(b)** pigs vaccinated with ST3-tetrasaccharide CRM197 glycoconjugate vaccine and Alum control group with CRM197 (40 μg/mL) and ST3-tetrasaccharide CRM197 conjugated (40 μg/mL) as well as unstimulated group; **(c)** animals injected with Prevnar13® and Alum control group with CRM197 (40 μg/mL) as well as unstimulated group. The values for a single animal per each group were plotted, and the mean ±SD of n = 6 (and n = 3 for the second batch of Alum control group) was calculated. The statistical analysis was performed by two-way ANOVA; * $p<0.01$, ** $p<0.001$.

4 Conclusions and Outlook

Streptococcus pneumoniae remains a deadly disease in small children and the elderly, even though polysaccharide and conjugate vaccines based on isolated capsular polysaccharides (CPS) are relatively successful. The most common serotypes that cause infections are used in vaccines around the world, but differences in geographic and demographic serotype distribution compromise protection by leading vaccines. The isolation of CPS from cultured bacteria for obtaining polysaccharides for conjugate-vaccine production is operationally challenging. Thus, we use the chemical synthesis approach to produce the minimal protective epitope of diverse oligosaccharides resembling the CPS of different serotypes. *S.pneumoniae* serotype 3, although included in the commercial vaccines, has shown atypical immunogenicity. It is attributed to the high CPS expression by the bacteria and to a weakened booster response leading to the incapability of vaccinated individuals to mount an immune response after booster immunization (212). Thus, serotype 3 has still been one of the most prevalent serotypes. To improve the immunogenic properties of ST3, synthetic antigenic structures based on antibody binding specificities were designed and synthesized (104). The medicinal chemistry approach for creating glycoconjugate vaccines has helped to improve the stability and immunogenicity of synthetic vaccine candidates for several serotypes and has increased the production of specific protective antibodies against different pathogens (90, 98, 213).

Synthetic tetrasaccharide based on *S.pneumoniae* serotype 3 (ST3) CPS repeating units have been conjugated to a CRM197 carrier protein and characterized as immunogenic in a mouse model. However, the protection had to be improved (104). Serotype 3 was chosen to illustrate the principle of proper optimization of variable aspects of vaccine formulation, such as dosage, adjuvant, and carrier protein. Understanding the most important aspects of vaccine preparation helps to obtain a better immune response to semi-synthetic vaccine candidates and protection against pneumococcal disease.

No information on the optimal dosage of oligosaccharide antigen was available previously, I started my work by establishing the best vaccine quantity. The amount of 2.2 μg of naïve CPS is used in commercial Prevnar13® vaccine. However, taking into account the impurities of the isolated CPS and the size of the mouse model the amount of synthetic oligosaccharide for animal

study can be reduced. I proved that the amount of synthetic antigen can be significantly decreased while maintaining sufficient protection shown by *in-vitro* opsonophagocytic killing assay. In defiance of the general notion “the more, the better”, the higher dose did not improve the protective effect of immunization but could even diminish the final success of the vaccination. However, with the very low amount of antigen, the immune response was delayed and an additional boost was needed. For my subsequent study, I decided to follow the best dose of the ST3-tetrasaccharide CRM197 conjugate equal to 0.4 μg of synthetic ST3-tetrasaccharide. The data is relevant only for the mouse model and it has to be evaluated in clinical studies for human use. Nevertheless, utilizing highly pure synthetic oligosaccharides in precisely optimized dosages is one of the fundamental aspects for successful vaccination.

Serotype replacement is a major issue in the case of glycoconjugate vaccines, that has prompted big efforts to develop a universal vaccine. The usage of conserved membrane proteins has been investigated. Unfortunately, as a bacteria capsule is the first target for the immune system, bacterial proteins cannot provide sufficient protection against infection. Therefore, combining the approach of using the synthetic oligosaccharide from the capsules of the most prevalent serotypes in conjugation to conserve bacterial protein has been a goal of my study. The carrier protein derived from *S.pneumoanie* can serve as “double-action bullet”, being both a carrier essential for glycan presentation and an additional vaccine antigen providing broader protection, so-called "additional valency". Hence, ST3-tetrasaccharide was conjugated to pneumolysin and PspA protein. Immunization with ST3-pneumolysin glycoconjugate triggered the production of protective anti-pneumolysin and anti-carbohydrate antibodies. Neutralizing antibodies against protein inhibited the hemolysis of human red blood cell by whole bacteria cell lysate and prevented alveolar epithelial cell permeability *in vitro*. The *in-vivo* model showed that ST3-tetrasaccharide Pneumolysin conjugates decrease the bacteria load in blood and lungs as well as reduce the disease severity in mice challenged with *S.pneumoniae* serotype 3. Additionally, nasal immunization with ST3-tetrasaccharide and ST2-hexasaccharide conjugated to pneumolysin and PspA inhibited colonization of the nasopharynx after infection with S.*pneumoniae* serotype 2. I was able to prove for the first time that the semi-synthetic oligosaccharide vaccine, utilizing conserved proteins pneumolysin and PspA, acts as a double-action bullet by triggering both local protection against bacteria colonization and systemic defense by the antibody-mediated opsonophagocytic killing of the bacteria during the infection.

The use of conservative protein needs to be further investigated. Combining different bacteria serotypes and various carrier proteins to extend vaccine protection will be the next step. Employing a chimeric protein, e.g. a combination of PspA and PspC for glycoconjugate vaccine seems to be also very attractive approach towards broadening a vaccine spectrum. Furthermore, investigating the differences in antigen recognition and presentation based on the carrier protein will be the question to answer.

An adjuvant is employed in vaccines to help to create a stronger and more efficient immune response and provide the protection from the disease. Therefore, the adjuvant is an essential part of a vaccine. Several adjuvants characterized by various mechanisms and different immune profile (cell-mediated or humoral and T-helper 1 or T-helper 2 (T_h1/T_h2) immune responses) are currently used (113, 214). The adjuvant formulation influence the magnitude of the immune response within the time course and the functional activity of the antibody response upon vaccination with ST3-tetrasaccharide CRM197 conjugate vaccine was tested. In order to improve the immunogenicity of semi-synthetic ST3-glycoconjugates, I used several commercially available adjuvants and incorporated them into biodegradable poly-lactic-acid (PLA) microparticles. This approach allowed for the physical combination of adjuvants and antigens, and it ensured the targeted uptake of the vaccine by professional phagocytes. Screening experiments in mice yielded promising results for agonists of TLR7/8, namely resimiquimod (R848) and bacterial RNA, as well as MPLA, a TRIF-biased TLR4 agonist used in several commercial vaccines. However, the OPKA results indicated that the RQ-848 and MF59^{TM} are the best candidates for the formulation of the ST3-tetrasaccharide CRM197 conjugated vaccine. No correlation was observed between antibody titer and opsonophagocytic protective activity in groups immunized with ST3-tetrasaccharide CRM197 conjugate and the different adjuvant formulation. A detailed investigation of the antibody has to be performed to fully understand the vaccine formulation effect generated after immunization as their avidity and character of immunocomplexes formed.

Antibody repertoires displayed by mice differ from other mammals, including humans (215), and species-specific variations of the immune response to glycan antigen have been observed (67). Pigs are very similar to humans in terms of anatomy, immunology, and physiology, and have been described as suitable models for different human infectious disease (206) as well as vaccine development (199). Thus, piglets were chosen as an animal model to evaluate the

immunogenicity of semi-synthetic ST3-tetrasaccharide CRM197 and pneumolysin conjugated vaccines. The study provides the first evidence for the immunogenicity of the synthetic glycoconjugate vaccine in a swine model. The generated antibodies were able to kill pneumococci and neutralize the toxic effect of pneumolysin *in-vitro.* However, the protective activity of the glycoconjugate vaccines in the swine *in-vivo* infection model has to be further investigated. I was also able to prove the involvement of T cells in response to semi-synthetic carbohydrate conjugated vaccine as well as the specific carbohydrate T cells subpopulation, namely T_{carbs}. The successful outcome of the study facilitates the possibility of advancing the ST3-tetrasaccharide conjugate vaccine for human clinical trial and opens new opportunities in a field of synthetic carbohydrate vaccine research for veterinary purposes.

In summary, I combined a series of innovations, which will not only enhance the efficacy and applicability of glycoconjugate vaccines but will also help to further clarify the principles of anti-carbohydrate- and anti-bacterial immunity. Therefore, my thesis work will open the road for efficacious, synthetic vaccines against bacterial pneumonia.

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