Towards glycoconjugate vaccines against carbapenem-resistance *Klebsiella pneumoniae*

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By

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This work was performed between April 2017 and April 2021 under the guidance of Prof. Dr. Peter H. Seeberger in the Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces Potsdam, and the Institute of Chemistry and Biochemistry, Freie Universität Berlin.

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Declaration

This is to certify that the entire work in this thesis has been carried out by Bruna M. S. Seco. 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

1- B. M. S. Seco, D. Shen, P. H. Seeberger. *Fighting urgent threats: Semi-synthetic glycoconjugate vaccine against carbapenem-resistant Klebsiella pneumoniae*. Manuscript in preparation.

2- Heidepriem J, Dahlke C, Kobbe R, Santer R, Koch T, Fathi A, Seco BMS, Ly ML, Schmiedel S, Schwinge D, Serna S, Sellrie K, Reichardt N-C, Seeberger PH, Addo MM, Loeffler FF, on behalf of the ID-UKE COVID-19 Study Group. *Longitudinal Development of Antibody Responses in COVID-19 Patients of Different Severity with ELISA, Peptide, and Glycan Arrays: An Immunological Case Series*. Pathogens. 2021; 10(4):438.

3- B. M. S. Seco, F.-F. Xu, A. Grafmüller, N. Kottari, C. L. Pereira and P. H. Seeberger. *Sequential Linkage of Carbohydrate Antigens to Mimic Capsular Polysaccharides: Toward Semisynthetic Glycoconjugate Vaccine Candidates against Streptococcus pneumoniae Serotype 14.* ACS Chemical Biology 2020 15 (9), 2395-2405.

4- S. R. Sanapala, B. M. S. Seco*, J. Y. Baek, S. I. Awan, C. L. Pereira, P. H. Seeberger. *Chimeric oligosaccharide conjugate induces opsonic antibodies against Streptococcus pneumoniae serotypes 19A and 19F.* Chem. Sci., 2020, 11, 7401-7407. (*Co-Author).

5- F. Bröcker, E. Wegner, B. M. S. Seco, P. Kaplonek, M. Bräutigam, A. Ensser, F. Pfister, C. Daniel, C. E. Martin, J. Mattner, P. H. Seeberger. *Synthetic Oligosaccharide-Based Vaccines Protect Mice from Clostridioides difficile Infections*. ACS Chem. Biol. 2019, 14, 12, 2720–2728.

6- E. Diago-Navarro, M. P. Motley, G. Ruiz-Perez, W. Yu, J. Austin, B. M. S. Seco, G. Xiao, A. Chikhalaya, P. H. Seeberger, B. C. Fries. *Novel, Broadly Reactive Anticapsular Antibodies against Carbapenem-Resistant Klebsiella pneumoniae Protect from Infection*. mBio. 2018: 9 (2).

Scientific Conferences and Prizes

Scientific Conferences and Symposia:

1- Good Scientific Practice Minisymposium. Center of Infection Biology and Immunity, Berlin. Germany, 2020.

2- Keystone eSymposia meeting on Vaccinology in the Age of Pandemics: Strategies Against COVID-19 & Other Global Threats. Online, 2020.

3- Summer symposium – Ecology of Infectious Diseases. Germany, 2019.

4- 14th Advanced Immunology Summer School, ENII (European Network of Immunology Institutes). Italy, 2019 (Poster).

5- Symposium Infectious Diseases in the 21st Century – Global Challenges for Health and Society. Germany, 2018 (Poster).

6- Biomolecular System Conference. Max Planck for Colloids and Interfaces. Ringberg, Germany, 2017. (Oral presentation).

Prizes:

1- Outstanding Poster Award. Glycan-conjugate vaccine against antimicrobial resistant *Klebsiella pneumoniae*. 14th Advanced Immunology Summer School, ENII (European Network of Immunology Institutes). Italy, 2019.

2- Speed Lecture Awards 2021 – 1st Place. Three minutes presentation about my PhD research. Berlin, Germany, 2021.

Contents

List of Figures and Tables

List of Abbreviations

AGA	Automatic Glycan Assembly
ALI	Acute Lung Injury
APC	Antigen-Presenting Cell
a.u.	Arbitrary Units
BALF	Bronchoalveolar Lavage Fluid
BCR	B Cell Receptor
BSA	Bovine Serum Albumin
CDC	Center for Disease Control and Prevention
CCL	CC chemokine ligand
CFU	Colony-Forming Units
CLR	C-type Lectin Receptor
COVID-19	Corona Virus Disease-19
CPS	Capsular Polysaccharide
CR	Complement Receptors
CRE	Carbapenem-Resistant Enterobacteriaceae
CRKP	Carbapenem-Resistant *K. pneumoniae*
CRM_{197}	Corynebacterium diphtheria Mutant CRM_{197}
CXCL	CXC chemokine ligand
Da	Dalton
DAMPs	Danger Associated Molecular Patterns
DC	Dendritic Cell
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DT	diphtheria toxoid
DTT	1,4-Dithiothreitol
ECVP	European College of Veterinary Pathologists
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
GC	Germinal Center
HE	Hematoxylin and Eosin
HIS	Human Inactivated Serum
HPLC	High Performance Liquid Chromatography
HRP	Horse Radish Peroxidase
IgG/IgA	Immunoglobulin G/A
IC_{50}	The half maximal inhibitory concentration
ICUs	Intensive Care Units
IL	Interleukin
IFN	Interferon
i.p.	Intraperitoneal
i.m.	Intramuscular
KPC	*K. pneumoniae* Carbapenemases
LB	Luria Broth
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAC	Membrane Attack Complex
MAIT	Mucosa-Associated Invariant T cells
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization With Time-Of-Flight Detection MS Mass Spectrometry
MASPs	MBL-associated serine proteases
MBL	Mannan-Binding Lectins
MDR	Multi-Drug-Resistant
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex

MOI	Multiplicity of Infection
MW	Molecular Weight
NET´s	Neutrophil Extracellular Traps
NHS	Normal Human Sera
NK	Natural-killer
NLRs	NOD-like Receptors
NOD	Nucleotide-binding Oligomerization Domain
OD	Optical Density
OPKA	Opsonophagocytic Killing Assay
ORFs	Open Reading Frames
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate-Buffered Saline
PBST	Phosphate Buffered Saline with 0.1% Tween-20
PFA	Paraformaldehyde
PMT	Photomultiplier Tube
PNP	*p*-nitrophenyl adipate ester
PRR	Pattern-Recognition Receptor
ROS	Reactive Oxygen Species
RT	Room Temperature
RU	Repeating Units
SDS-PAGE	Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis
SLO	Secondary Lymphoid Organs
ST258	Sequencing Type 258
TCR	T Cell Receptor
TD	T cell Dependent
Tfh	T follicular helper
Th	T cell helper
THB	Todd Hewitt Broth
TI	T cell Independent

TMB	3,3',5,5'-Tetramethylbenzidine
TT	Tetanus Toxoid
TLRs	Toll-like receptors
UTI	Urinary Tract Infection
WHO	World Health Organization
XDR	Extensively-Drug-Resistant
ZPS	Zwitterionic Polysaccharides

Summary

Antimicrobial resistant bacteria account for high mortality rates due to the lack of effective methods to combat the pathogens. Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is a bacterial group of huge concern that is resistant to nearly all available antibiotics. CRKP has been classified as an "urgent threat" to public health with a dire need to develop new drugs alternative treatments. Antibody therapy or even better, a vaccine, targeting this group would be a viable alternative to resolve this threat. Current strategies to target CRKP rely mostly on the bacterial capsular polysaccharides as antigens. However, the high variability in the CPS composition and low epidemiological correlation of clinical isolates would lead to vaccines with limited target spectrum and numerous antibodies for each CPS variant would be needed for acute treatment, making it a very costly approach. Contrary, O-antigens that are part of the bacterial LPS, account with just four serotypes for the majority of clinically relevant strains, with the O2afg serotype as the most frequent in the CRKP group. The use of native LPS-based antigens for vaccine development is hampered by the low immunogenicity, the lack of a T-cell dependent immune answer, and endotoxin contaminations in LPS batches, which can lead to lethal side effects. Thus, no LPS based vaccines are currently in development. In my thesis I address this bottleneck by exploring a semi-synthetic glycoconjugate vaccine using a highly pure and well-characterized synthetic oligosaccharide that mimics the native O2afg antigen of CRKP. The antigen was designed based on a hexasaccharide repeating unit of O2afg and synthesized by Dr. Dacheng Shen. The glycan was subsequently conjugated to CRM_{197}, a carrier protein, and adsorbed into alum as adjuvant, creating a semi-synthetic glycoconjugate vaccine against CRKP based on approved and validated technology.

I provide conclusive evidence that the novel glycoconjugate vaccine induces a strong T-cell dependent supported by an immune response with a long-term memory effect at very low antigen concentrations. The resulting antibodies activate complement deposition, thereby activating the opsophagocytic pathway. The generated antibodies are well suited for passive immunisation and result in a significant improvement of several physiological parameters in a mouse model for acute pneumonia. This included lower levels of cytotoxic enzymes, neutrophil

infiltration and the reduction of permeability and lung edema. Hospital-acquired pneumonia is the major problem caused by *K. pneumoniae,* especially among individuals with critical illness, for example HIV and cancer in intensive care units. Recently, the pathogen has also been associated with SARS-CoV-2 pulmonary co-infections contributing to chronic obstructive pulmonary diseases, a severe COVID-19 condition with high mortality rates. Thus, the antibodies would significantly reduce the burden as a result of co-infection and increase the chances of survival. I show that a highly effective and low cost glycoconjugate vaccine targeting conserved LPS structures can be developed based on proven technology and opens up for a safer alternative to fight antimicrobial resistant pathogens without the effect of evolutionary selection of resistant strain caused by the currently treatment with antibiotics.

Zusammenfassung

Antibiotikaresistente Bakterien sind für eine hohe Sterblichkeitsrate verantwortlich, da wirksame Methoden zur Bekämpfung der Krankheitserreger fehlen. Carbapenem-resistente *Klebsiella pneumoniae* (CRKP) ist eine sehr besorgniserregende Bakteriengruppe, die gegen fast alle verfügbaren Antibiotika resistent ist. CRKP wurde als „akute Bedrohung“ für die öffentliche Gesundheit eingestuft, die dringend die Entwicklung neuer Medikamente erfordert. Eine Antikörpertherapie oder noch besser ein Impfstoff, der auf diese Gruppe abzielt, wäre eine praktikable Alternative, um diese Bedrohung zu beseitigen. Gegenwärtige Strategien zur Bekämpfung von CRKP beruhen hauptsächlich auf den bakteriellen Kapselpolysacchariden (CPS) als Antigenen. Die hohe Variabilität in der CPS-Zusammensetzung und die geringe epidemiologische Korrelation klinischer Isolate würden jedoch zu Impfstoffen mit begrenztem Zielspektrum führen und für die Akutbehandlung würden zahlreiche Antikörper für jede CPS-Variante benötigt, was dies zu einem sehr kostspieligen Ansatz macht. Im Gegensatz dazu machen O-Antigene, die Teil des bakteriellen Lipopolysaccharids (LPS) sind, mit nur vier Serotypen die Mehrheit der klinisch relevanten Stämme aus, wobei der O2afg-Serotyp der häufigste in der CRKP-Gruppe ist. Die Verwendung von nativen LPS-basierten Antigenen für die Impfstoffentwicklung wird durch die geringe Immunogenität, das Fehlen einer T-Zell-abhängigen Immunantwort und Endotoxin-Kontaminationen in LPS-Chargen erschwert, die zu tödlichen Nebenwirkungen führen können. Daher befinden sich derzeit keine Impfstoffe auf LPS-Basis in der Entwicklung. In meiner Dissertation adressiere ich diese Herausforderung, indem ich einen halbsynthetischen Glykokonjugat-Impfstoff unter Verwendung eines hochreinen und gut charakterisierten synthetischen Oligosaccharids erforsche, das native O2afg-Antigen von CRKP nachahmt. Das Antigen wurde basierend auf einer sich wiederholenden Hexasaccharideinheit von O2afg entwickelt und von Dr. Dacheng Shen synthetisiert. Das Glykan wurde anschließend an das Trägerprotein CRM197 konjugiert und als Adjuvans an Alaun adsorbiert, wodurch ein halbsynthetischer Glykokonjugat-Impfstoff gegen CRKP basierend auf genehmigter und validierter Technologie geschaffen wurde.

Ich liefere schlüssige Beweise dafür, dass der neuartige Glykokonjugatimpfstoff bei sehr niedrigen Antigenkonzentrationen eine starke T-Zell-abhängige, unterstützte Immunantwort mit

Langzeitgedächtniseffekt induziert. Die resultierenden Antikörper aktivieren die Komplementablagerung, wodurch der opsophagozytäre Weg aktiviert wird. Die erzeugten Antikörper eignen sich gut für die passive Immunisierung und führen zu einer signifikanten Verbesserung mehrerer physiologischer Parameter in einem Mausmodell für akute Pneumonie. Dazu gehörten niedrigere Konzentrationen an zytotoxischen Enzymen, verringerte Neutrophilen-Infiltration und Permeabilität sowie eine geringere Anzahl an Lungenödemen. Eine im Krankenhaus erworbene Lungenentzündung ist das Hauptproblem, das durch *K. pneumoniae* verursacht wird, insbesondere bei Patienten auf Intensivstationen mit kritischen Erkrankungen wie HIV und Krebs. Kürzlich wurde der Erreger auch mit pulmonalen SARS-CoV-2-Koinfektionen in Verbindung gebracht, die zu chronisch obstruktiven Lungenerkrankungen beitragen, einer schweren COVID-19-Erkrankung mit hohen Sterblichkeitsraten. Somit würden die Antikörper die Belastung durch eine Koinfektion deutlich reduzieren und die Überlebenschancen erhöhen. Ich zeige, dass ein hochwirksamer und kostengünstiger Glykokonjugat-Impfstoff, der auf konservierte LPS-Strukturen abzielt, basierend auf bewährter Technologie entwickelt werden kann und eine sicherere Alternative zur Bekämpfung antibiotikaresistenter Krankheitserreger darstellt – ohne den Effekt der evolutionären Selektion resistenter Stämme durch die aktuelle Behandlung mit Antibiotika.

1 Introduction

1.1 Infectious Diseases and the role of *Klebsiella pneumoniae*

According to the world health organization (WHO), the impact of bacterial infectious diseases has caused the death of more than 8.4 million people worldwide in 2016, affecting mainly newborns, children, and elderlies from low and middle-income countries (1). *K. pneumoniae* has been responsible for thousands of deaths alone, especially because of a substantial decrease of treatment options (2). The increase of untreatable infectious diseases could kill more than ten million people in 2050, surpassing diseases like cancer, diabetes and measles, if no urgent actions are taken (3).

Klebsiella pneumoniae is a gram-negative, encapsulated, non-motile, facultative anaerobic, rod-shaped bacterium, belonging to the Enterobacteriaceae family. *K. pneumoniae* grows on MacConkey agar at 37°C as a mucoid lactose fermenter and is commonly found in water, soil, and animals. The bacteria express a prominent polysaccharide capsule (K-antigen) as well as lipopolysaccharides (O-antigen) on the cell surface providing resistance against the host defense mechanisms, thereby contributing for pathogenicity and virulence (4).

In humans, *K. pneumoniae* colonizes the gastrointestinal tract (GI) where they normally do not cause diseases, but when invading other parts body, these bacteria are responsible for a wide range of diseases including pneumonia, urinary tract infection, sepsis, liver abscesses, and meningitis (4). *K. pneumoniae* has become a pathogen of concern, due to the rise in the number of severe infections and increasing treatment failures in hospitals and in the community. The pathogen ability of acquiring genetic traits, has produced hypervirulent, as well as multi-drug resistance strains, contributing to an increased rate of deaths around the globe (5).

Today, *K. pneumoniae* is one of the most common causes of hospital-acquired pneumonia and nosocomial infections, especially among individuals with critical illness in intensive care units (ICUs). Recently, the pathogen has been associated with SARS-CoV-2 pulmonary co-infections contributing to chronic obstructive pulmonary diseases, a severe COVID-19 condition (6, 7). The evolution of the infectious disease often results in sepsis (Fig. 1) where the mortality rate reaches 50% in severely ill patients (8). The high mortality rate combined with an increased antimicrobial

resistance in in *K. pneumoniae* has raised an worldwide alert and created an urge for novel treatment options and actions in order to restrain the uncontrolled spread of infections (2).

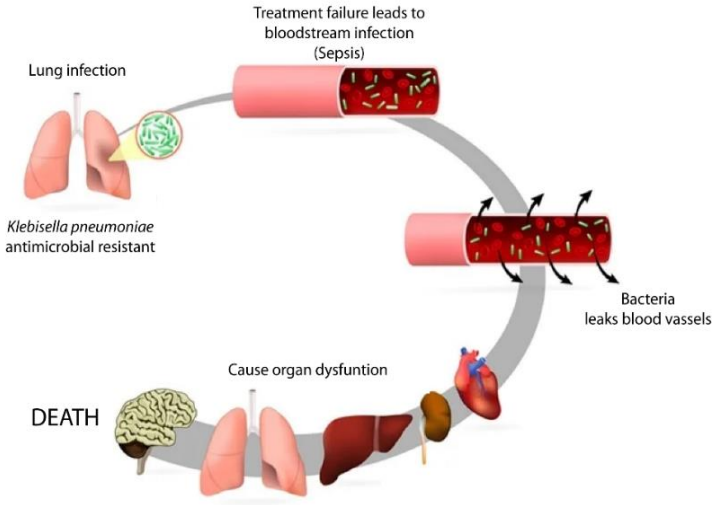

Figure 1. Evolution of pneumonia caused by *Klebsiella pneumoniae* resistant strains. The progress of the disease often leads to sepsis causing the death of more than 50% of the patients (Adapted from news-medical.net).

1.1.1 Carbapenem-resistant *Klebsiella pneumoniae*

Carbapenem-resistant *K. pneumoniae* (CRKP) strains have the ability to hydrolyze carbapenems, a sub-class of β-lactam antibiotics, usually used for the treatment of infections caused by multi-drug-resistant (MDR) Enterobacteriaceae (9). Concerning, over the past ten years, CRKP has become endemic worldwide and its success is attributed to the production of a variety of *K. pneumoniae* carbapenemases (KPC), enzymes capable of breaking down most β-lactams, and thus conferring resistance to the drug (10). KPCs are encoded on plasmids that are easily disseminated between strains via horizontal gene transfer under antibiotic selective pressure, contributing to the high environment persistence of CRKP (11, 12).

The few therapeutic options left, like colistin and ceftazidime-avibactam either cause nephrotoxicity and neuronal side effects in patients, or strains have already developed resistance (13-15). The accumulation of multiple resistance genes to last resource antibiotics and the successful dissemination of the resulting bacterial variants led to the emergence of a novel strain, now termed “Extensively-Drug-Resistant” (XDR) *K. pneumoniae* (16). This development

prompted the Center for Disease Control and Prevention (CDC) to categorize Carbapenem-resistance Enterobacteriaceae (CRE) as an “Urgent Threat” to public health, aiming for strict monitoring to establish alternative therapies to combat these pathogens (2).

A specific CRKP lineage belonging to the sequencing type 258 (ST258) plays a major role in the dissemination of KPC in the USA, Europe, and worldwide and was therefore termed “high-risk lineage” (17-20). ST258 strains are divided into two characteristic clades, based on the distinct forms of capsular polysaccharide (CPS) and plasmid gene content. Clade I ST258 typically carry a plasmid-encoded KPC-2, while clade II ST258 genomes harbor a plasmid-encoded KPC-3 (21). The dispersion success of high-risk ST258 clones is complex and includes numerous routes of transmission involving hospital patient-patient spread, acquisition following contact with colonized hosts, as well as transmission by medical staff, food chain, animals, and environmental sources. Although the success of ST258 CRKP due to antibiotic resistance is evident, additional virulence factors in this group augment the difficulty in fighting this pathogen (22).

1.1.2 Virulence factors in *K. pneumoniae*

Virulence factors enable bacteria to replicate and disseminate within a host by subverting or escaping host defenses. *K. pneumoniae* uses a range of structures such as capsular polysaccharide (CPS), lipopolysaccharide (LPS), porins, fimbria, siderophores and efflux pumps, to evade host immune defenses (23). Thereby they escape neutrophil-mediated intracellular killing, resist complement-mediated killing and the action of antibiotics, down-regulate inflammatory cytokines, thus resulting in successful replication and dissemination in the host (24). From this group of virulence structures, two play a very important role upon infection, the CPS and LPS, because they are the primary interaction of the bacteria within the host, ranking these structures as the most important targets for vaccine development (25).

1.1.2.1 *K. pneumoniae* capsular polysaccharide

The capsular polysaccharide (CPS) located on the outside of the bacterial cell, attached to the outer membrane (26) is composed of four to six glycan repeating units and is encoded by the highly variable cps gene cluster. As a result one can observe a broad range of linear and branched capsular phenotypes (27). In *K.* pneumoniae, the capsular antigen (K-antigen) is a result of a

combination of D-mannose, D-glucose, D-galactose, L-fucose, and L-rhamnose building blocks and are negatively charged due to the presence of uronic acid and pyruvate substitutions. Other substitution like *O*-acetyl, *O*-lactosyl, *O*-formyl and glutamate also contribute to the antigen diversity (28). The K-antigens are species-specific and used for serotyping *K. pneumoniae* strains. To date, 79 capsular serotypes have been described based on their sugar composition and glycosidic linkage (29). Serological techniques used have resulted in frequent cross-reactivity between capsular groups, and the inability to type acapsular strains (30). Additionally, the CRKP strains are mostly non-typable and the epidemiological correlation between clinical isolates and K-antigen is poor because of the broad variety of serotypes (31, 32).

Recently, new molecular techniques discriminate *K. pneumoniae* serotypes based on the cps gene cluster. This method identified 135 distinct types and showed that many of these open reading frames (ORFs) are conserved while other ORFs and alleles are very variable, depending on the capsule type. Some types are indistinguishable based on their sequence alone and it can be also difficult to determine the capsular types when sequence variation exists inside of a given serotype group (29, 33). The *cps-1* and *cps-2* are associated with the ST258 group and are likely involved in the global success of this group (34). More than eleven different cps loci have been identified in this group with several insertion sequences that brings further diversification into the genome (35). The high variability in the cps operon is advantageous for *K. pneumoniae* because it allows for a rapid change in the polysaccharide composition as a mean to evade host defense mechanism. The capsule switch is a species-specific strategy used by bacteria to escape immune cells recognition from the host (36).

The CPS is essential for bacteria to survive harsh environmental conditions, like dissection and detergents. However, the CPS is also an important virulence factor used by *K. pneumoniae* to protect against the host immune response. The CPS may help the bacteria to circumvent opsonophagocytosis and serum-mediated killing by macrophages, neutrophils, epithelial cells, and dendritic cells (Fig. 2) (37, 38). Specially, the overproduction of CPS is associated with increased mucoviscosity in *K. pneumoniae*, thereby mediating resistance to antimicrobials as well as biofilm formation (39). The presence of fucose in the capsule has been associated in the evasion of bacterial phagocytosis (40). This is further underlined by comparison with acapsular *K.*

pneumoniae, where the phagocytosis by innate immune cells is improved and the virulence is reduced considerably (41).

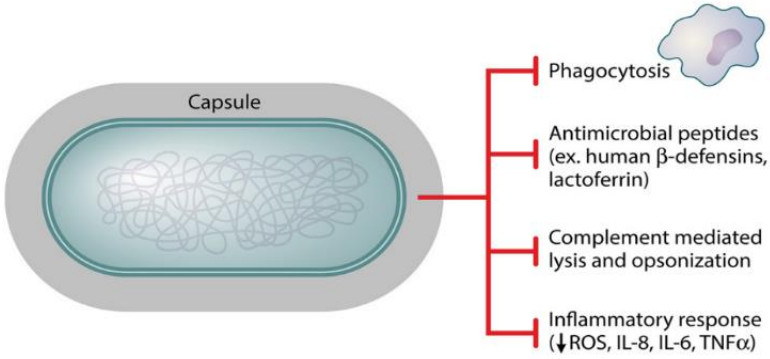

Figure 2. Capsular polysaccharide mechanism of resistance in *K. pneumoniae*. The bacterial capsular polysaccharide (CPS) helps to escape the host immune responses such as phagocytosis and complement mediated killing (Adapted from Paczosa and Mecsas, 2016).

The suppression of early inflammatory responses is another factor caused by capsulated *K. pneumoniae* (4). Lung epithelial cells produce toll-like receptors (TLRs) that are stimulated by pathogen molecules resulting in a downstream signaling cascade pathway with the production of antimicrobial defensins, cytokines, and chemokines. The CPS generates an anti-inflammatory effect by inhibiting TLRs, specially TRL-2 and TLR-4, and consequently inhibiting Interleukin 8 (IL-8), a very important molecule responsible for neutrophil recruitment and activation (41, 42). It also lowers the production of pro-inflammatory cytokines like TNF-α and IL-6. In addition to the reduction of pro-inflammatory cytokines, the CPS induces the production of anti-inflammatory cytokine IL-10, reducing inflammation and immune cells recruitment (43, 44). Moreover, CPS also impairs the maturation of dendritic cells, resulting in lower production of IL-12 and TNF-α, consequently preventing T cell helper-1 (Th1) signaling pathway activation (25). The CPS acts also as an efficient shield against host antimicrobial peptides, which under normal conditions are responsible for destabilize membranes of bacteria and enhance immunity in the host. The resistance mechanism follows the release of CPS from bacteria cells that can trap the peptides, thus preventing the interaction of these molecules with the bacteria surface (24, 45).

The prominent role of *K. pneumoniae* CPS as virulence factor to escape host-immune responses and its surface localization made it early on an obvious target for vaccine and antibody therapy development (46). However, the high variability in the cps gene cluster shows the plasticity of K-antigen, making it difficult to mount specific immune response against the pathogen or to design CPS-based vaccines that can cover the high diversity and broad epidemiological range of clinical isolate serotypes.

1.1.2.2 Lipopolysaccharide of *K. pneumoniae*

An additional important virulence factor in *K. pneumoniae* is the lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria. The structure of LPS is composed of lipid A, core oligosaccharides, and the O-antigen. The O-antigens are the most exposed portion of LPS and vary between strains of *K. pneumoniae* (Fig. 3) (24).

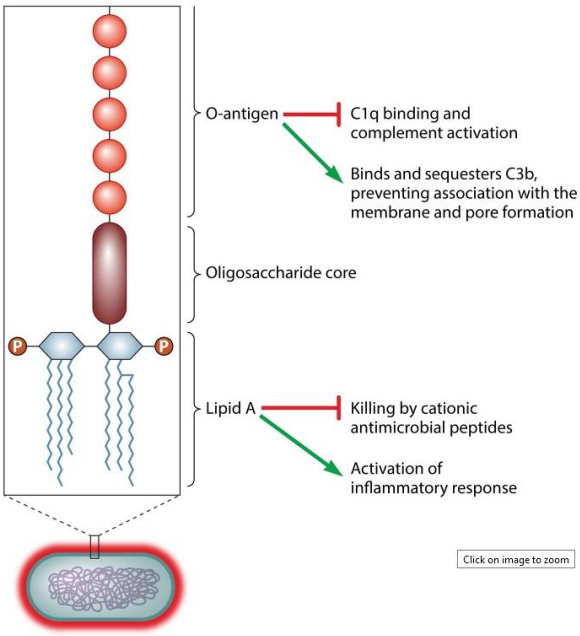

Figure 3. LPS structure and mechanism of resistant in *K. pneumoniae*. LPS is a virulence factor responsible for evading the host immune response using different strategies such as blocking complement deposition. (Adapted from Paczosa and Mecsas, 2016).

The lipid A part and the core oligosaccharide are highly conserved in bacteria because they are crucial for the integrity of the outer-membrane structure, acting as a barrier against external

environment molecules. Lipid A, a well-known endotoxin, can trigger the immune system to release pro-inflammatory mediators causing sepsis and septic shock already at picomolar levels. The structure is known to activate TLR-4 receptors leading to an inflammatory cascade that culminates in an exacerbated immune reaction, often fatal (47). The core of LPS is composed of highly conserved heptose and Kdo residues that links the core to the lipid A. Both structures are normally shielded by the bacterial CPS thus no immune response to the Lipid A and the LPS core is usually observed (48).

In contrast, the O-antigen is the most exposed part of LPS and also more variable due to the mediation of interactions with the environment and host defenses (49). The synthesis of O-antigens occurs in the cytosol and the periplasm of gram-negative bacteria and it is further transported to the outer membrane leaflet via transmembrane protein transporters. The variation of glycosyltransferases in the cytosol and periplasm of the bacteria leads to the variability of O-antigen oligosaccharide chains on the bacterial surface (50).

The O-antigen plays a main role in evading innate immune responses such as complement-mediated killing, since shortened or absent O-antigen structures in bacteria lead to its killing in the presence of human sera (51). The main mechanism of O-antigen mediated immune evasion is via blocking of C1q binding to the bacterium or by intersection of the C3b protein far away from the bacterial membrane, thus preventing the classical complement pathway activation and subsequent formation of the membrane attack complex (MAC) (Fig. 4). The effective abortion of the complement pathway by the bacteria leads to a higher chance of successful proliferation and colonization of the patients (51). In addition, O-antigens are essential to confer the ability to surpass organ barriers and access the bloodstream, causing septicemia and eventually death (52).

Although there is a variation in O-antigen polysaccharides, only nine serotypes have been described compared to more than 79 CPS serotypes (32). From this, the four serotypes, O1, O2, O3, and O5, are responsible for more than 90% of all *K. pneumoniae* infections worldwide (53). Apart from the high prevalence, the serotypes O1 and O2 are particularly important, as they are associated with high virulence and antimicrobial resistance rates (54-56). Both serotypes share a D-galactan-I antigen that is composed of → 3)-β-D-Galf-(1 → 3)-α-D-Galp-(1 → repeating units, while the O1 antigen is further capped by a D-galactan-II [→ 3)-α-D-Galp-(1 → 3)-β-D-Galp-(1 →]

repeating unit. Recently, a modification of D-galactan-I → 3)-α-D-Galp by a branching terminal α-D-Galp was identified and nominated as D- galactan-III (→ 3)-β-D-Galf-(1 → 3)-[α-D-Galp-(1 → 4)]-α-D-Galp-(1 →) (54) or O2afg serotype (Fig. 4) (57). This modification is possible due to the presence of a glycosyltransferase encoded by the *gmlABC* operon in the O-antigen gene cluster of the bacteria (57).

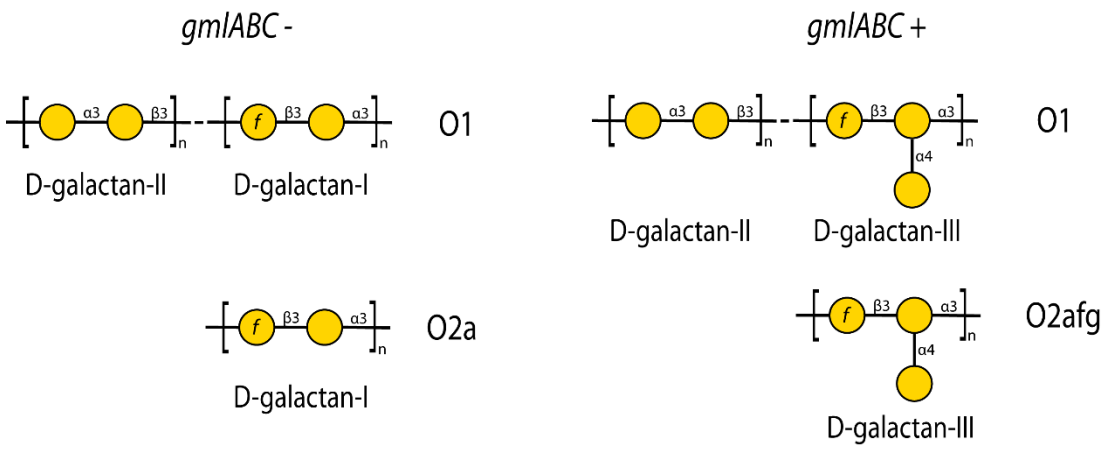

Figure 4. LPS structures of different *K. pneumoniae* serotypes. The O1 and O2 serotypes share a D-galactan-I antigen while the O1 serotype is further capped by D-galactan-II antigen. The D-galactan-I antigen can be modified by a branching terminal α-D-Galp when the bacteria express the gmlABC operon, generating a D-galactam-III antigen, also known as O2afg serotype.

Interestingly, more than 80% of CRKP isolates encode the *gmlABC* operon, highlighting the importance of D-gal III for the *K. pneumoniae* global dissemination (32). Although O2afg *K. pneumoniae* are markedly more serum sensitive than O1 strains, analysis of human sera showed only very low numbers of specific B cells against O2afg antigens. The poor immunogenicity of gal-III antigen may explain the success of CRKP dissemination worldwide and underwrites carbapenem-resistance propagation through this group (56).

With only a few O-serotypes covering the majority of *K. pneumoniae* clinical isolates, it is conceivable that a polyvalent glycan-conjugate vaccine will confer protection against more than 90% of the most common clinical isolates. This scenario is even more impressive when compared to the relative success of pneumococcus glycan-conjugate vaccines, that contain up to 13 CPS-serotypes, covering only 75% of clinical isolates worldwide (58). More importantly, the use of

gal-III as a key vaccine antigen may result in a reduction of carbapenem-resistance dissemination and may contribute to an enhanced activation of immune cells against CRKP.

1.2 Host immunity against *K. pneumoniae*

Bacteria are constantly improving resistance to antibiotics and refine their virulence factors to escape the immune system. As a result, persistent infections arise due to the inability of the host immune system to clear the causative microorganism (59). *K. pneumoniae* uses different strategies to surpass host defenses: firstly, it avoids the innate immunity including mechanical, chemical, and cellular defenses, and secondly, the bacteria eludes the cellular response of the adaptive immunity that is responsible for the formation of memory-B cells against specific bacterial antigens (22).

The respiratory and urinary tract are the preferable sites of *K. pneumoniae* as entry point for an infection. For successful colonization, the microorganisms have to break through the mechanical barriers that usually prevent pathogen establishment on site. In the respiratory tract, the mechanical mucociliary elevator barrier is a layer of mucus that traps microbes and then transports them out, using the ciliary coating. In the urinary tract, the urinary mechanical force and the low urine pH acts as a physical and chemical barrier respectively, preventing the bacterial entry into the bladder (60). After *K. pneumoniae* passes the mechanical barriers, it has to overcome cellular and humoral innate defenses, and finally the adaptive immunity (61).

1.2.1 Innate immunity against *K. pneumoniae*

The innate cellular defense works in a dependent and independent manner of humoral and mechanical defenses. Innate immune cells are able to recognize large numbers of pathogens or danger associated molecular patterns (PAMPs and DAMPs) through a limited number of germ line-encoded pattern recognition receptors (PRRs), such as the 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) (62). PAMPs do not vary among entire classes of pathogens because they are essential for the survival of the pathogen, but luckily they are distinguishable from human self-antigens (63). Once PRR from innate cells recognize PAMPs of bacterial pathogens, they trigger pro-inflammatory responses by activating

intracellular signaling pathways resulting in the activation of gene expression and synthesis of a broad range of molecules, including cytokines and chemokines , that coordinate the early host response to infection (64).

The Lipid A portion of the LPS is a noticeable feature of gram-negative bacteria, as one of the most potent PAMPs. It is responsible for the inflammatory response observed during endotoxic shock (62). *K. pneumoniae* is a gram-negative bacterium that expresses LPS on the cell membrane. However, inside of the gram-negative group, the lipid A molecules vary in phosphorylation, numbers of acyl chains, and fatty acid composition that affect the bacterial virulence and immune stimulatory capacity (65). For LPS recognition, the PAMP teams up with the extracellular acute-phase-LPS-binding protein and then binds to the co-receptor CD14 expressed at the cell surface of immune cells. Thereby the transfer to the accessory molecule MD2, that is associated with the extracellular domain of TLR4 that is finally activated and results in a downstream signaling pathway (66). Besides TLR4, gram-negative bacteria, like *K. pneumoniae,* can activate several other PRRs. Lipoproteins and bacterial membrane proteins for example stimulate TLR2 (67) while flagellin stimulates TLR5 (68). Bacteria have also unmethylated CpG DNA that is recognized by TLR9 (69), while the peptidoglycan activates NOD1 and NOD2 in the cytosol (70, 71).

If *K. pneumoniae* successfully breaks through the mechanical barriers and reaches the lungs, the first cell lines of defense are the resident alveolar macrophages. They recognize bacterial PAMPs via TLRs and are responsible for the direct-phagocytosis and amplification of the immune response through the production of chemokines and cytokines such as interleukin-8 (IL-8), IL-17, IFN-γ, leukotriene B4, inducible protein 10 (CXCL10), CXCL9, CXCL11 and CXCL1 (24). These effector molecules recruit neutrophils, a very important cell of the innate immune system. Neutrophils are broadly recognized as the first-line responders to bacterial infections. These cells have superior phagocytic and killing capacity than alveolar macrophages, and are important for the containment and clearance of *K. pneumoniae* infections (72). The mechanism of action of neutrophils against bacteria include not only phagocytosis and opsonophagocytosis but also the production of a range of inflammatory cytokines. Furthermore, the release of antimicrobial substances such as reactive oxygen species (ROS), serine proteases, myeloperoxidase, as well as

neutrophil extracellular traps (NET´s) (73-75). Although the innate cellular defense is very efficient against *K. pneumoniae* in healthy individuals, the pandemic ST258 group are resistant to neutrophil mediated clearance in the lung of infected mice (76).

Another important group of immune cells to contain *K. pneumoniae* infections are the dendritic cells (DC) that are specialized in the screening, processing and presentation of foreign particles in the body (77). DC use toll-like receptor 9 (TLR-9) to detect Cpg motifs and double stranded DNA that once recognized by the receptor, leads to the docking of adaptor molecules like MyD88 to TLRs and recruitment of proteins, belonging to the IRAK family (78), this leads to NFκb activation and gene expression for the production of inflammatory cytokines like IL-6, IL-12 and TNF-α which further recruits immature DCs and monocytes to the site of infection (79). TLR-4 is present on the surface of neutrophils and DCs, and upon binding to bacterial LPS it stimulates the production of IL-23 and IL-17 by these cells (80). IL-17 production itself depends on IL-23 generation mainly from alveolar macrophages and DCs. Furthermore, IL-17 is a very important cytokine that creates a feedback signal responsible for the enhanced recruitment of neutrophils to the lungs during *K. pneumoniae* infection (73).

The importance of IL-17 as signaling molecule to recruit immune cells is underscored by the fact that it is also produced by other cell types, including T cells, CD4 T cells, CD8 T cells and NK T cells. During *K. pneumoniae* lung infections, T cells are necessary for the early upregulation of certain inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and IFN-γ, controlling the dissemination of the pathogen in the lungs (81). In addition to IL-17 cytokine, IL-22 has shown to be very important for the control of *K. pneumoniae* by reducing the bacterial load in the lungs and inducing a Th17 response in an IL-23 dependent manner. Another function of IL-22 is the induction of lipocalin-2 expression in the blood to sequester iron and prevent its use by bacteria to limit their growth (82). Recently, mucosa-associated invariant T (MAIT) cells have also been reported as an important group for *K. pneumoniae* infection control, as mice lacking these cells show an increased mortality rate (83).

The innate cells are very specialized in fighting bacterial infection with sophisticated integrated systems, receptors, and effector molecules. However, *K.* pneumoniae has evolved strategies to avoid its recognition by innate immune cells resulting in lower activation of the immune system

and consequently reduced signaling pathway activation (44). Nevertheless, the human body has developed additional strategies to fight bacterial threats that rely on proteins found in humor or body fluids, classified as humoral response, with the complement system as one of the most important humoral defenses against *K. pneumoniae*.

1.2.1.1 The Complement System

One very reliable component of the humoral innate immunity against *K. pneumoniae* is the complement system. It comprises a network of soluble and membrane bound proteins, present in blood and fluidics of humans that in a synchronized mode activate the classical, lectin and alternative pathway (Fig. 5) (84). The activation of different paths leads to two main killing mechanisms. One involves phagocytosis via C3 protein fragments deposition onto bacterial surface causing opsonization and activation of immune cells, and the other lysis of the bacterial cell wall by the formation of the C5b-C9 protein complex, creating the membrane attack complex (MAC) (85).

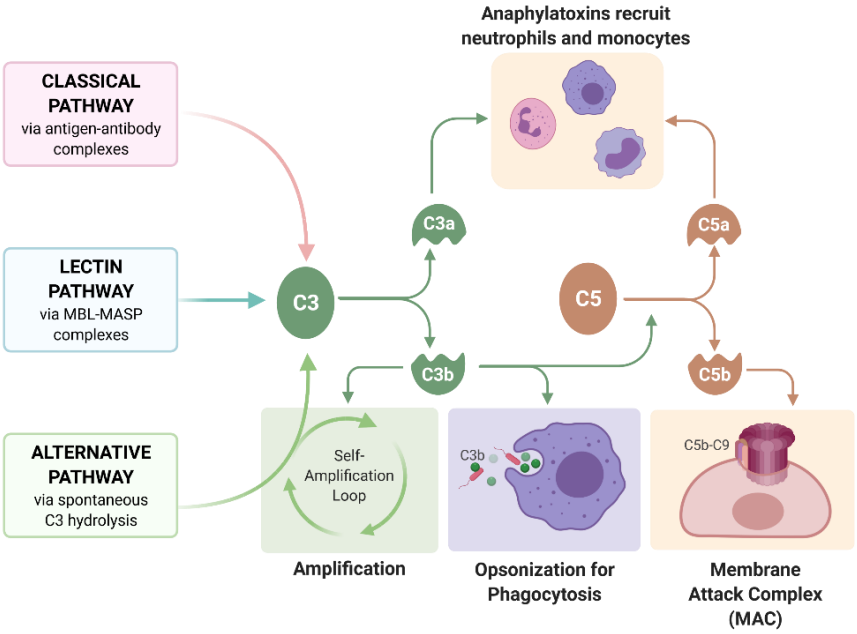

Figure 5. Complement pathways of the immune system. The classical, lectin, and alternative pathways generate the major complement opsonin C3b and the membrane attack complex (MAC, C5b-9). The C3b protein act as an opsonin causing phagocytosis of the bacteria while the MAC forms a pore in the cell membrane resulting in the lysis of the pathogen (Modified from Biorender).

In plasma, the complement system is constantly being activated via an alternative pathway, in order to control any invasion by pathogenic microorganism like *K. pneumoniae*. This process occurs due to a spontaneous hydrolysis of a labile thioester bond that converts C3 to a bioactive form C3(H_2O) in the fluid phase (86). Upon hydrolysis, the C3 undergoes a dramatic structural change that exposes a binding site for the Factor B. The C3(H_2O)-factor B is then cleaved by a serine protease Factor D, forming the C3 convertase complex C3(H_2O)Bb. The C3(H_2O)Bb is able to interact and cleave native C3 molecules to C3a and C3b (Fig 6A). The bound C3b onto the surface of bacteria intruders act as opsonins causing the phagocytosis of the microorganism by immune cells. (87).

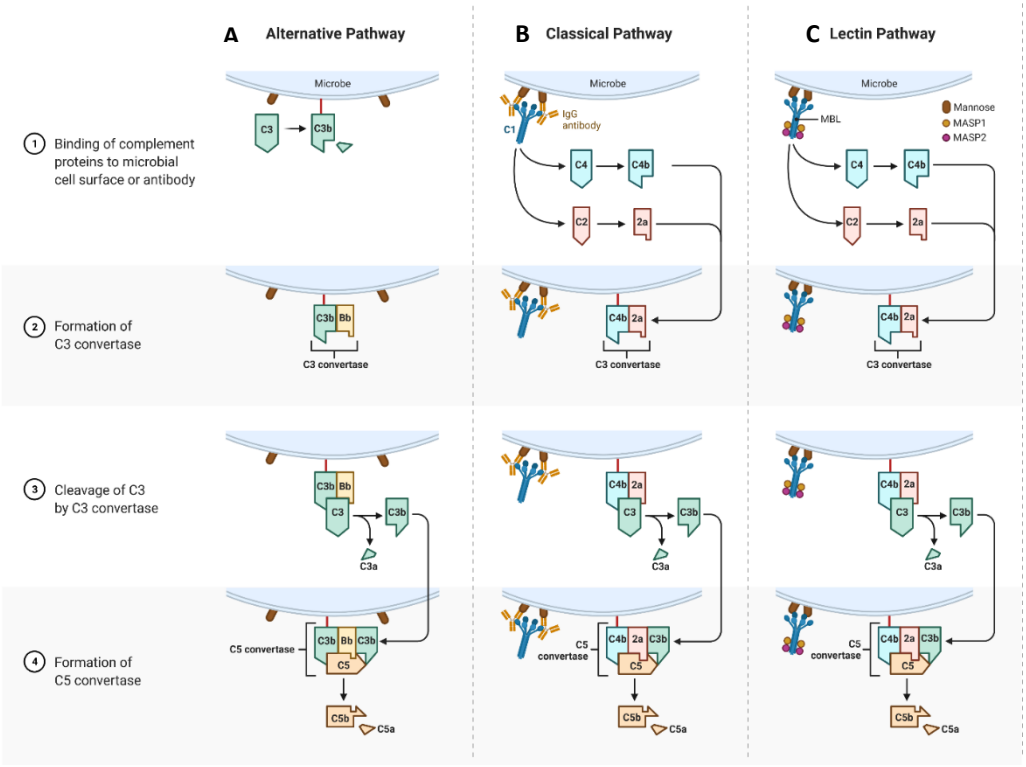

Figure 6. The three major activation pathways of the complement system. **A-** The alternative pathway occurs due to a spontaneous hydrolysis of C3 into C3(H_2O) that rapidly engages factors B and D to form a C3 convertase C3(H_2O)Bb triggering more C3b. The C3b binds a C3 convertase to generate the C5 convertases (C3bBbC3b). **B-** The classical pathway is a result of the engagement of the C1 complex (C1qrs) with antigen-antibody complexes causing the cleavage of C2 and C4 complement proteins and formation of a C3 convertase (C4b2a). This cleaves C3 to C3b that binds to the C3 convertase generating the C5 convertase (C4b2a3b). **C-** The lectin pathway forms the same convertases as the classical pathway. However its activation depends on mannan-binding lectins (MBL) and MBL-associated serine proteases (MASPs) that recognize mannose structures on the surface of the pathogen. All three pathways form C3b that act as opsonins on the bacterial cell surface resulting in phagocytosis. C3b also leads to the production of C5 convertase, producing C5b and MAC, causing pathogen lysis (Modified from Biorender).

Bacteria and viruses contain a variety of surface glycoproteins with terminal monosaccharide exposing horizontal 3'- and 4'-OH groups (glucose, mannose, and *N*-acetyl-glucosamine) that are rarely present on host proteins and healthy cell surfaces. These structures activate the complement via the lectin pathway and are recognized by the innate immune system by pattern-recognition receptors (PRR), such as mannan-binding lectins (MBL), collectins, and ficolins, in a Ca-dependent manner (88). Each of them complexes with MBL-associated serine proteases (MASPs), that exists in three forms (MASP1, MASP2, and MASP3). After target recognition, the MASPs associated to MBL are activated and result in the cleavage of C4 and C2 complement proteins, thus forming the C3 convertase that produce C3b complement protein (Fig. 6C). The C3b attaches to the cell surface and act as an opsonin causing the phagocytosis by specific immune cells. In *K. pneumoniae,* MBL and collectin 11 (CL-11) are the PRRs responsible for activating the lectin pathway, while Ficolin-1, Ficolin-2 and Ficolin-3 are targeting gram-positive bacteria, *Mycobacterium tuberculosis*, and influenza virus (89).

Finally, *K. pneumoniae* can be cleared out via activation of the classical complement pathway, where the complement activation is antibody-dependent. The C1q protein recognizes the antigen-antibody complex on the cell surface and activates the C3 convertase enzyme that cleaves the C3 complement protein into C3b. This cleavage exposes a thioester that links itself covalent to the bacterial surface (Figure 6B) (84). Once the C3b is attached to the cell surface, it causes opsonization of the bacteria, which is the signal to be recognized by neutrophils, monocytes and macrophages via complement receptors (CR1, CR3, CR4 or CR5) triggering phagocytosis and internalization of the opsonized bacteria. Subsequently, the bacteria are killed by several antimicrobial peptides and ROS species (90). The complement-mediated phagocytosis by CRs recognition is enhanced when the recognition of bacterial surface antigens by IgG/IgM also occurs (88). The enhancement occurs because additionally to CRs, phagocytic immune cells have also Fcγ receptors on the cell surface that can recognize the Fc portion of bound antibodies to the bacterial surface. When both complement and antibody receptors are loaded, they act in synergy increasing binding affinity of the immune cells, thus increasing phagocytosis of the targeted bacteria (91).

Apart of phagocytosis as a mechanism of bacterial clearance, another very important downstream complement pathway strategy against *K. pneumoniae* is the activation of complement-mediated cell lysis. This mechanism is achieved due to the high levels of C3b that when associated with C4b2a or C3bBb complement proteins forms a C5 convertase (C4b2aC3b or C3bBbC3b), which cleaves C5 into C5b (Figure 6). C5b activates a downstream cascade pathway resulting in the formation of MAC that consists of multiple copies of the C5b-9 and other complement proteins: C6, C7, and C8. When these proteins assemble onto the bacterial surface, they form a 10 nm wide pore resulting in bacterial death via cell lysis (figure 6) (92). The mechanism involving MAC formation is exclusively effective against gram-negative bacteria, because gram-positive bacteria have a very thick cell wall in which MAC cannot penetrate rendering them resistant to complement-mediated lysis (91).

Even tough the complement system is very efficient in keeping infections under control, *K. pneumoniae* has developed specific strategies to avoid complement activation. The thick polysaccharide capsule and long O-antigen polysaccharide chains play a major role in avoiding the deposition of C3b and MAC proteins on the bacterial surface (28). As a result, the bacterial infection remains, escaping phagocytosis and cell lysis, the two major mechanims of complement dependent defense mechanisms against bacterial pathogens.

1.2.2 Adaptive immunity against *K. pneumoniae*

Pro-inflammatory signaling pathways induced by PRRs after encountering *K.* pneumoniae not only activate the innate immune response but also play a vital role in the activation, maturation, and shaping of the adaptive immune response (93). Adaptive immunity strategies rely on the activation of clonal gene rearrangements from a broad repertoire of antigen-specific receptors on lymphocytes, specifically B cells, producing antibodies against bacterial targets and generating a memory response that fight future infections in a faster manner (94).

To activate the adaptive immune response, an interaction of the pathogen with innate immune cells like DCs, macrophages, and neutrophils is necessary. The DCs and macrophages work as antigen-presenting cells (APCs) that bridge the innate with the adaptive immunity (95). The APCs capture and process the pathogen loading specific bacterial antigens via MHC-II on the cell

surface, while the neutrophils produce important pro-inflammatory cytokines and chemokines that attract monocytes and DC's to the site of infection. Recent evidence shows that TLR receptors influence the MHC loading pathway, indicating the importance of TLR in the activation of adaptive immunity (96). After antigen uptake, the APCs become activated and migrate to secondary lymphoid organs (SLOs) to present the surface loaded peptides into MHC II molecules to T cells. During this process, the upregulation of costimulatory molecules, chemokine receptor expression, and cytokine secretion are activated and will be important for the interaction with T cells receptors in the SLOs (97). Spleen, LNs, and mucosal associated lymphoid tissues are SLOs that efficiently trap foreign antigens entering via bloodstream, peripheral tissues, and mucosal sites. They facilitate interactions between antigen-bearing DCs, B cells, and T cells due to cell compartmentalization in different zones of SLO follicles that will initiate the adaptive immune responses (98).

B cells are originally from bone marrow derived cells and are responsible for the long-lasting immunity after migrating to SLOs. For the generation of a memory response, naïve B cells that live in the B cell follicles of SLOs, recognize bacterial antigens via B cell receptors (BCR) that results in downstream signaling, leading to antigen internalization, processing, presentation via MHC-II, and cell activation. Antigen-activated naïve B cells express chemoattractant receptors (99) that direct them to the border of the T cell zone of SLOs follicles where they interact with antigen-specific T helper cells (Th cells) that have been primed by DCs and differentiate towards T follicular helper cells (Tfh cells) (100). Following the interaction with T cells via CD40 receptors, naïve B cells can differentiate into short-lived or long-lived plasma cells, or memory B cells (Fig. 7). The short-lived plasma cells produce bacteria-specific antibodies with relatively low affinity. However, B cells can also differentiate into germinal center (GC) B cells and further differentiate into long-lived plasma cells and memory B cells, with the last one circulating throughout the body, with high-affinity BCRs ready for a rapid response to the antigen (101). Interestingly, naïve B cells can also generate short and long-lived plasma cells independently of T cells, however the generated antibodies show very low levels of somatic hypermutation and isotype switching (102).

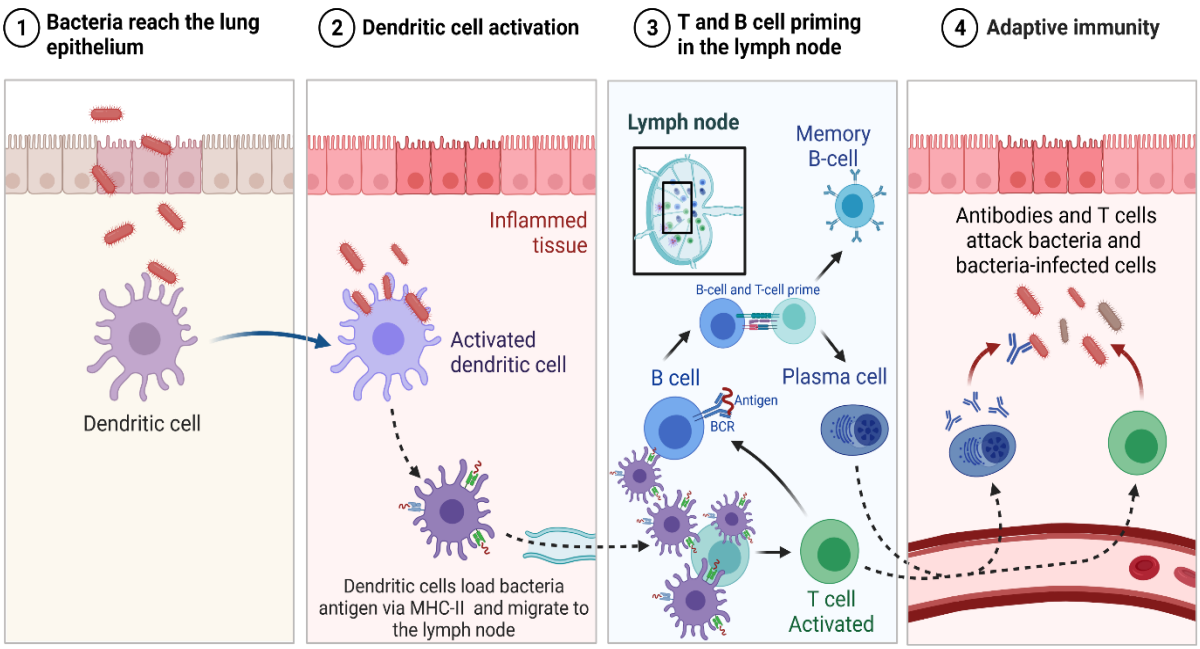

Figure 7. Adaptive immunity against bacterial pathogens. Upon bacterial infection, the invaders are recognized and phagocytosed by innate immune cells, like dendritic cells (DCs). After bacterial uptake and processing, specific bacterial antigens are loaded via MHC-II causing the activation and migration of DCs into secondary lymphoid organs (SLOs), like lymph nodes, to present the loaded peptide to naïve T cells and B cells. The naïve B cells recognize the bacterial antigens that are soluble or presented on DCs via B cell receptors (BCR) resulting in a downstream signal that leads to antigen internalization, processing, presentation via MHC-II, and cell activation. The activated B cells interact with antigen-specific T helper cells that have been primed by DCs. This prime interaction causes the differentiation of B cells into short-lived or long-lived plasma cells, or memory B cells. Plasma cells and activated T cells then enter the blood stream and reach the site of infection, producing specific antibodies and cell-mediate-killing of the pathogen, respectively (Modified from Biorender).

Although B cells are very important in generating specific antibodies and memory against bacterial pathogens, T cells also play vital role in adaptive immunity, especially in the activation of antigen-specific naïve B cells, cytokine production, and cell mediating killing (103). T cells are originally from bone marrow progenitors that migrate to the thymus for maturation and selection, and subsequently circulate in the blood and lymphatic vessels where they encounter APCs and B cells (104). There are two main effector T cells, the helper T cells (Th), denominated as $CD4^+$ T cells, and the cytotoxic T cells (Tc), known as $CD8^+$ T cells.

The $CD4^+$ T cells fight microbial infections by releasing a variety of interleukins that generate specific T cell subsets, while $CD8^+$ T cells have the ability to kill infected cells (104). The maturation

of both effector T cells depends on the interaction between the T cell receptor (TCR) and antigenic peptides. The TCR is generated by recombination of genomic DNA sequences during T cell maturation in the thymus. It is composed of two chains (α and β) that recognizes exclusively peptides that are loaded onto MHC molecules (105). The receptor is linked to the CD3 membrane protein that is responsible for transmitting an intracellular signal after TCR loading. Additionally, each TCR is associates with either the CD4 ($CD4^+$ T cell) or CD8 ($CD8^+$ T cell) co-receptor. $CD8^+$ TCR interact with peptides loaded exclusively via MHC class I, while $CD4^+$ TCR engage to peptides loaded via MHC class II. All nucleated cells express MHC class I, however the MHC class II molecules are present only on APCs culminating in the activation of $CD4^+$ T cells, the crucial immune cell for the adaptive immunity against bacterial infection (106).

Before T cells are able to stimulate B cells, resulting in long-lasting immunity, they have to be activated. For that, naïve CD4+ T cells in the SLOs encounter MHC II-antigen complex on the surface of APCs, which loads its TCR (107). Additionally, to TCR loading, the T cell requires another two signals in order to achieve full activation. The second signal comes from co-stimulatory molecules like CD28 in helper T cells that bind to one of two receptors on the APC, the CD80 or CD86, or the CD40 in B cells. Finally, the third signal comes from released cytokines that determine the functional subclasses of T cells (108). The release of specific cytokines together with the expression of transcription factors cause the CD4+ T cell differentiation into Th1, Th2, Th17, or Treg subsets. The Th1 cells secrete IL-12, IFN-γ, and tumor necrosis factor (TNF) are important for the control of intracellular virus and bacteria, and tumors. The Th2 cells secrete specific interleukins such as IL-4, IL-5, and IL-13 and fight parasitic infections, while Th17 cells are specialist in controlling extracellular bacterial and fungal infections by secreting IL-17 and IL-22. While Th1, Th2 and Th17 cells leads to effector mechanism against the pathogen, the Treg cells leads to the control and down-regulation of T cells activation to keep the levels of inflammation under control, which is mediated by IL-10 cytokine (Fig. 8) (109).

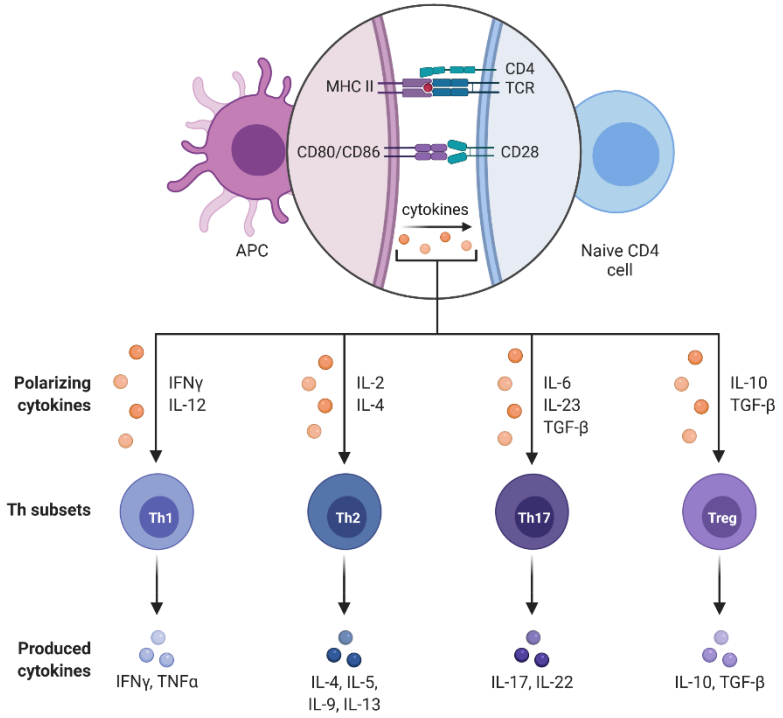

Figure 8. T cell activation and differentiation. Naïve CD4 T cell recognize MHC II-antigen complex on the surface of APCs via TCR, which is the first signal for T cell activation. The second signal comes from the interaction of the CD28 receptor in the T cells with one of two receptors on the APC, the CD80 or CD86. Finally, the third signal occurs after the stimulation of the cell by cytokines. The integration of the three signals cause the CD4+ T cell differentiation into Th1, Th2, Th17, or Treg cellular subsets responsible for the control of microbial infections (Modified from Biorender).

Adaptive immunity relies on a very complex machinery and depends on innate immune cell activators. Since *K. pneumoniae* has developed strategies to avoid innate immunity, especially with modification of PAMPs, adaptive immunity is further impaired (28). In addition, when the body encounters bacteria that are part of the human microbiota, such as *K. pneumoniae*, it promotes the down-regulation of pro-inflammatory cytokine that impair the activation of the adaptive immunity by reducing B cell and T cell signaling activation (110). Even more concerning, the O2fg LPS in *K. pneumoniae* from ST258 group is very poorly immunogenic. Therefore, only very low levels of specific B cells against the target antigen are produced and the cells fade away before mounting a memory response. This explains the success of CRKP dissemination worldwide and underwrites carbapenem-resistance propagation through this group (56). Thus, the development of strategies that can enhance the activation of the innate and adaptive immune

answer against this pathogen might be a promising strategy to fight ST258 and antimicrobial resistance dispersion.

1.3 Strategies to combat *K. pneumoniae*

Antimicrobials are the most frequently used therapy to treat infections caused by *K. pneumoniae.* As mention before, currently, β-lactam antibiotics are the largest class of antibiotics administered to patients to treat a *K. pneumoniae* infection (111). The mechanism of action of β-lactam antibiotics is the irreversible covalent reaction with an active site residue in transpeptidases of the bacteria cell wall synthesis apparatus. They are divided into sub-classes, the penicilins, monobactams, cephamycins, cephalosporins, and carbapenems (112). The carbapenems have been used successfully for more than 40 years against gram negative infections, due to their broader antimicrobial effect, compared to penicillins or cephalosporins. Carbapenems are the preferred choice to treat severe infections, especially the ones caused by multi-resistant gram-negative bacteria (113). Concerningly, CRKP has developed the ability to produce a variety of enzymes that breaks down even carbapenems and thus confers resistance to the drug. The excessive use or mismanagement of antibiotics has led to a positive selection towards the rise of resistant strains (10). To date, there are only few antibiotics left to treat CRKP. One of them is colistin and the other ceftazidime-avibactam, but both cause either nephrotoxicity and neuronal side effects in the patients or strains have already developed resistance to it (13-15).

The increasing failure to treat *K. pneumoniae* infections with antibiotics has given space for new strategies to fight the pathogen. As discussed above, the human immune system has unique strategies to fight bacterial infections, consequently scientists have tried to exploit the host immune system to combat *K. pneumoniae*, without generating antibiotic resistance (62). The therapeutic strategies include passive and active immunization that boosts the host immune response against the pathogen. For passive immunization, antibodies targeting bacterial antigens, such as proteins or polysaccharides, are used directly for the treatment or prevention of infections, generating a temporary protection. In contrast, active immunization involves the use of specific antigens to vaccinate the host and stimulate the immune system to develop a memory response, thus reaching long-lasting protection (51).

1.3.1 Passive immunization

Passive immunization relies on the transfer of ready-made antibodies into the host that target the microorganism in a specific manner. It can occur naturally via maternal transfer of antibodies to the fetus via placenta and to babies via breast milk, or artificially by injection (114). The artificial passive immunization is achieved when antigens from pathogens are used to immunize hosts, such as humans, horses, mice, or rabbits, to generate high levels of specific antibodies that can be transferred into a non-immune host in the form of purified immunoglobulins or antiserum therapy (115). Another very useful tool for the production of antibodies is the monoclonal antibody (mAb) technology. The technique relies on the immunization of mice with specific antigens, followed by the isolation of specific B cells. Subsequently, they are immortalized by fusion with myeloma cells, forming a hybridoma, which produces large amounts of antibodies *in vitro.* These mAb's are further purified and prepared for human use (116) . Passive immunity is very important for patients that encounter the pathogen and have insufficient time to mount an immune response. Therefore, such patients are at a higher risk of infection and complications, such as bacteremia. Passive immunity can also provide protection in immunosuppressive and immunocompromised patients, who cannot generate an immune response against the bacteria themselves (117). Many antibodies have been used to reduce *K. pneumoniae* counts in blood and lungs, consequently, avoiding the progress of the infection. So far, the most targeted antigens for passive immunotherapy are carbohydrates such as capsular polysaccharides (CPS) and O-antigens because of its high surface exposure (118).

Several studies have shown that anti-CPS antibodies confer protection in animal models. For example, anti-CPS antibodies generated in rabbits or in humans protected mice against sepsis in murine burn models (119, 120). Another group demonstrated that murine antibodies, targeting K2 CPS, protected rats against *K. pneumoniae* pneumonia. Rats that received antibodies have shown a reduced number of bacteria in the lungs, showed less lung inflammation, kept stable body weight, and had better motor activity, when compared to the control group (121). Studies using passive immunization in humans are known for more than 25 years. Healthy individuals received a 24-valent CPS *K. pneumoniae* vaccine. Following blood collection and processing of the plasma into hyperimmune globulin antisera, that contained anti-CPS antibodies specific

against *K. pneumoniae* (122), the sera have been administered to patients admitted to the intensive care units (ICU) in a preventive approach. The goal was to avoid *K. pneumoniae* infection in severely ill and weakened patients. Although the study showed reduction in the infection incidence and patient severity, there was no statistically significant improvement, when compared to a control group that did not receive the treatment (123). Recent studies have used a mAb that targets CPS2 from the ST258 strain. The anti-CPS2 monoclonal antibody was administered passively into a murine pulmonary infection model, enhanced serum bactericidal activity against ST258 by increasing the C3 complement deposition and MAC formation (124). In 1994, Trautman *et. al.,* produced anti-O-antigen antibodies from *K. pneumoniae*, serotypes O1, O2ab, O2ac, O3, O4 and O12, using uncapsulated strains, but there was a limited binding on strains producing CPS (125). A recent study showed that mAbs raised against O1 and O2 (but not specifically against O2afg) serotypes protected mice against capsulated *K. pneumoniae,* enhancing the clearance of the pathogen by opsonophagocytic killing. Interestingly, when combining the mAb with carbapenems it potentiates the antibiotic effect, raising the opportunity for using lower doses of antibiotics, thereby limiting the toxic side effects (126).

It is reasonable to suggest that passive immunization, using a mixture of antibodies, targeting different antigens is key to generating broader protection, due to the high variety of strains worldwide. Thus, using antibodies raised against O-antigens are more suitable for passive immunization therapy than using capsular antigens due to the lower number of O-antigens (nine serotypes), compared to the large number of capsular antigens (more than 77 serotypes).

1.3.2 Active immunization

Active immunity is the induction of antibody production by the host, after being exposed to a specific pathogen or parts of it. It also generates specific cells that can last for years or even be permanent, called memory B cells. The development of the active immunity may take days or weeks after the first contact to the pathogen and can occur naturally or artificially. The natural active immunity develops when people encounter pathogens in the environment, but it can be disadvantageous when coming across deadly microbes that can decease the host before reaching an immune response. The creation of an artificial active immunity happens when dead microbes

or parts of them are injected into a person, having the advantage of not causing the disease as a result of it. (115).

Vaccines, targeting *K. pneumoniae,* have been studied for many years, using whole cell preparation or subunits (127). However, currently there are no licensed vaccine and there are no reported clinical trials ongoing. In 2008, a study using a vaccine based on live attenuated *K. pneumoniae*, showed that the vaccine elicited IgG production and protected mice challenged with a sub-lethal dose of the pathogen (128). Another study, using an inactivated whole cell vaccine, containing *K. pneumoniae* and other Enterobacteriaceae bacteria was tested against urinary tract infections (UTI) in humans via vaginal immunization. It reached phase 2 clinical trials with 78% reduction in reinfection in woman (129). Even though the vaccines showed encouraging results, whole cell vaccines caused by different PAMPs expressed on the cell surface of the bacteria. It also leads to the generation of a polyclonal immunity, where multiple antigens are recognized by the immune system. Additionally, lipid A (endotoxin) contaminations in vaccine preparations, even at picomolar level, can trigger the immune system to release pro-inflammatory mediators causing sepsis and septic shock. Therefore, such vaccines are not optimal for immunization in humans, especially in immunocompromised, elderly, and children (47).

To overcome the problem, subunit vaccines have been an alternative strategy, used to develop vaccines against *K. pneumoniae*, in an effort to make new vaccines safer. The use of only parts of the microorganism has also its down side, mainly a lower immune system stimulation. Therefore, subunit vaccines are often combined with adjuvants to improve their ability to induce a strong immune response (130). The CPS and LPS are the most common subunits, used to generate vaccines against *K. pneumoniae*, due it its high cell surface exposure. Patients that have been exposed to the bacteria produce antibodies that can recognize CPS and O-antigen structures, showing that they can be recognized by the immune system (51). A polyvalent-CPS vaccine containing 12 different CPS antigens protected mice upon challenge with the pathogen but the immunity was short-lived (131). Following the same approach, the Swiss Serum and Vaccine Institute developed a monovalent vaccine containing K1 antigen and a hexavalent CPS vaccine containing K2, K3, K10, K21, K30 and K55. The vaccines were well tolerated in healthy humans

and induces a strong immune response in more than 80% of the individuals. (132, 133). A 24-polyvalent CPS vaccine (Klebvax®) reached clinical trials. The vaccine was safe, induced a good immune response in humans, and generated antibodies with opsonic activity. This promising vaccine never exceeded coverage of 70% of *K. pneumoniae* strains due to the complexity of vaccine formulation and it was never licensed. Additionally, the short-lived immunity generated by the chosen polysaccharides have not encouraged the continuation of clinical trials (134). To achieve long-lasting immunity, different groups have conjugated the CPS to a carrier protein with the aim to stimulate T cells and therefore provoking a memory response. Zigterman *et. al.,* used the K11 CPS from *K. pneumoniae* and conjugated it to either keyhole limpet hemocyanin or bovine serum albumin (BSA). The conjugated K11 CPS formulation induced the production of IgG's, pointing to the maturation of B cells, while the CPS alone produced only IgM's (135). Recently, the Seeberger laboratory conjugated synthetic hexasaccharide CPS, common to the ST258 group, to CRM_{197}. Mice and rabbits vaccinated with the glycoconjugate vaccine produced a robust immune response and long-last immunity. However, the lack of cross-protection to other strains from this group makes it difficult to control the spread of resistant strains expressing other CPS types (136).

For a long time, it was believed that CPS would mask LPS from recognition by the immune system, yet studies have proven that O-antigens extend from the majority of CPS (137). The reduced number of O-antigens serotypes, when compared to CPS serotypes, has driven scientists to focus on O-antigens for vaccine production against *K. pneumoniae* (46). A LPS conjugate vaccine reduced the bacterial load in the lungs of rats, but the level of the immune response or a boost regime was not evaluated (138). The O1 antigen, loaded into liposomal (139) or alginate microparticles (140) enhanced the immune protection. The formulations were less toxic compared to free LPS immunization trials. The most recent O-antigen vaccine has been designed as a multivalent vaccine containing O1, O2, O3 and O5 serotypes that were conjugated to flagellin A or B from *Pseudomonas aeruginosa,* in order induce immunity against both pathogens. The vaccine resulted in high IgG-titers in rabbits against all antigens including the carrier proteins and protected mice against *K. pneumoniae* infection (141). Although the lower number of O-antigen serotypes makes vaccine development simpler, the main drawback, remains on the adverse toxic

effect to the lipid A from LPS. The use of liposomal formulations or chemical treatment of the LPS is used to reduce its toxicity. However, this leads to extra purification steps that renders the vaccine process demanding, costly, and reduces the yield. Furthermore, chemical procedures may damage the antigen structure, affecting its immunogenicity (127). Different strategies are still on need to improve vaccines against *K. pneumoniae* and more importantly, the ST258 subgroup.

1.4 Improving vaccines against *K. pneumoniae*

1.4.1 Vaccine types and mechanism

Vaccines are the best known artificial means to generate active immunity. They are composed of antigens that either are derived from the pathogen or produced synthetically to mimic parts of the pathogen. Different technologies lead to a variety of vaccines (Fig. 9), which are broadly categorized as live and non-live. These categories separates vaccines that contain attenuated replicating microorganism from those that have dead whole cell pathogens or just components of it. Over the past decades, further technologies have been developed, such as viral vectors, nucleic acid based RNA and DNA vaccines, and virus-like particles, expanding the non-live category considerably (130).

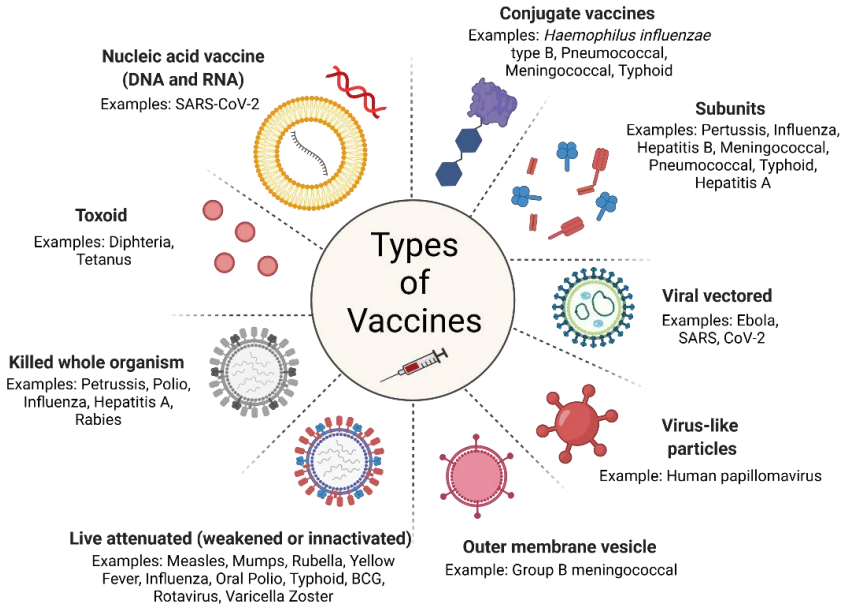

Figure 9. Different types of vaccine technologies targeting a broad variety of microorganism (Modified from Biorender).

The goal of vaccination is mainly to confer long term protection through the induction of antibodies (IgG, IgA, IgM) mediated by B cells and T cells. Conventional protein vaccines contain antigens that are recognized by APCs that process and load it into MHC class II. Further adjuvants in the vaccine formulation stimulate APCs via PRR enhancing cell activation. Activated APCs drive the loaded vaccine antigen to the lymph node, where it is presented to T cells via TCR receptor engagement. Concomitantly, soluble vaccine antigens engage BCR receptors from B cells that are further processed and loaded via MHC class II molecules. The activated B cells with loaded MHC II, encounter the activated T cells via TCR and the interaction causes the release of important cytokines, resulting in B cell proliferation and subsequent differentiation into short-lived and long-lived plasma cells and memory B cells. The short-lived plasma cells secrete antibodies two weeks after vaccination while the long-lived plasma cells travel to reside in the bone marrow and can produce antibodies for decades when reencountering the pathogen (Fig. 10) (130).

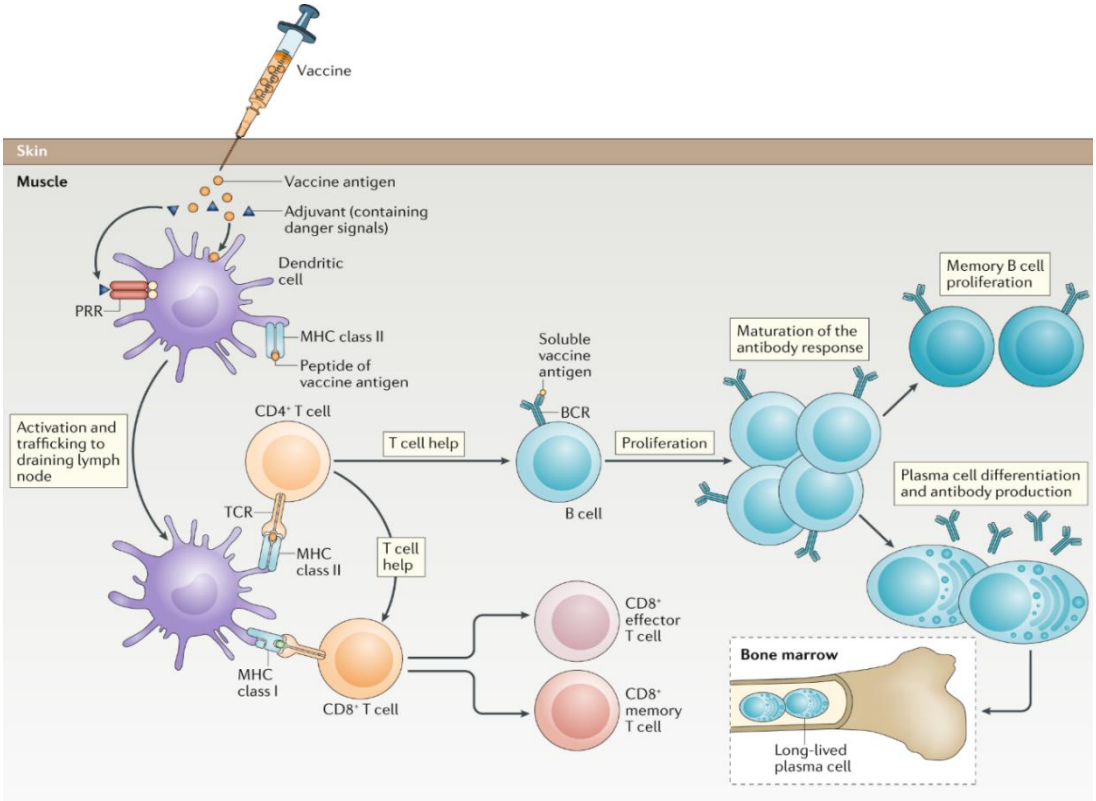

Figure 10. Generation of an immune response after vaccination. After APCs recognizes the vaccine antigen, it is drained to the lymph node where it activates T cells that will further stimulate B cells that have recognized the vaccine antigen via BCR, processed and loaded the antigen via MHC II. The cells engagement causes B cell proliferation and differentiation into memory B cells and plasma cells. The plasma cells migrate to the bone marrow and live for decades in the host (Adapted from Pollard & Bijker, 2020).

The use of *K. pneumoniae* as whole-cell vaccine formulation is not the optimal approach since gram-negative bacteria contain lipid A (endotoxin) on the outer membrane that can trigger the release of pro-inflammatory mediators causing sepsis and septic shock (see chapter 1.2.1) (47). Therefore, many researchers have explored the use of purified CPS or O-antigen structures for vaccine development against *K. pneumoniae.* However, they do not follow the conventional protein activation of the immune system.

1.4.2 T cell independent carbohydrate antigens

The use of purified carbohydrates like CPS or LPS for vaccine development against *K. pneumoniae* do not follow the conventional protein activation of the immune system, because carbohydrates do not induce a T cell response. Thus, carbohydrates are classified as T cell independent (TI) antigens (142, 143). This category is further divided into TI-1 for recognition of LPS, which produces a polyclonal non-antigenic B cells response, (144) and TI-2 for recognition of polysaccharides (145).

The recognition of bacterial polysaccharides by the immune system relies on B cells, more specifically BCR receptors as a first signal and TLR interaction as a second signal. B cell stimulation depends on multivalent binding of high molecular weight polysaccharides that have at least 460 nm in length or a sufficient size to cross-link 10 to 20 membrane receptors on the cell surface (146). The cross-linking of BCRs induces intracellular Bruton's tyrosine kinase (Btk) accumulation on the membrane side that leads to intracellular calcium mobilization. This first signal is sufficient to induce B cell proliferation, but a second signal from the TLR interaction with PAMPs is necessary, to stimulate antibody secretion (147). The resulting antibody secretion by B cells is dominated by low-affinity IgM's, because TI activation is not sufficient to induce class switching and consequently impairs the production of IgG's and a memory response (148). Interestingly, TI-2 antigens are not suitable for immunizations in children below two years or adults above 65 years of age since they do not mount an immune response against them (149). Apart of B cells, other APCs can also engulf polysaccharides and process them inside of endosomes by oxidative agents such as reactive oxygen species (ROS). However, the APCs cannot activate T cells because the processed polysaccharides cannot interact with MHC II, thus the TCR receptors on T cells will not be loaded (150).

In summary, the main characteristics of a T-independent response are the lack of IgG (class switch), no rapid immune answer after re-stimulation with the antigen (lack of boost and memory effect), and the lack of memory T and B cells (148). Consequently, the use of isolated CPS or O-antigens as antigens for vaccine development against *K. pneumoniae* would not induce long-lasting immunity and would be ineffective in high-risk group like children and the elderlies.

1.4.3 T cell dependent carbohydrate antigens

Although polysaccharides are known to elicit a TI response, there is one type in particular that can activate T cells, called zwitterionic polysaccharides (ZPS). They are a distinct class of carbohydrates that carry positively charged amines and negatively charged carboxylate and phosphate groups. These charges play an essential role in immune system activation (151). ZPS are found in *Bacteroides fragilis, S. pneumoniae* serotype 1, and *Staphylococcus aureus* type 5 and 8 (152). The activation of T cells by ZPS is dependent on APCs, which engulf the antigen, process it into smaller fragments via oxidative reactions, and load them on MHC II (153). The engagement of ZPS to the MHC II is mediated via electrostatic interactions with the peptide-binding grove in the receptor that occurs due to molecule charges (154). Once the APC is loaded, it presents the ZPS to CD4+ T cells via TCR-loading which culminates into cytokine production and T cell activation. The mechanism, by which the ZPS can activate T cells opened a window for the development of carbohydrate vaccines without the need of protein interactions. Even though ZPS induces a T cell response, the majority of polysaccharides, found on bacteria, such as *K. pneumoniae*, are non-ZPS and therefore cannot engage via the above described mechanism with MHC II (155).

To overcome this problem, a strategy linking covalently CPS or O-antigens to carrier proteins, forming a glycoconjugate, is currently used to convert TI carbohydrates into T-dependent (TD) carbohydrates. (156). Commercial vaccines, using this strategy, have successfully prevented infectious diseases caused by *S. pneumoniae, Neisseria meningitdis* and *Haemophilus influenza* (149, 157). The mechanism of activation of the immune system by glycoconjugates rely on the uptake of the antigen by APCs and internalization in an endosome that leads to a depolymerization of the glycan-protein-conjugate by ROS, reactive nitrogen species, and acidic proteases, resulting in smaller fragments. After processing, the remaining glycan-peptides bind

to the MHC-II via the protein domain and the glycan part is presented to the TCR of the CD4+ T cells, priming carbohydrate specific T cells (T carb cells).

For the activation of B cells, the carbohydrate portion of the glycoconjugates cross-link the BCR following an internalization of the glycoconjugates into the endosome that process the glycan by ROS into smaller repeating units (~10K da) and the protein by acidic proteases into peptides. The processed glycan-peptide binds MHC II and allow for the presentation of the carbohydrate to the TCR of CD4+ T cells that have been primed by APCs. This interaction activates T cells to produce cytokines such as IL-4 and IL-2 inducing B cell maturation forming plasma cells that induce carbohydrate-specific IgG antibodies and memory B cells (Fig. 11). Thus, glycoconjugates induce polysaccharide-specific IgM-to-IgG class switching, memory B cell, and long-lived T carb cell memory. More importantly, glycoconjugate vaccines induce high-affinity antibodies in children under the age of two and the elderly, the two main groups at risk (158).

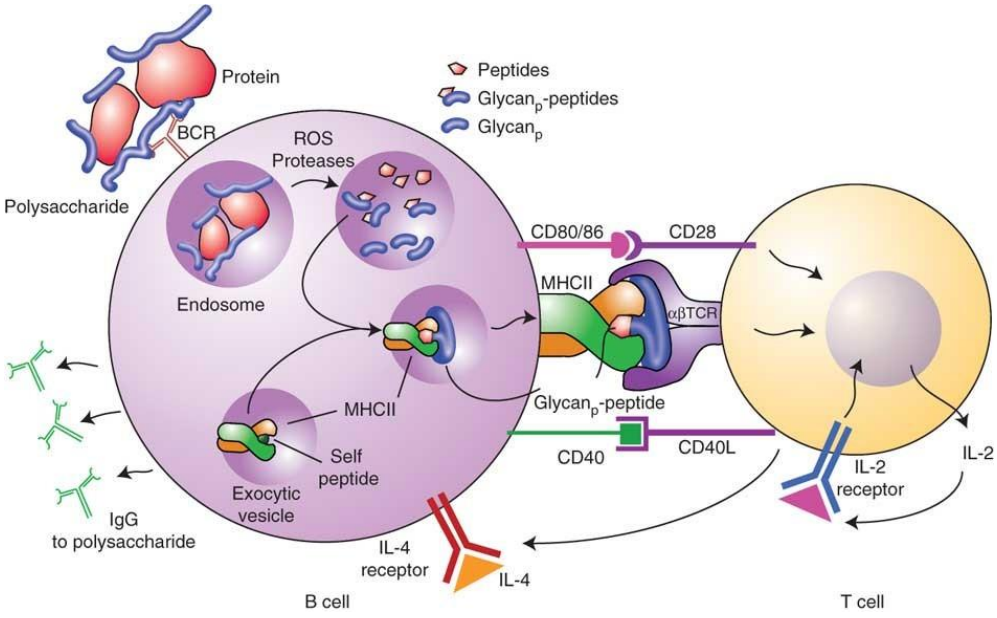

Figure 11. Immune system activation mechanism by glycoconjugate antigens. The carbohydrate portion of the glycoconjugate binds the BCR following an internalization, processing, and loading via MHC II. The loaded glycan-peptide is presented to T cells by loading the TCR, which is the first signal for T cell activation. The engagement of CD28 and CD40L T-receptors to CD80/86 and CD40 B-receptors, respectively, are the second signal for T cells activation. The third and final signal comes from the induction of cytokines such as IL-4 and IL-2 that stimulates the B cell maturation, production of anti-carbohydrate IgG antibodies and generation of B memory cells, and T memory cells (Adapted from Avci et.al., 2011).

The production of glycoconjugate vaccines begins with the isolation of the native polysaccharide from bacterial cultures. Heat and chemical treatment is performed for the isolation of the polysaccharide, followed by purification steps via ultracentrifugation, ultrafiltration and enzymatic treatment to reduce the contamination by nucleic acids and proteins. The purified polysaccharide is then depolymerized by enzymatic degradation and the size of the fragments are determined by size exclusion chromatography (159). The selected polysaccharide fragments are further activated by chemical reactions and conjugated to the carrier protein. Typical carrier proteins are the tetanus toxoid (TT), diphtheria toxoid (DT), or CRM 197 a detoxified variant of DT (160).

Although the coupling of O-antigens or CPS from *K. pneumoniae* to a carrier protein is the rational for enhancing a long-lasting immunity against the pathogen, isolated polysaccharides from the native bacteria contain traces of impurities, such as endotoxin, after the isolation process. Further purification steps to decrease the amount of impurities from the isolated antigen make the process demanding, costly, and lower yielding. Chemical procedures used during these steps may damage the carbohydrate structure affecting its immunogenicity (127).

1.4.4 Synthetic carbohydrate antigens and glycoconjugate vaccine design

The development of glycoconjugate vaccines, based on the isolation of native polysaccharides is challenging and limited to pathogens that can be cultured. Further complications arise from highly labile polysaccharides (161) or ultrastable variants, that cannot be depolymerized (162). The polysaccharide preparations unsurprisingly contain traces of impurities, since the isolation starts from bacterial batches, where different proteins, lipids, carbohydrates, DNA and RNA are part of the crude material (163).

To overcome these problems, methods to synthesize glycan epitopes have been explored. A marketed semisynthetic glycoconjugate vaccine against *Haemophilus influenza* type B relies on the use of synthetic oligosaccharides, generated by polymerization of synthetic building blocks. The conjugation of the synthetic glycan epitopes to a TT protein produces a semisynthetic glycoconjugate vaccine (QuimiHib) that has been proven to produce a robust immune response in humans (164). So far, no other semisynthetic vaccines have been licensed.

The development of synthetic glycoconjugate vaccines depends primarily on the identification of the glycan composition on the pathogen surface. With this information, chemical synthesis is used to generate a minimal protective epitope that can be recognized by the immune system. Chemical and enzymatic reactions, or a combination of both, can be employed to produce synthetic glycans. Chemical solution-phase synthesis has produced a variety of epitopes used in glycoconjugate vaccines, but the challenges rely on the stereochemical control of the glycosidic bond formation and the necessary time to synthesize complex structures (165). Automatic glycan assembly (AGA) improved the chemical synthesis of complex glycans considerably (166). AGA can be used to generate defined carbohydrate antigens with up to 100 monomers (167) in a fast and reliable manner.

Multiple factors are important for a rational antigen design such as length, frameshift of the repeating unit (RU), stereoselectivity, and covalent modifications. Therefore, multiple synthetic glycans are generated in order to identify the best antigen to elicit an strong and reproducible immune response (163). The generated glycan library is screened with sera from patients or animals to provide insights into the potential minimal epitope that induces the production of IgG's, indicative for B cell immune recognition. Glycan microarrays are a useful tool for a high throughput screening of sera, since hundreds of glycans can be immobilized on a glass slide, using minimal amounts of the synthetic glycan (168).

After the definition of the minimal epitope, the glycan is conjugated to a carrier protein forming a glycoconjugate. CRM_{197}, DD, or TT are the most commonly used carrier since they are approved for human use (169). For conjugation, thiol linkers are used to attach glycans to maleimide groups on CRM_{197}, or oligosaccharides with a terminal amine are converted into *p*-nitrophenyladipate esters and covalently attached to lysine side chains in CRM_{197} (170). The ratio glycan:protein is determined by a mass shift using MALDI-TOF MS and usually reaches four to ten glycans per protein (171). Additional quality control using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) may be used to confirm the increase in molecular weight of the glycoconjugate.

The use of subunits, instead of the whole pathogen reduces the strength of the stimulation of the immune system. Therefore, the immunogenicity of the antigen needs to be enhanced by formulation with an adjuvant, which increases the stimulus of the immune system (172).

Aluminium salts (alum) are the most common adjuvants in marketed vaccines since it has been approved for human use more than 70 years ago. The most popular alum adjuvants are aluminium phosphate $Al(PO)_4$, that is negatively charged, and aluminium Hydroxide $Al(OH)_3$, that is positively charged. Antigens adsorb onto alum particles via electrostatic, Van der Waals, or hydrophilic interactions(173). The stimulation of the immune system by alum relies on the activation of innate immune cells via TLR, presentation of particulate antigen format that improves APC uptake and delays antigen clearance from the injection site (174). It also modulates the production of IL-4, IL-10, IL-13, that are responsible for the enhancement of a Th2 immune response, $CD4^+$ T cells proliferation, IgG1 and IgE production and formation of the inflammasome (175-177). Recently, the adjuvant system 04 (AS04), that combines alum with monophosphoryl lipid A (MPLA), was approved for use in human papilloma virus (HPV) vaccine formulations. The immune modulation by alum is enhance by MPLA that produces a Th_1 response with mostly IgG_2 and IgG_3 isotypes. Surface expression of MHC II and co-stimulatory molecules on the APCs are enhanced by the adjuvant and therefore increase the interactions with DCs, macrophages, and B lymphocytes, boosting antigen presentation (178).

The formulated glycoconjugate vaccine follows testing *in vivo* to determine the immunogenicity of the glycan antigens. Mice are the most commonly used animal models for vaccination, however the rabbit immune system is closer related to the human response, thus, drawing conclusions about the potential efficacy in humans is more likely correct in the case of the rabbit model system. (163). The development and evaluation of an immunization schedule is important, since booster vaccination usually enhances antibody production and the development of a long-term memory response. Most glycoconjugate vaccines follow a scheme that includes a first immunization followed by two boosts within a 14-day interval (179).

1.5 Aims of this thesis

K. pneumoniae is a pathogenic bacterium that causes pneumonia and sepsis. It has spread globally, and conventional antibiotic treatments have failed to control it, due to sophisticated antimicrobial resistance mechanisms. More concerning, the CRKP bacteria have developed resistance to the antibiotics of last resort, making them an"urgent threat", as classified by the CDC. Hence, alternative solutions to combat this pathogen are imperative.

Virulence factors like CPS and LPS on the bacteria cell surface down-regulate the activation of the innate and adaptive immune system, and therefore, many groups have tried to boost the immune recognition of *K. pneumoniae* using passive immunization and vaccines. However, attempts to combat *K. pneumoniae*, especially ST258, have relied on CPS as antigen target. The high variability of CPS antigens in this group makes it hard to develop a universal vaccine against most or even all circulating strains. To overcome this problem, *O*-antigens have been thought to be an alternative antigen, because only nine representative serotypes are present to date. However, the presence of endotoxins in *O*-antigen preparations, raises concerns about the safety of native *O*-antigens in vaccine formulations for human use.

The use of synthetic chemistry to generate fully synthetic *O*-antigens would be an attractive solution to generate the antigen without traces of endotoxin. So far, there is no report of a synthetic glycoconjugate vaccine targeting *O*-antigens of *K. pneumoniae.* In particular, there is no report on monoclonal antibodies or vaccines, targeting the O2afg antigen that is expressed on more than 80% of CRKP strains.

The primary objective of this thesis is:

- Formulation of a vaccine that contains the synthetic O2afg antigen and determination of the immune response in a rabbit model.

The secondary objectives of this thesis are:

- Evaluation of antibody binding specificity to the synthetic antigen as well as to the ST258 subgroup of *K. pneumoniae*.

- Definition of an antibody effector mechanism *in vitro* via opsonophagocytic killing and complement activation assays.
- Determination of antibody protection efficacy and immune cell activation via passive immunization in a murine pneumonia infection model, using polyclonal anti-sera of rabbits, immunized with the glycoconjugate vaccine.

2 Experimental Section

2.1 Vaccine design

2.1.1 Glycan antigen synthesis

The O2afg *K. pneumoniae* serotype is composed of multiple repeating units (RU) of Gal-III epitope, which is a trisaccharide branched frame composed of two galactopyranose and one galactofuranose (180). The design and synthesis of the antigen was performed by Dr. Dacheng Shen as part of his PhD thesis performed at the Max Planck Institute of Colloids and Interfaces. The synthesis was based on the assembly of single glycan building blocks containing protective groups, following a global deprotection as a final step. Two antigens were produced, one containing one RU (trisaccharide) and a second containing two RU (hexasaccharide) (Fig. 12). Both glycan antigens had an additional aminoalkyl linker attached to its anomeric carbon from the hydroxyl end in order to use the amine group in the opposite end of the linker for glycan immobilization on microarrays and further conjugation to the carrier protein.

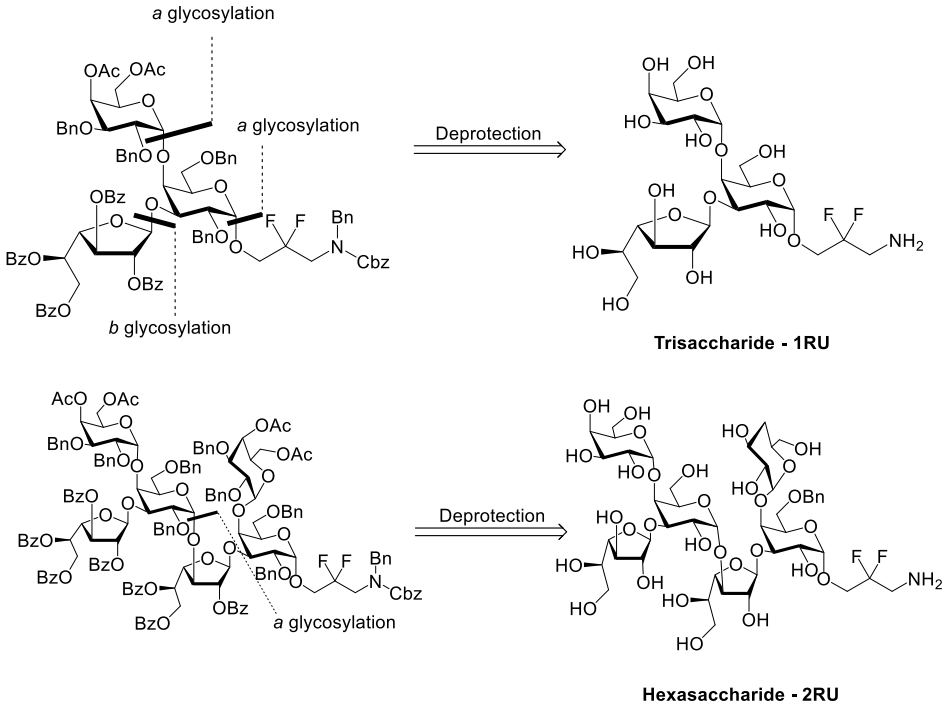

Figure 12. The synthesis of O2afg antigen (Gal-III) was based on the assemble of single building blocks containing protective groups. A final global deprotection of the synthetic antigen resulted in the final antigen product. Two antigens were generated, one containing only one repeating unit (RU) – trisaccharide, and the second one with two RU - hexasaccharide. The synthesis was performed by Dr. Shen.

2.1.2 Minimum glycan epitope screening

In order to determine the minimum glycan epitope, the designed trisaccharide and hexasaccharide were incubated with serum of patients infected with *K. pneumoniae* resistant to carbapenems (CRKP). Recognition of the glycan antigen by human antibodies present in sera was measured using glycan microarrays (181). A total of 20 human sera samples from patients infected with CRKP was provided by the group of Dr. Bettina Fries from Stony Brook University, New York. Two non-infected sera and one pooled human serum from 287 individuals (WHO 007 sp., NIBSC) were used as negative controls and served as a reference for the determination of antibody binding threshold. Patients were consented under institutional review board (IRB) and SBU Human Subjects Committee approved protocols (IRB# 896845 and 851803). The health information was deidentified. Healthy donors gave written informed consent for blood donation under IRB# 718744. After blood collection, the samples were centrifuged, and the sera were transferred to Eppendorf tubes and sent by plane under cooling conditions. Samples were kept at -20°C until further use.

Microarray slides were produced by coupling the synthetic glycans containing an amine linker (figure 12) to the CodeLink *N*-hydroxysuccinimide-activated glass slides (SurModics Inc., Eden Prairie, USA). Primarily, the synthetic oligosaccharides were suspended in sterile printing buffer (50 mM sodium phosphate buffer, NaPi, pH 8.5) to the final concentration of 200 µM in a printing plate and it was loaded into a contact-free piezoelectric microarray robotic spotter (Scienion, Berlin, Germany). The glycans were printed in triplicate under humidity and temperature controlled conditions. The BSA-adipic linker and carrier protein were also printed in the same slide for unspecific binding controls. The printed slides followed an incubation for 16-24h in a humidity chamber at room temperature to enhance the coupling reaction efficiency. After, the slide was quenched for 1h with ethanolamine (100 mM in 0.1 M NaPi, pH 9) for the inactivation of reactive groups on its surface, washed 3x for 2min with ddH_2O, centrifuged for 5 min at 300 x g, and stored at 4°C until use. For the screening, the slide was blocked with blocking buffer (1% (w/v) BSA in PBS) for 1h and followed a centrifugation for 5min at 300 x g. A FlexWell grid (FlexWell 64, Grace Bio-Labs, Bend, US) was placed, generating 64 testing fields, where 30 µL of the serum samples (diluted 1:100 in blocking buffer) were applied into individual wells, in

duplicate. After incubation for 1h at 37°C in a light-protected humidified box, the samples were washed 3x with 50 µL of washing buffer (PBS + 0.1% Tween-20) and incubated with 30 µL of secondary fluorescently labeled antibodies (Goat anti-Human IgG (H+L) Alexa Fluor 647, Thermo Fisher, diluted 1:400 in blocking buffer) following incubation for 1h at 37°C. Finally, the samples were washed 3x with 50 µL of washing buffer and 1x with ddH_2O and dried by centrifugation (5 min at 300x g) and proceeded to fluorescent read-out. The Axon GenePix 4300A microarray scanner was used for the fluorescent read-outs and the photomultiplier tube (PMT) voltage was adjusted such that scans were free of saturation signal. The GenePix Pro 7 software (Molecular Devices, Sunnyvale, CA, USA) produced the mean fluorescent intensity values (MFI) that were used for data analysis with R (RStudio Version 1.2.5033) or GenePix Pro 8 software (Graphpad Software Inc., La Jolla, USA) for graphical visualization. After selection of the minimum epitope, the glycan antigen was conjugated to a carrier protein in order to enhance a T-dependent immune response.

2.1.3 Antigen conjugation to CRM_{197} and characterization

The conjugation of the synthetic glycans to the CRM_{197} as a carrier protein occurred in two steps and individually for each compound. First, the oligosaccharide was attached to the spacer *p*-nitrphenil adipate ester (PNP) and the structure followed the conjugation to the protein completing the second step of the reaction. The chemical reaction was performed in PBS solution at pH ~8 (Fig. 13). MALDI-ToF MS (Autoflex Speed, Brucker Daltonics) determined the mass of pure CRM_{197} and the conjugated structures and the information was used to determine the glycan:protein loading ratio. Briefly, glycoconjugates and protein containing 2,5-dihydroxyacetophenone (DHAP) and 2% trifluoracetic (TFA) as matrix were spotted onto MTP 384 steel plates. The machine was set in linear positive mode and the spectra were acquired over an m/z range between 30.000 and 210.000. The generated data was analyzed with the Flexanalysis software. The protein:glycan ratio was established by subtracting the glycoconjugate mass from the carrier protein and the resulting value was divided by the mass of the glycan containing the adipic linker.

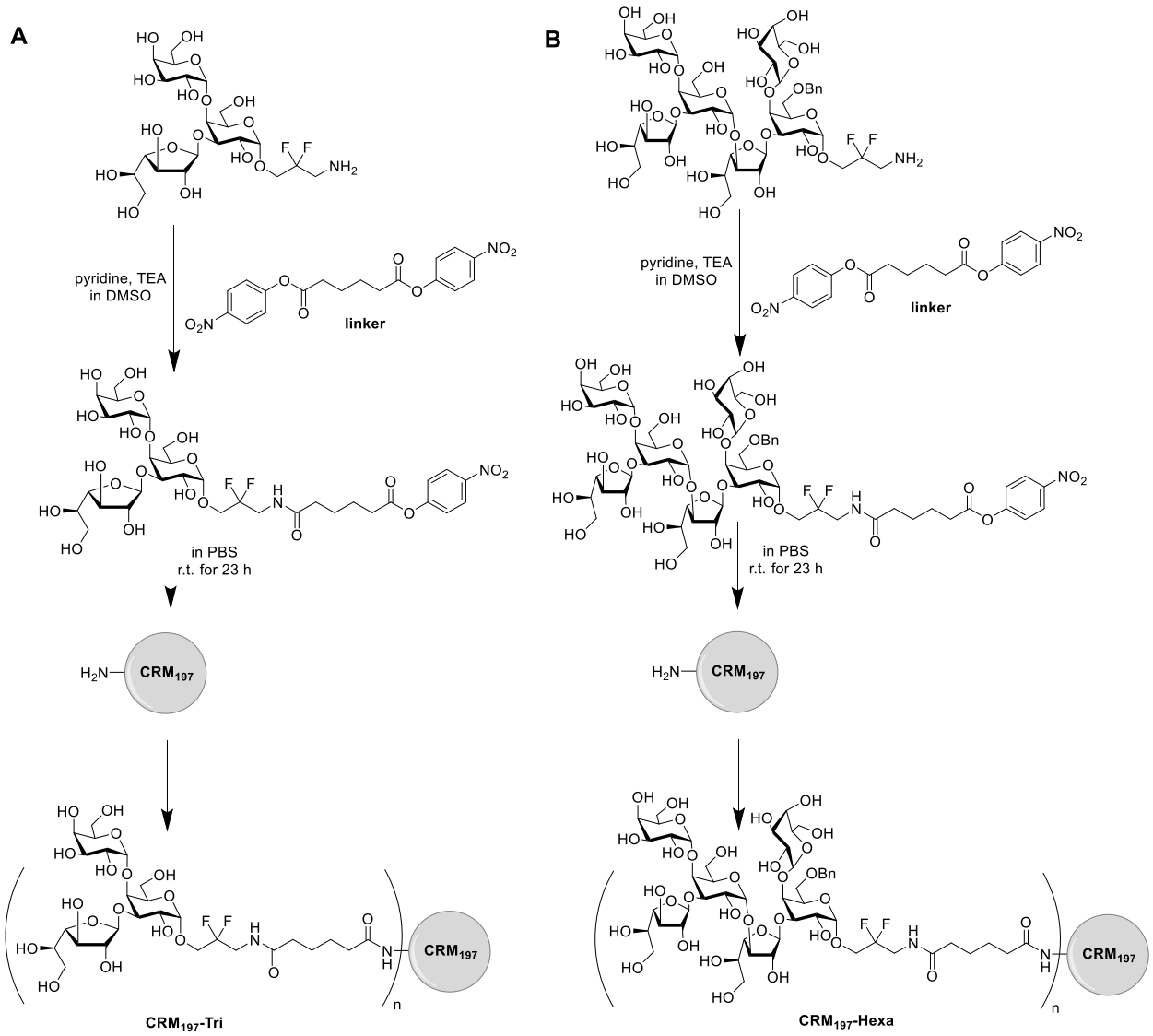

Figure 13. Coupling of the trisaccharide (A) and the hexasaccharide (B) to the carrier protein CRM_{197} resulted in the glycoconjugate CRM_{197}-tri and CRM_{197}-hexa respectively. The chemical reaction was performed by Dr. Shen.

The glycoconjugates were also characterized by SDS-PAGE. The SDS-PAGE gel was prepared using 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. The glycoconjugates were suspended in loading buffer (200 mM Tris-Cl (pH 6.8), 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% (v/v) glycerol) and an amount of 1-2 µg of the samples was loaded in the gel. The CRM_{197} was used as

positive control and the page ruler Plus Prestained ladder 10 to 250 kDa (Thermo Scientific) was used as a size marker. The gel ran at 120 V and 25 mA for 90 min and followed a staining for 30 min in a solution composed of 0.5 % (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid. For the gel visualization a distaining with 50% (v/v) methanol and 10% (v/v) acetic acid for 15 min, or O/N was performed and the gel was photographed for data record.

2.1.4 Glycoconjugate formulation with adjuvant

After glycoconjugate characterization, the vaccine formulation was carried on. The selected glycoconjugate was formulated with aluminum hydroxide ($Al(OH)_3$) (Alhydrogel, Brenntag, Denmark) and the final vaccine contained a total of 1 µg of sugar per 500 µL dose that was estimated based on the protein:glycan mass ratio of the glycoconjugate provided by MALDI-ToF. Briefly, the glycoconjugate suspended in PBS (PBS pH 7.4, PAN-Biotech, Germany) was sterilized using a 0.22 µm filter and an aliquot containing a total of 1 µg of sugar was mixed with 125 µg of Alum, following addition of PBS up to 500 µL final volume per dose. The solution was rotated O/N at 4°C to allow the adsorbtion of the glycoconjugate to the alum matrix.

The glycan antigen adsorbtion was measure by bicinchoninic acid assay (Pierce™ BCA Protein Assay Kit, Thermo Fischer). After O/N rotation, the vaccine samples were centrifuged for 10 min at 3000 x g allowing the precipitation of Alum particles. The supernatant was carefully transferred to an Eppendorf and analyzed by BCA assay in triplicate, following manufacturer instructions. A vaccine with no O/N rotation was used as control reference (non-adsorbed protein in the vaccine supernatant). The reduction of more than 80% of protein in the supernatant after O/N incubation when compared to the control was the indication of successfully adsorbtion to the adjuvant matrix. After formulation, the vaccines were used to evaluate the activation of the immune system *in vivo*.

2.2 Animal immunization and hyperimmune sera collection

The glycoconjugate vaccine was tested in rabbits in order to determine the immune response against the synthetic antigen *in vivo*. The study design included two groups, the glycoconjugate vaccine testing group with five 8-week-old Zika rabbits (n = 5) and the control group with three rabbits (n = 3). All animals were kept according to the governing laws of DIN EN ISO 9001:2000

standards, German guidelines according to law 8a Animal Welfare Act of 18. May 2006 (BGBI. I, p.1206), European Union guidelines 86/609/EWG of 24.11.1986 and according to the European Agreement of 18.3.1996 for protection of animal trials and other for scientific purposes used vertebrates of 11.12.1990 (BGBl.ll S. 1486). Further, a veterinarian inspected the animals regularly. The immunization was performed in strict accordance with the NIH/OLAW Animal Welfare Assurance, identification number F16-00178 (A5755-01) and was authorized by LALLF MV (Landesamt für Landwirtschaft, Lebensmittlelsicherheit und Fischerei Mecklenburg-Vorpommern) in accordance to TierSchG and Tierschuutz-Versuchstierverordnung (project #49062 and #49406).

Rabbits were immunized intramuscularly (i.m.) with CRM-Hexa-Alum vaccine (0.5 mL), that contained the glycoconjugate in the formulation, or PBS-Alum as a negative vaccine control. Iimmunizations were performed on day 0 (primary immunization) and boosted with the same vaccine formulation on days 14 and 28. Blood was collected at days 0 (pre-immune), 14, 21, 28, and 35 for hyper-immune sera analysis. Memory response was accessed by following a long resting period and boosting the animals at day 133. The rapid antibody generation at day 144 was determined by analyzing the hyperimmune sera from blood collected at this time point (terminal-bleed) (Fig. 14).

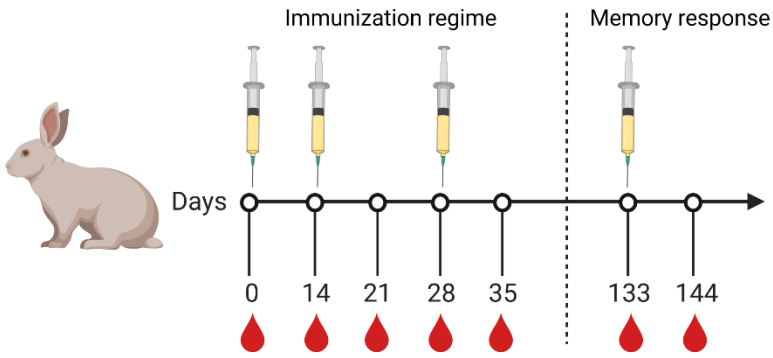

Figure 14. Rabbit Immunization schedule. Zika rabbits were immunized with either CRM-HEXA-Alum or PBS-Alum (negative control) vaccine. The primary immunization was followed by two boosts with 14 days interval. Blood was collected throughout the time points. Animals were boosted at day 133 after a long resting period followed by blood sampling after 11 days (day 144) in order to evaluate the presence of memory response.

For serum extraction, the collected blood (15-50mL, per animal) from different time points was left to coagulate for at least 30 min at RT and then centrifuged for 15 minutes at 2000 x g to separate blood cells from the serum containing antibodies. The serum was transferred to clean vials, aliquot, and stored at -20°C until the performance of antibody evaluation assays.

2.3 Antibody binding target assays

2.3.1 Glycan microarray

The recognition of the glycan antigen by the antibodies present in the hyper-immune sera of vaccinated animals was measured by glycan microarray (181). The production of microarray slides containing the glycan epitopes of interest is described in section 2.1.2 of this thesis. Collected rabbit sera from different time points were diluted 1:50 in blocking buffer (1% (w/v) BSA in PBS) in order to avoid unspecific binding. A total amount of 50 µL of diluted sera was added to the microarray slide in duplicate for the tested group and single measurement for the control group and followed an incubation at 37°C for 1h in a humidifier chamber. After three washing steps with PBS-T (PBS + 0.1% Tween-20), the samples were incubated with 30µL of fluorescent labeled anti-rabbit IgG (Goat anti-Rabbit IgG (H+L), Alexa Fluor Plus 594, Thermo Fisher, catalog # A32740) or anti-rabbit IgA (Goat Anti-Rabbit IgA alpha chain (DyLight® 488), ABCAM, # ab96975), diluted 1:400 in blocking buffer, in order to evaluated long-term immune response and mucosal antibody production, respectively. The slide was incubated at 37°C for 40 min, washed three times with washing buffer and once with ddH_2O, and dried by centrifugation (5 min at 300 x g).

The sample fluorescent signals were scanned by Axon GenePix 4300A microarray scanner (Molecular Devices, USA) and the photomultiplier tube (PMT) voltage adjusted to avoid fluorescent saturation. The GenePix Pro 7 software (Molecular Devices, Sunnyvale, CA, USA) produced the mean fluorescent intensity values (MFI) that were used for data analysis with R (RStudio Version 1.2.5033) or the GenePix Pro 8 software (Graphpad Software Inc., La Jolla, USA) for graphical visualization.

2.3.2 Enzyme-Linked Immunosorbent assay (ELISA)

The evaluation of antibody binding to the carrier protein was determined by Enzyme-Linked Immunosorbent assay (ELISA). High-biding 96-well plates (Corning, USA, #3361) were coated with 50 µL of CRM_{197} diluted in PBS at a final concentration of 5 µg/mL and incubated at 4°C O/N. Plates were washed once with PBS-T and blocked with 1% BSA-PBS for 1 h at 37 °C. Primary serum dilutions in 1% BSA-PBS were added and plates were incubated for 1 h at 37 °C. After washing three times with PBS-T, secondary antibody (anti-rabbit IgG Fc HRP, Dianova, #111-035-046) diluted 1:10.000 in 1% BSA-PBS was added and plates were incubated for 1 h at 37 °C. Wells were washed three times with PBS-T and revealed by colorimetric detection by incubating with 50 µL of TMB substrate (BD Biosciences, San Jose, USA) for 5-20 min. The reaction was stopped by the addition of 50 µL of 2% sulfuric acid and absorbance values were determined at 450 nm with an Infinite M200 microplate reader (Tecan).

2.3.3 Flow cytometer for bacteria-surface-specific antibody binding

The evaluation of antibody binding to the native bacterial antigen was performed by flow cytometer (FACS) using well-characterized strains of *K. pneumoniae* expressing O2afg or O1 antigen as positive and negative control, respectively, which were kindly provided by Prof. Dr. Chris Whitfield form the University of Guelph (57) and commercially bought from SSI Diagnostica. The binding of antibodies from immunized animals to the bacteria was assessed following a well stablished protocol with modifications (182).

Briefly, bacteria were grown on agar LB plates at 37°C for 12-16 h and a single colony was transferred into 5 mL Luria Broth (LB) (Roth, art. No. X968.3 or Sigma art. No. L3522) and incubated O/N at 37°C with shaking (300 rpm). An aliquot of 5 µL was transferred into 4.95 mL of LB in a 50 mL tube and incubated at 37 °C, until reaching exponential growth phase (~2-3h). Bacterial density was determined by measuring the optical density (OD) of the samples where an OD of 0.1 contains 2 x 10^8 CFU/mL (based on bacteria growth curve). A total of 1 x 10^5 bacteria cells was transferred to an Eppendorf, centrifuged (13.000 rpm 5 min), and washed three times with PBS. The bacteria pellet was suspended with 200 µL of sera diluted 1:10 in PBS-BSA 1%, incubated for 1h at RT or 4°C, and followed by two washing steps with 0.5 mL of PBS-BSA 1% and

centrifugation (13,000 rpm, 5 min). The bacterial pellet was resuspended in 200 µL of fluorescent labeled secondary antibody diluted 1:100 (Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 635, Invitrogen) and incubated at 4°C for 1h. Washing steps were repeated and the bacterial pellet was resuspend with 1 mL of 4% (wt/vol) PFA following incubation at 20 min at RT for bacteria fixation. After centrifugation at 13,000 g for 5 min, the samples were resuspended in 500 µL of PBS-BSA 1% and used for FACS analysis. The FACS CANTO II (BD) was used for the acquisition and the threshold was set at FSC = 200 and SSC = 200 combined with an "AND" logic gate. Positive control single-stained samples were used to set photomultiplier tube (PMT) voltages and 10.000 events were recorded per sample. The acquisition of pure water or 0.22-µm-filtered 1× PBS with records of < 100 events/min ensured that the machine was cleaned. The software FlowJo 10.7.1 (BD) was used for data analysis. The positive binder quantification was based on the product of the percent of gated events that passed the fluorescence threshold and the median fluorescence intensity (MFI) of those events that passed the threshold.

2.4 Antibody activity assays

2.4.1 *In-vitro* opsonophagocytic killing assay (OPKA)

The assay was performed as described previously (183) with modifications for *K. pneumoniae* since gram-negative bacteria are more prone to complement killing in comparison to gram-positive bacteria. Concisely, the effector HL-60 cell line (a human origin leukemia cell line) was used as phagocytic cell. For granulocyte differentiation, approximately 4×10^5 cells/mL were seeded in tissue culture flasks (Corning, N.Y.) in complete medium (90% RPMI 1640, 10% FCS, 1 mM L-glutamine and penicillin-streptomycin solution; PAN Biotech, Germany) containing 0.8% *N,N*-dimethylformamide (DMF; 99.8% purity; Fisher Scientific, Fair Lawn, N.J.) 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 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 1 x 10^7 cells/mL. For the opsonophagocytic killing assay, a ratio of 400:1 effector to target cells was used. Glycerol stock of *K. pneumoniae* O2afg grown to mid-log phase (OD600= 0.2–0.3) were gently thawed and diluted in opsonophagocytic buffer to a final density of 1000 CFU per 20 µL. Pooled heated inactivated (56°C, 30 min) rabbit sera samples (10 µL) were aliquot

in round bottom non-treated 96-well plates in triplicate at four-fold dilution intervals. The bacterial suspension (20 µL) was added to each well and incubated for 15 min at 37°C. After, 2.5 % (v/v) baby rabbit complement (Rabbit complement, Cedarlane) and 4×10^5 differentiated HL-60 cells (in 40 µL) were added to each well. The plates were incubated for 45 min at 37°C in 5% CO_2 with intermittent shaking. The phagocytic reaction was stopped by putting the plate on ice for 15 min. Viable extracellular bacteria were determined by plating aliquots (5 µL) from each well on LB agar plates and incubating at 37°C until visualization of colonies (~12-16 h) for CFU counting. Negative control containing bacteria with complement, HL-60 cells, and buffer, but without antibody, was used as reference to calculate the percent killing of *K. pneumoniae*. The assay was repeated twice independently and in duplicate each. The percent killing was calculated as % phagocytic killing = [(CFU at 0 min − CFU at 120 min)/CFU at 0 min] × 100 and reported as means ± SD of CFU. The opsonic index values where 50% bacterial killing occurs was obtained from four parameters logistic regression of individual opsonic curves (184).

2.4.2 Complement deposition assays

The activation of classical complement pathway was evaluated by detecting the deposition of complement proteins C3c and C5b-9 on *K. pneumoniae* surface after incubation with rabbit sera and normal human sera (NHS, Pan Biotech, Germany, cat no. P30-2901). The deposition was measured by FACS and it has been previously described elsewhere (124). Bacteria were grown on agar LB plates at 37°C for 12-16h and a single colony was transferred into 5 mL Luria Broth (LB) (Roth, , art. No. X968.3 or Sigma art. No. L3522) and incubated O/N at 37°C with shaking (300 rpm). An aliquot of 5 µL was transferred into 4.95 mL of LB in a 50 mL tube and incubated at 37 °C, until reaching the exponential growth phase (~2-3 h). Bacterial density was determined by measuring the optical density (OD) of the samples where an OD of 0.1 represents 2×10^8 CFU/mL. The bacteria were diluted to 1×10^8 CFU/ml in 1 mL PBS-BSA 1% or 20% fresh human serum (in PBS-BSA). PBS or hyper-immune pooled rabbit sera heat inactivated (10% final volume) was then added, and bacteria were incubated either 20 min or 2 h at room temperature for C3c and C5b-9 deposition, respectively. Bacteria were washed with PBS-BSA1%, resuspended in PBS-BSA1%, and incubated with either FITC-conjugated anti-human C3c (Abcam Rb pAb to C3c – ab4212 FITC) at 1:500 or 635-conjugated mouse anti-C5b-9 (Bioss, anti C5b-9 bs2673R-A647) at 1:100 or

without antibody for 20 min at 4°C. After incubation, the bacteria were washed and resuspended 500 µL of PBS-BSA 1% and analyzed for fluorescence by FACS Canto II as described at 2.3.3 section.

2.5 Passive immunization in murine pneumonia model

2.5.1 Rabbit hyperimmune sera preparation

The hyperimmune sera was prepared by pooling the rabbit sera from day 35 (time point after full immunization) from five animals for CRM_{197}-HEXA-Alum group and three from PBS-Alum group, separately. The pooled sera were heat at 56°C for 30min for complement deactivation, sterilized with 0.22µm filtration, and stored at -20 °C until usage.

2.5.2 Bacteria strain characterization and infective sample preparation

Bacteria characterization. The *K*. *pneumoniae* strain used in the infection study was donated by Prof. Dr. med. Bastian Opitz from Charité – Universitätsmedizin Berlin (Berlin - Germany), and belonged to the ST258 clonal group expressing KPC-2, TEM-1 & SHV-11 carbapenemase genes. In order to determine whether the strain belongs to O2afg serotype, a PCR amplifying specific O-antigen genes (185) and the regions of the *gmlABC* operon (57, 180) was performed. A strain expressing O2afg antigen was used as positive control. The boiling method was used for DNA extraction. One single bacterial colony grown on LB agar plate was inoculated in 5 mL LB broth (Roth, art. No. X968.3 or Sigma art. No. L3522) and incubated at 37°C until exponential phase was reached (~2-3h). The sample (1 mL) was transferred to an Eppendorf tube and centrifuged at 13.000 rpm for 5 min following two washing steps with RNAse free water (Qiagen, Cat. No. 129112). The tube was then placed at 100 °C in a boiling water-bath for 15 min and immediately frozen at 0 °C on ice. Subsequently, the suspension was centrifuged at 13,000 rpm at 4 °C for 15 min and the supernatant was transferred to a clean 500-µL tube and stored at − 20 °C for PCR analysis. For PCR, 50 µg of extracted DNA was mixed with 4 µL of PCR Master Mix containing Mg^{2+} (Thermo Fisher Scientific, Waltham, MA, United States, cat no. F-530XL), 0.2 µL of Taq polymerase (Phusion™ High-Fidelity DNA Polymerase, Thermo Fisher, cat No. F-530XL), 0.5 µM primers (Table 1), and 200 µM of each dNTP (Invitrogen, cat. No. 18427013) in a final reaction volume of 20 µL. The following cycling conditions was used: 98°C for 30 s, followed by 20 cycles

of 98°C for 10 s, 57.7°C for 30 s, and 72°C for 3 min, then a final extension at 72°C for 10 min. The samples were stored at -20°C until use. The DNA fragment size was determined on a 1% agarose gel and the 1Kb plus ladder (Invitrogen, cat. No. 10787018) was used for size reference.

Table 1. Nucleotide sequence of primers used for O-serotyping *K. pneumoniae*.

Serotype	Primer Name	Nucleotide sequence (5' - 3')	Product lenght (Kb)
O1/O2	wb O1/O2-A-F	CGCTATAAGAGCAGCATGCTAG	1.3
	wb O1/O2-A-R	CGATATCACCTACTGCCAGA	
	wb O1/O2-B-F	TTGTTGAGCCTGACAGGATC	1.6
	wb O1/O2-B-R	GCCATTGCTTGCTTGTACAG	
O3	wb O3-A-F	CTATCGCTACCGTGGCTTTA	0.8
	wb O3-A-R	TCTCGTCCACAATATCAGCG	
	wb O3-B-F	GCCTACAGTATCTACCTCTG	0.9
	wb O3-B-R	CGGTAAAGTCAGGATGGAAG	
O4	wb O4-A-F	CAGAAGCGCGAGTTAATCTG	0.7
	wb O4-A-R	GGTCCAGTTAGGCTCAATTC	
	wb O4-B-F	GTCAGCGGGAATTATTGGAC	1.2
	wb O4-B-R	CTTGAGATCCAGAATGCCAC	
O5	wb O5-A-F	GCTACCAAACCAGTATGCTG	1.8
	wb O5-A-R	AGGTGCGTACTGGAAGTATG	
	wb O5-B-F	GGTGATGAAAGCCAGAATGC	1.4
	wb O5-B-R	CAGTGCCTGAAACAGTTTGC	
O8	wb O8-A-F	CGTGGCAATGGTTTGCTAGT	1.2
	wb O8-A-R	TCAATCCACACAACTCGGTC	
	wb O8-B-F	GCTAGTTCGGCAACTAACTCAC	0.8
	wb O8-B-R	AGTTCCAGCATCGAAGCAACTC	
O9	wb O9-A-F	CGCGCTCAGTTATTCCATTG	1
	wb O9-A-R	CTGGCTGATGACAGAGAATC	
	wb O9-B-F	GCATTCCTGTTCGTGTATGG	0.9
	wb O9-B-R	ATGTCACCGACAGCAAGTAC	
O12	wb O12-A-F	CTGCAGATGGCTAAACGTGA	0.6
	wb O12-A-R	CCGTTCGGGCTTGTTCAATA	
	wb O12-B-F	GAAGTCGACTTTGCTGCAGA	1
	wb O12-B-R	ACGTTGATCAAGCTCCTCTC	
O1	wbb O1-F	GATTTCACTTTCCGGGCAAC	1.1
	wbb O1-R	GGCTTGCTGAATCACAAGAC	
O2ac	wbb O2ac-F	AAACATCGCTGACTCGAGTC	1
	wbb O2ac-R	CGACTATGATCGTACCAACG	
O2afg ***gmlABC* operon**	gmlABC-F	ATGCCAAGTTCAGGCCCATTATG	2.8
	gmlABC-R	CTAATAATTTATCGTTGACCTTCGCATTG	

Bacteria infective inoculum. The bacteria inoculum used for infecting the mice was prepared as follows. The day before infection, a small amount of bacteria was collected from frozen medium (20% glycerol (Merck, Germany) in Todd Hewitt Broth (THB) (BD, Germany) supplemented with 0.5% yeast extract (THY)) and plated on LB or THB agar plates (BD, Germany) and allowed to grow for 8-10 h at 37°C. Viable colonies were transferred to 10 mL of THY medium added to 10% fetal calf serum (FCS; Gibco, USA) until an optical density (OD) of 0.03-0.04 at 600 nm was achieved. Bacteria were grown to mid-log phase at 37°C in water bath for around 2h until an optimal OD of 0.3-0.4 that corresponds to an amount of 0.3-0.4 x 10^9 colony-forming units (CFU) per mL. Total bacteria number in solution was calculated and the solution was centrifuged at 3100 rpm without breaks at RT. The pellet was resuspended in phosphate-buffered saline (PBS; Gibco, USA) for a final concentration of 1 x 10^9 CFU/mL. The final solution was kept on ice until murine infection was performed. A ten-fold serial dilution of the final bacterial solution in PBS was made and four final grade dilutions were plated on LB agar plates, in duplicate, in order to validate the cell density.

2.5.3 Mice and Ethical Approval

Animals used in this study were obtained from Charles River (Charles River Laboratories, Sulzfeld – Germany). Conditions for housing and handling of the animals were approved by the German Office for Health and Social Affairs in Berlin (LAGeSo), aiming to minimize animal stress. Mice were kept under specific pathogen-free conditions at the internal animal facility at Charité – Universitätsmedizin Berlin (Berlin - Germany) prior to experiment, with a 12/12 hours (h) light/dark cycle, with temperature around 23°C and humidity close to 40%. The animals were wild type (WT) C57BL/6N, 9-10 week old female mice, weighting between 19-22g.

2.5.4 *In vivo* murine pneumonia model

Study design. Three groups with twelve C57BL/6N mice each were divided into cages containing four animals each from mixed groups. The first group was passively immunized with CRM-HEXA-Alum hyperimmunne sera and infected with *K. pneumoniae* (testing group), the second group was passively immunized with PBS-Alum hyperimmune sera and infected with *K. pneumoniae* (negative control group), and the third group was passively immunized with CRM-HEXA-Alum

hyperimmune sera but infected with PBS (antibody control group, PBS-Mock). The study design was performed in a 48 h window with the followed schedule. Blood was collected from the animals that were then intra nasally infected with 1x 10^8 CFU of *K. pneumoniae*. A total amount of 100 µL of hyperimmune sera was administered via intraperitoneal 2 h after infection and the animals were monitored for body weight and temperature variation every 12 h until final preparation to characterize disease progression. At the 48 h time point, mice were sacrificed and blood, bronchoalveolar lavage fluid (BALF) and lungs were collected for the measurement of CFU density, immune cells counting, cytokines level, myeloperoxidase (MPO) activity, and histopathology of lungs (Fig. 15).

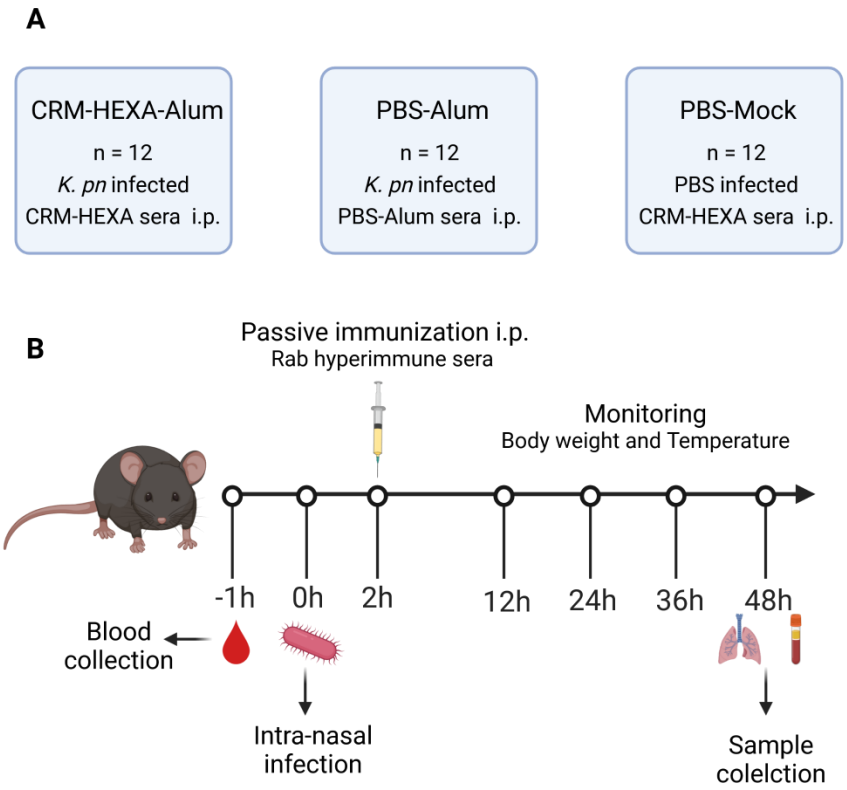

Figure 15. Passive immunization study design. **A** - Three groups of twelve C57BL/6N mice each were passively immunized with either CRM-HEXA-Alum or PBS-Alum, after infection with CRKP (K. pn) or PBS (mock group). **B** - Blood from mice was collected following an intranasal infection with 1 x 10^8 CFU K. pn. After 2 h, the animals were passively immunized with rabbit hyperimmune sera and then monitored every 12 h until 48 h. At the last time point (48 h), lung, BALF, and blood form the animals were collected in order to predict infection rate, cell parameter and lung tissue injured.

Sera titration and mice blood antibody titer. In order to establish the optimal hyperimmune sera dose for passive immunization, three groups of mice with three mice each were passively immunized i.p. with either 1 µL , 10 µL or 100µL dose of CRM-HEXA-Alum hyperimmune sera. Blood from the animals was collect from the Vena cava caudalis at time 0h prior immunization and after 48 h post-immunization. Blood was let to sit for 30 min and RT and it was centrifuged for 15 min at 2000 x g to separate blood cells from the serum containing antibodies. The antibody titers against the glycan antigen were evaluated by glycan microarray.

Intranasal infection. Mice were anesthetized i.p. with 3 mL/kg of a mixture of 100 mg/mL ketamine (CP-pharma, Germany) and 20mg/mL xylazine (CP-pharma, Germany), diluted in 0.9% NaCl solution according to their weight. Under anesthesia and without reflexes, mice were hanged by their teeth on a line to reach a vertical position and allow exposure of their nostrils. To avoid eye dryness, a small portion of 3 mg/g Thilo-tears gel (Alcon, USA) was applied in the ocular region of the animals. The bacteria stock solution (described at section 3.2) was diluted 1:1 with hyaluronidase (Sigma-Aldrich Chemie GmbH, USA) to facilitate bacterial colonization of the lungs and mice were infected intranasally with 20 µL of bacteria. The mock infected control group was inoculated with the same volume of PBS. Infection was performed in two steps, to avoid blockage of the nasal cavity of the mouse. Altogether, mice were infected with 1 x 10^8 CFU of *K. pneumoniae* and placed back in cages heated with infrared light, for the waking phase after infection.

Animal preparation and sample collection. At the 48 h time point, the animals were prepared for sample collection (blood, BALF, lung) as follows. Mice were anesthetized with 80 µg/g bodyweight ketamine and 25 µg/g bodyweight xylazine. Under deep narcosis, animals were fixed on a working surface with needles. The stomach area was opened parallel to body length until trachea region and skin were fixed with needles. Blood was collected from the vena cava caudalis in a tube containing ethylenediaminetetraacetic acid (EDTA)-K (Sarstedt, Germany) or nothing for serum colelction. Small blood aliquots were kept for bacteria counting and for flow cytometry with the fluorescence-activated cell sorting (FACS) analysis. The remaining blood was centrifuged at 4°C for ten minutes with 4000 x g and the plasma collected was frozen for quantification of cytokines and lung permeability parameters. For BALF collections, after death of the animals, the

diaphragm was cut to release the lungs and the thorax was opened through the sternum. The connective tissue was removed for the detachment of the lung. The muscle tissue covering the trachea was carefully separated with forceps, avoiding collapse of the arteries and the trachea was released. To fix the BAL tube, a surgical thread was passed around the trachea. Using fine dissection scissors, a small incision was made at the posterior part of the trachea, the tube was inserted into the trachea and fixed by the surgical knot. The BAL tube was attached to the trachea and a solution of 1000 µL of PBS containing complete Mini protease inhibitors (Roche Diagnostics GmbH, Germany) used for the acquisition of the bronchoalveolar lavage fluid (BALF). A small aliquot was used for bacteria counting and FACS analysis and tube was centrifuged for ten minutes at 4°C with 300 x g. The supernatant was frozen in liquid nitrogen for later analysis of antibody level, cytokines expression, and MPO activity. For lung collection and bacteria count, the lungs were perfused with PBS injected through the heart right ventricle after cutting the inferior vena cava in the thorax region. Lungs were washed until the eluent became whitish to avoid blood cell contamination of the sample. The lung lobes were prepared and kept in a solution of PBS and protease inhibitors. Then cut into smaller pieces and homogenized through a 100 µm filter for cell analysis and CFU counting.

2.5.5 Measurements of immune cells in blood and BALF

Forty-eight hours post-infection, mice were anesthetized (160 mg/kg ketamine and 75 mg/kg xylazine) and blood was collected in a tube containing EDTA (Sarstedt, Germany). The EDTA-blood was used for hemogram (measured with Scil Vet abc™) and the levels of eosinophils, monocytes, granulocytes, lymphocytes, and platelets were measured. Additionally, flow cytometry was used to identify specific cell phenotypes from blood and BALF of passively immunized mice. For FACS analysis, erythrocyte lysis was performed prior to staining with red cell lysis buffer (containing 0.01M $KHCO_3$, 0.155M NH_4Cl and 0.1 mM EDTA diluted in distillated water). The cell populations were labeled with the following monoclonal antibodies: phycoerythrin (PE) anti-F4/80 (BM8), PE-cyanine 7 (PE-Cy7) anti-CD11b (M1/70), Alexa Fluor 700 (A700) anti-MHC Class II (I-A/I-E) (M5/114-15-2) (purchased from eBioscience, USA), A700 anti-CD3 (17A2) (Invitrogen, USA), fluorescein isothiocyanate (FITC) anti-Ly-6G (1A8), Brilliant Violet 510 (BrV510) anti-Ly-6C (HK1.4) (purchased from BioLegend,USA), BrV510 anti-CD19 (1D3), Brilliant Violet 421 (BrV421) anti-

Siglec-F (E50-2440), Pacific Blue V450 anti-Ly-6C (AL-21), peridinin chlorophyll protein (PerCP)-Cyanine 5.5 anti-Ly-6G (1A8), anti-Siglec-F (E50-2440), APC anti-NK1.1 (NKR-P1B/NKR-P1C) (PK136), anti-CD11c (HL3) (purchased from BD Bioscience, Germany). Quantification of cell numbers from the *in vivo* samples was performed by addition of counting beads (Invitrogen, USA). Then, cells were analyzed for size (FSC) and granularity (SSC) and doublets were excluded. Then, the followed strategy was used: CD3, CD19 and NK1.1 were used as lineage markers; neutrophils identified as Ly-6G+CD11b+ cells; eosinophils as siglec-F+CD11b+Ly-6C-/lowF4/80+ SSChigh; inflammatory monocytes as Ly-6ChighCD11c-Ly-6GCD11b+ F4/80+; monocytes and macrophages as Ly-6C+CD11b+MHC-classII+F4/80+ and alveolar macrophages as siglec-F+CD11c+F4/80+ cells. Stained cells were analyzed with a BD FACSCanto™ II employing FACSDiva Software (BD Biosciences, Germany). The data obtained was analyzed using the FlowJo software (Tree Star Inc., USA).

2.5.6 Cytokine and Chemokine Quantification

Sera collected from animals after preparation (see section 3.4) were quantified for cytokine and chemokine levels using beads in a multiplex assay (LEGENDPlex^{TM} multiplex assay, Biolegend, San Diego, CA). A customized panel containing the following cytokines and chemokines capture antibodies was used: MIP-1α, CCL-2 (MCP-1), which belongs to the macrophage activation pathway, CXCL1 (Gro-α), produced by epithelial cells in response to LPS and TNF-α stimulation and a chemoattractant of neutrophils, TNF-α and IFN-γ, which are pro-inflammatory cytokines, IL-1β, an anti-inflammatory cytokine, IL-6 and IL-12, cytokines involved in B cells and T cell activation pathway. The assay was performed following manufacturer instructions and the data was analyzed using the software provided by the manufacturer.

2.5.7 Permeability assay

The permeability of the pulmonary vascular barrier was assessed by quantifying the total amount of proteins present in the alveolar space that increases upon breakdown of the alveolar/vascular barrier. The DCTM Protein Assay (Bio-Rad Laboratories Inc., USA) reagents were mixed with BALF samples or standards, according to the manufacturer's instructions and incubated for 15 minutes

at RT. After reaction, the plate was analyzed at 750 nm using a plate reader SpectraMax M2e (Molecular Devices, USA).

2.5.8 Myeloperoxidase (MPO) activity assay

The level of neutrophil activation and inflammation can be estimated by the activity of the granulocyte enzyme called myeloperoxidase (MPO) produced by these cells. To quantify MPO activity present in BALF samples, a quantification assay was performed based on the incubation of the samples with 0.75 mM H_2O_2 and TMB for five minutes at 37°C for the break of the TMB substrate by the enzyme. The TMB reaction was stopped with 2M H_2SO_4 and the plate was measured with 450nm wavelength using a plate reader SpectraMax M2e (Molecular Devices, USA).

2.5.9 Histopathology

Lungs isolated from infected mice (see section 3.4 for animal preparation and sample collection) were fixed in 4% buffered formalin (pH 7.0) and sent to the Department of Veterinary Pathology at Freie Universität Berlin for histopathology analysis. The lungs were embedded in paraffin, and cut into 2-5 µm thick sections followed by hematoxylin and Eosin (HE) staining. Tissue samples were immersed three times in xylene (Chemsolute Xylol Technisch, Th. Geyer, Germany) for twice two min, and three 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, washed in water for 5 minutes and dyed in eosin (Waldeck, Germany) for 30 seconds. Samples were immersed in increasing ethanol concentrations (70%, 80%, 96%, and 100%) to eliminate the water. Finally, the ethanol was replaced by xylene by four immersions of one minute each in xylene solution. Scanning of HE-stained slides was performed by Aperio CS2 slide Scanner (Leica Biosystems Imaging Ins., CA, USA). Trachea ligation was performed to avoid alveoli collapse and lungs were carefully harvested and fixed in 4% paraformaldehyde solution (pH 7.0). The degree of edema formation was assessed semiquantitatively (0 = no edema, 1 = minimal edema, 2 = mild edema, 3 = moderate edema, 4 = severe edema). Three evenly distributed sections per lung were microscopically evaluated to assess edema formation. Histopathology examination was performed by a European

College of Veterinary Pathologists (ECVP) board-certified pathologist, who were blinded to study groups.

3. Results and Discussion

3.1 Antibodies from CRKP infected patients recognize designed synthetic *O*-antigens

The determination of epitope specificity is very important for the design of carbohydrate-based vaccines since the target antigen will be responsible for the generation of specific antibodies after immune stimulation (186). Human serum from infected patients contains anti-glycan antibody repertoire that reflects the interaction between the host immune system and the main antigens from the pathogen (168). Therefore, human sera from CRKP infected patients were used to screen antibodies against the synthetic glycan epitopes to determine whether the designed trisaccharide and hexasaccharide-*O*-antigens would be potential leads for vaccine development.

To assess the interaction of human serum antibodies with the synthetic O2afg trisaccharide and hexasaccharide glycan epitopes, we analyzed 20 patients infected with CRKP and three samples of healthy individuals (one sample consisted of a pool of more than 287 healthy individuals), using glycan microarrays. The synthetic glycans were immobilized on the surface of a glass slide and a positive glycan control containing the 6-deoxy hexose l-rhamnose (Rha) was used in the assay, because most humans develop antibodies against this pathogen-associated glycan structure (Fig. 16A) (186). The glycan array analysis showed that patients, recovered from a CRKP infection, have developed antibodies recognizing the hexasaccharide-repeating unit (RU), as well as the trisaccharide. Based on these results we concluded that the minimal epitope for antibodies against O2afg strains is Gal-III (Fig. 16B).

Higher molecular weight glycan antigens are known to elicit a better immune response than lower molecular weight fragment due to the mechanism of activation of B cells by glycans in a T-independent manner (142, 147). The B cell receptors (BCR) are able to evoke a down-stream signaling when multivalent binding cross-link between 10 to 20 receptors occurs (146). Therefore, antigens with higher molecular weight are more prone to induce B cell stimulation via BCR and elicit antibody production than lower molecular weight ones. This supports our results where the hexasaccharide epitope was significantly better recognized by human antibodies when compared to the trisaccharide structure. Since the hexasaccharide was the only epitope

exhibiting a significant increase in antibody binding titer when compared to the negative sera control, it was selected for further vaccine development and animal immunization.

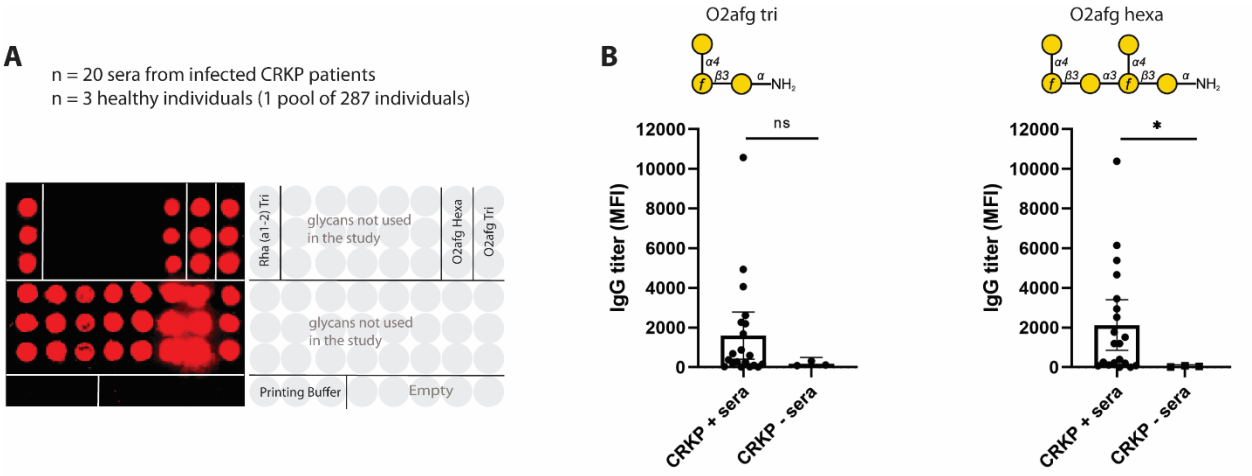

Figure 16. Determination of human antibody binding to synthetic O-antigens derived from O2afg serotype. **A** – Synthetic glycans (O2afg tri and O2afg hexa) were immobilized on a glass slide and the serum from CRKP infected patients and healthy individuals were incubated with the antigens. Antibody binding was determined by using a glycan microarray assay and the fluorescence intensity indicates the number of bound antibodies. **B** – The graphs shows the IgG titer of bound human IgG antibodies to either O2afg trisaccharide (on the left) or the hexasaccharide (on the right). Both antigens showed positive antibody binders, but only the hexasaccharide showed a significant increase in fluorescence intensity, when compared to the negative control group. A trisaccharide containing the 6-deoxy hexose l-rhamnose (Rha) was used as human antibody positive control. The analysis was performed using the unpaired t-test of individual serum mean values from triplicate printed glycan spots. CRKP – carbapenem-resistant K. pneumoniae, MFI – mean fluorescence intensity.

3.2 Coupling of O2afg hexasaccharide to a carrier protein

Although humans infected with CRKP produce antibodies against O2afg glycan structures, the generated immune response is short lived and the antibodies normally exhibit low affinity to the target glycan antigen, because glycan antigens stimulate T-cell independent immune responses, without the formation of germinal centers. As a result, affinity maturation, somatic hypermutations, and memory B cell production are aborted (148). To generate a long-lasting immune response against glycan antigens after vaccination, the O2afg hexasaccharide epitope was conjugated to CRM_{197} as a carrier protein, forming a glycoconjugate. The addition of carrier protein to the glycan antigen enables the presentation of the glycan epitope by B cells via MHC-

II and further stimulation by T cells via TCR-MHC-II engagement (T-cell dependent immune response). The T cells retro stimulate B cells to became long-lasting plasma cells and memory B cells that produce high affinity antibodies against the pathogen for a long period of time or even permanently (150).

The synthetic O2afg hexasaccharide was conjugated with CRM_{197} was achieved by coupling the oligosaccharide to the primary amine side chains of lysine residues and the *N*-terminus of the protein using a *p*-nitrophenyl adipate ester (PNP) as a linker (Fig. 13). CRM_{197} is a non-toxic mutant of the diphtheria toxin containing a single amino acid exchange from glycine to glutamic acid at position 52 (187) and it is the most used as carrier protein in glycoconjugate vaccines, because it is well-characterized and known to induce a robust immune response (188). The protein exhibits 39 lysine residues, but the conjugation reactivity of each individual residue depends on steric accessibility. Other factors such as glycan antigen size and reaction conditions also affect the conjugation reactivity (171). In order to improve the protein to glycan ratio, glycoconjugate reactions were performed at different pH. The optimal conjugation reaction to achieve higher 1:4 ratio using one mg of protein was at pH 8.

After antigen conjugation, the mass of the glycoconjugate and unconjugated protein was measured by MALDI ToF and the average number of attached glycans to CRM_{197} was estimated by the mass subtraction of CRM_{197} and the glycoconjugate, and the resulting value divided by the mass of the glycan antigen containing the linker (Fig. 17A). The glycoconjugate was further characterized by SDS-PAGE to visualize the increase of the mass on an SDS gel when compared to the unconjugated protein (Fig. 17B). The results showed that the O2afg hexasaccharide was successfully conjugated to CRM_{197} and the average loading was 5.2 glycan units per protein monomer (Fig. 17). The loading ratio was in accordance with other glycoconjugates described in the literature, where the average load vary between four to ten glycans per unit of protein (171).

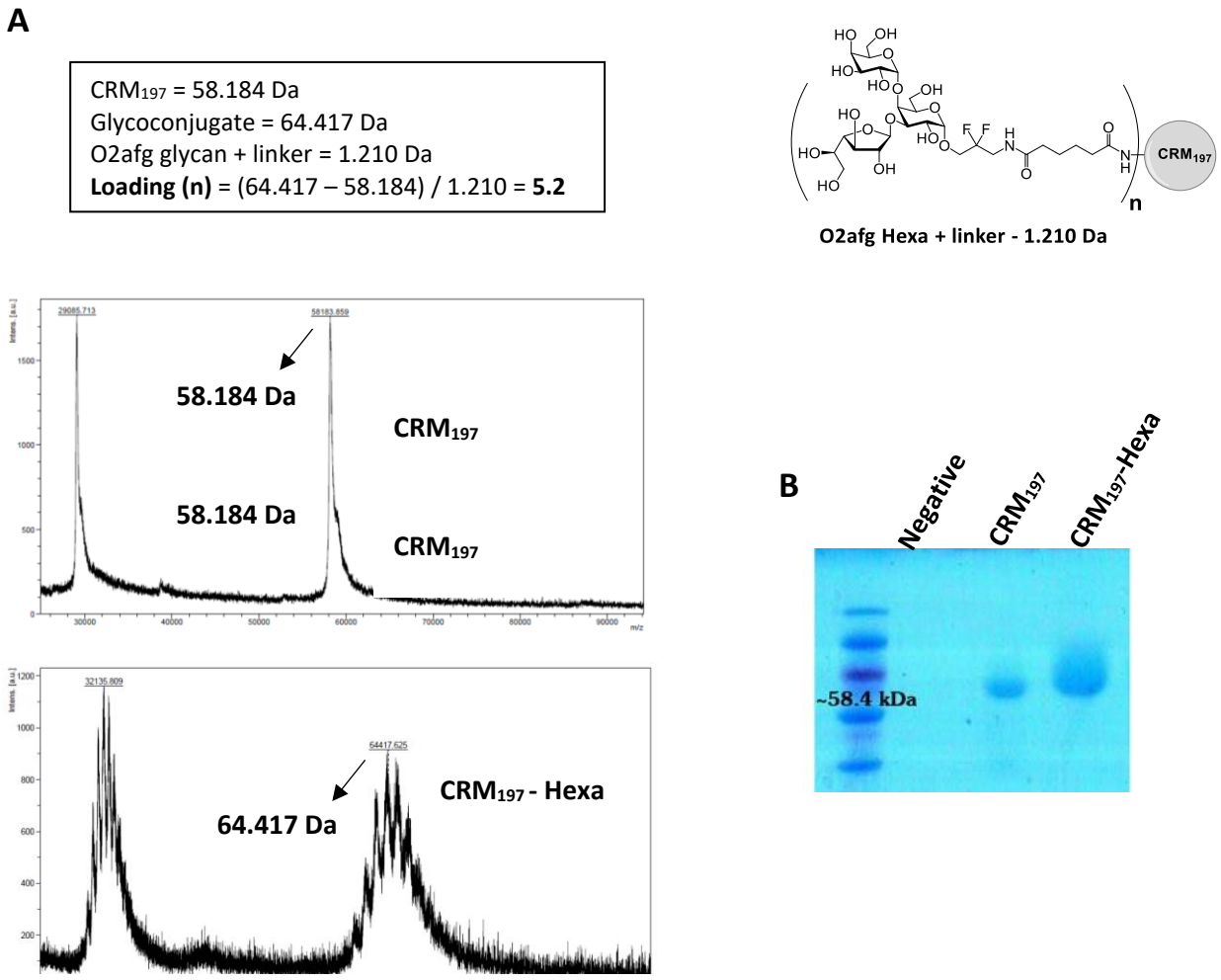

Figure 17. Characterization of O2afg Hexa-CRM_{197} glycoconjugate and the calculation of glycan loading of glycoconjugates. **A** – Calculation to estimate the glycans per protein. MALDi-ToF-MS spectra of CRM_{197} (on the top) and the glycoconjugate CRM_{197}-Hexa (at the bottom). Mass of O2afg hexasaccharide containing the linker. **B** – SDS-PAGE of glycoconjugate and protein alone. The amount of 2 µg of protein or glycoconjugate protein was loaded in each lane and the PageRuler Plus prestained protein ladder was used to estimate the molecular weight of the structures.

3.3 Glycoconjugate vaccine formulation with aluminum adjuvant

When using subunit vaccines, the immune response against the targets is reduced when compared to whole cell vaccines, because PAMPs from the microorganism are not present. The addition of adjuvant in the vaccine formulation helps to overcome the problem of lower immune system stimulation (172). There are several adjuvants approved by regulatory agencies, but alum

is the most commonly used adjuvant in human vaccines (189). The successfully marketed vaccine against pneumococcus, Prevnar13®, contains glycoconjugate antigens that are formulated with aluminum phosphate. Though, aluminum hydroxide has been demonstrated to have a more potent adjuvant effect than aluminum phosphate because of its higher adsorption capacity and better adsorption of antigens at neutral pH (190). Adjuvants also influence on the type of immune response after encountering the antigen. Alum, for example, is known to stimulate type 2 immune response, crucial to fight off extracellular microorganism (191), such as *K. pneumoniae*.

To enhance the immune stimulation of CRM_{197}-Hexa glycoconjugate, the glycoconjugate was formulated with aluminum hydroxide and the adsorption rate was measured after 24 h incubation. The antigen content in synthetic glycoconjugate vaccines varies between 0.1 µg to 2.2 µg of glycan per dose adsorbed into alum (192). To minimize glycoconjugate wsste, the chosen amount of one µg of the glycoconjugate, which lies in between the described range, was chosen. The gylcoconjugate was incubated with 0.125 mg of alum that is the amount of adjuvants present in the Prevnar13® glycoconjugate vaccine. The results showed that one µg of O2afg glycoconjugate was successfully adsorbed onto 125 µg of aluminum hydroxide, with more than 80% adsorption rate when compared to the control (Fig. 18). The high rate achieved in the study is important to ensure a high local concentration at the injection site and enhance the uptake by antigen-presenting cells (193). Alum itself stimulates the immune response at the injection site by enhancing direct or indirect the stimulation of DCs, activation of complement, and generation of inflammasome that results in the release of chemokines (190). The total amount of adjuvant for a single vaccine dose was defined based on the content present in the marketed glycoconjugate vaccine Prevnar13®. Moreover, the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) with the WHO, established a upper limit of 0.85 – 1.25 mg of alum per single vaccine dose for human use (194). Therefore the amount of alum used in our vaccine (0.125 mg) is well below safety limits, established by representative authorities.

Levels of adjuvants used in vaccines are a topic of debate. Especially for the use in children and the elderly adjuvant levels can be critical, due to activation of the immune system with accompanying high levels of inflammation. Thus, our formulation with low levels of alum adjuvant might encourage the use of our vaccines in clinical trials, as well as the approval for

human use, in particular in children and elderlies, which are at the most risk to contract a *K. pneumoniae* infection.

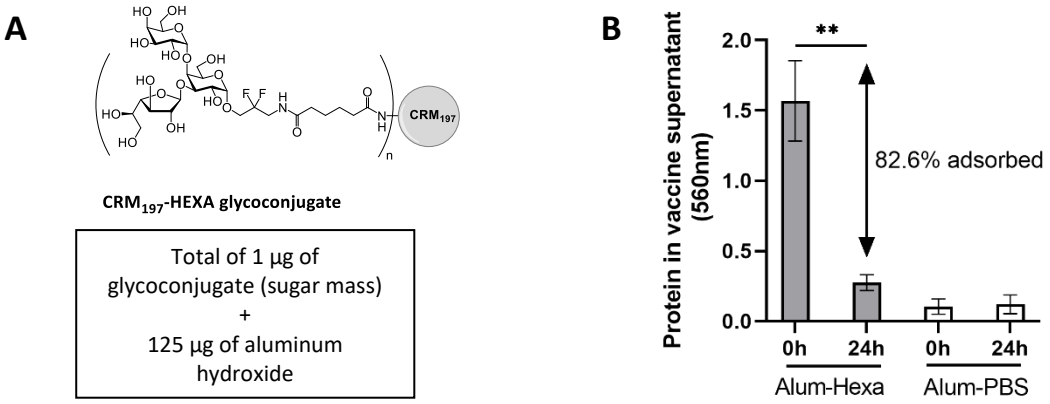

Figure 18. Glycoconjugate vaccine formulation with aluminum hydroxide in a final dose of 0.5 mL in PBS. **A** – Glycoconjugate (total of 1µg based on sugar present in the glycoconjugate) was adsorbed into 0.125 mg of aluminum hydroxide. The solution was rotated for 24 h at 4 °C to allow the adsorption of the antigen with the alum matrix. **B** – Quantification of protein in the supernatant of vaccines after incubation with alum, measured by ELISA. The adsorbtion rate was > 80% when compared to the same vaccine formulation without incubation. A negative control containing alum and PBS was used in the assay. The error bars are the SD of three independent assay performed with three different vaccines. Unpaired t-test was used for statistical analysis.

3.4 The formulated glycoconjugate vaccine evoke the production of anti-O2afg specific antibodies *in vivo*.

Once the O2afg glycan antigen was successfully conjugated to CRM_{197} and formulated into alum, the vaccine was used to establish the antibody response *in vivo*. One crucial point of vaccine development is to define the minimal amount of antigen that will generate a robust immune response (optimal dose). To date, there are no well stablished guidelines that determine the optimal dosage range, when using synthetic oligosaccharide antigens in vaccines. Although, marketed vaccines like Prevnar13® that contains native polysaccharides conjugates to CRM_{197} have been proven to evoke a robust immune response using an amount between 2.2 to 6.6 µg of each isolated capsular polysaccharides per vaccine dose (195). When using synthetic vaccines, the antigens are purer than isolated polysaccharides. Previous immunization studies with synthetic glycoconjugate vaccines usually contained 0.1 µg to 2.2 µg of glycan per dose to

generate a robust immune response in mice and pigs against pneumococcus (192). Therefore, in this study we used one µg of the glycan synthetic antigen, per dose, to immunize rabbits, based on the rational that it lays between the range of synthetic glycoconjugate vaccines that successfully mounted an immune response.

Another very important factor in vaccine development is the choice of animal models. Mice are the most commonly used animal model because it has the advantage of low cost and easier management. Contrary, rabbits are more expensive and demand larger facilities. However, their immune system is evolutionary closer to the human counterpart. Knowing that the rabbit immune system allows for somatic maturation that is recognized to be an important factor for the successful generation of anti-glycan antibodies (196), we decided to choose the rabbit as model system in this study.

The rabbits were divided in two groups: group one consisted of five animals that were immunized intra muscularly (i.m.) with 0.5 mL of glycoconjugate vaccine each (from now on denominated hexa-CRM) and a second group contained three animals that received the same vaccine formulation but with PBS instead of the glycoconjugate (from now on denominated PBS). The control group contained fewer animals to reduce the use of animals in research experiments, since PBS with alum formulations are known to not induce any immune response with specific antibodies. The vaccination regime was based on a well stablished three-dose vaccine schedule (179, 197), with one vaccine dose followed by two boosts with a 14 day interval between each injection. The long-term immune response was measured after a boost injection three and a half months after the last immunization and the analysis of the antibody response was measured after eleven days post-immunization (see section 2.2 Fig. 14). The antibody titer against the synthetic antigen was measured with glycan microarrays.

The results showed that the glycoconjugate vaccine containing one µg of the synthetic antigen (Hexa-CRM) induces a robust immune response in rabbits with increased IgG titer after the first boost (day 21). A significantly higher antibody titer was achieved after the second boost (day 35). After a resting period of three and a half months (day 133), the animals were immunized again with the same vaccine formulation. The concentration of antigen specific IgGs in the serum was rapidly restored, reaching the same level, observed after the second boost (day 35), within elven

days post-immunization (day 144) (Fig. 19 A). This rapid increase in antibody titer indicates the pre-existence of memory B cells that upon re-stimulation with the antigen, can promptly differentiate into plasma cells and secrete large quantities of antibodies (198). The anti-O2afg antibodies bound to both synthetic antigens, confirming that the O2afg trisaccharide is the minimal repeating unit recognized by the immune system (57) (Fig. 19B).

Interestingly, the glycoconjugate vaccine also induced the production of specific IgA that recognized both, the hexasaccharide (Fig. 19 C) and the trisaccharide (Fig. 19D). However, the second boost did not increase significantly the levels of IgA in the animals. Systemic immunizations (intramuscular, subcutaneous) are known to induce systemic humoral and cell-mediated immune response but thought to be incapable to elicit IgA antibodies that are important for mucosal protection. Thus, scientists have been focused on developing vaccine formulations that allow for the delivery of the antigen via mucosal surfaces, such as nasal and mouth route, in order to achieve high IgA titers and consequently mucosal protection. However, this many reports using systemic vaccine administration have shown the induction of IgA antibodies (199), rendering this hypothesis unlikely. Our vaccine candidate supports the evidence that intramuscular vaccination may stimulate the production of IgAs as well, although the levels of generated IgAs were much lower than IgGs (Fig. 19). The IgA antibody class is extremely important to combat pathogens that cause respiratory infection and residents of human microbiota, which is the case of *K. pneumoniae*. A subclass of IgA called secretory IgA (SIgA) highlights the importance of this class because it can transpose the mucosal membrane and access the lumen of different organs, while IgG and IgA alone cannot (200). Unfortunately, the evaluation of the SIgA content was not possible in this study, due to non-availability of commercial secondary antibodies against rabbit SIgA.

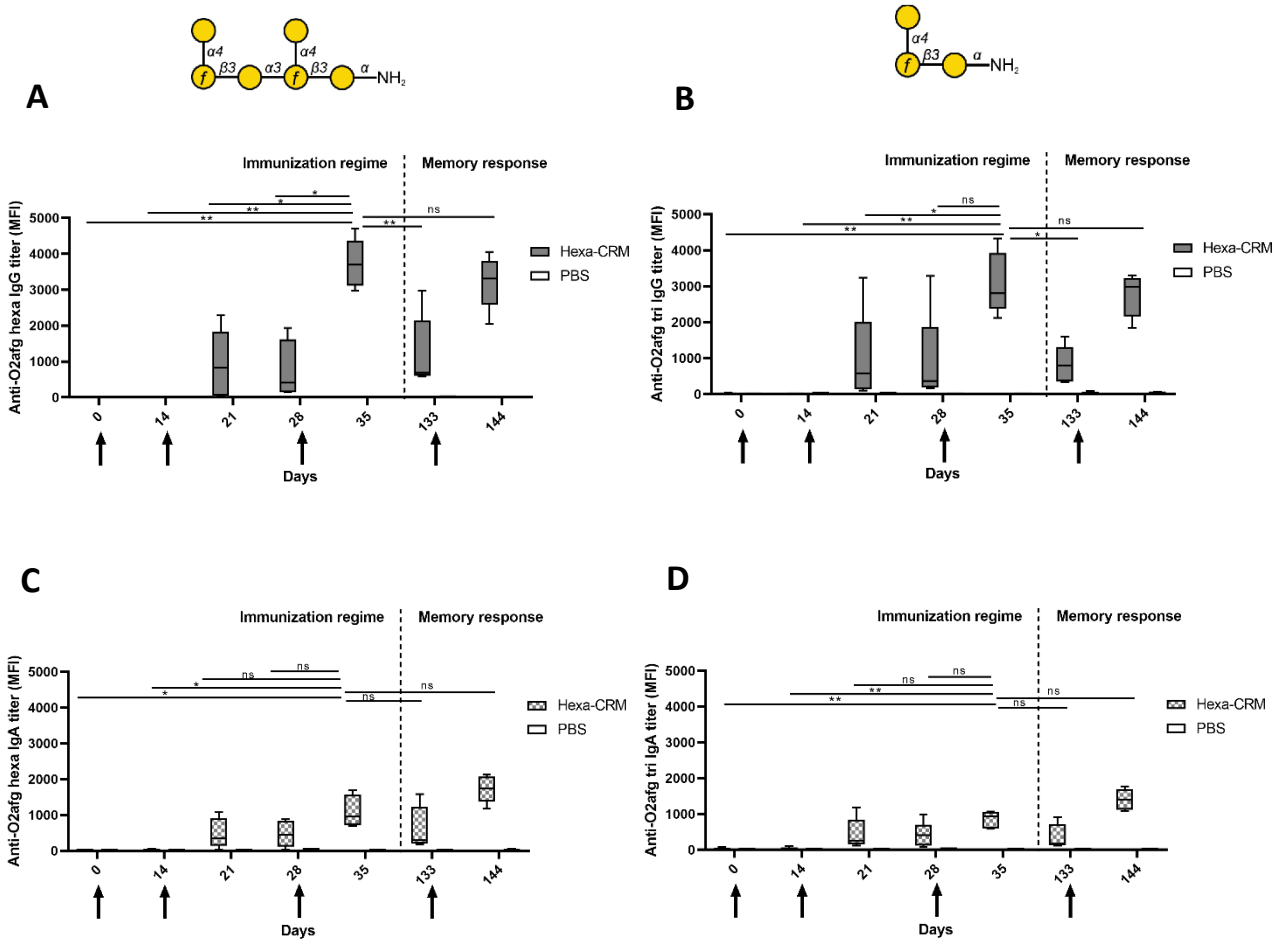

Figure 19. IgG and IgA antibody titer in rabbit sera after vaccine immunization. Rabbits were immunized intramuscularly (i.m.) with either glycoconjugate vaccine containing one µg of the O2afg hexasaccharide synthetic antigen (Hexa-CRM) or PBS within the same vaccine formulation (PBS). The first immunization occurred at day 0 and was followed by two boosts at day 14 and day 28 (black arrows). The memory response was evaluated with a boost at the day 133 (black arrow) followed by antibody response evaluation at day 144. **A** – Levels of IgG specific to O2afg hexa. **B** – Levels of IgG specific against O2afg tri. **C** – Levels of IgA specific to O2afg hexa. **D** – Levels of IgA specific antibody against O2afg tri. The data represents the 95% CI distribution of five animals in the Hexa-CRM group and three animals in the PBS group. Two-way ANOVA was used for statistical analysis. MFI – mean fluorescence intensity measured with glycan microarray. *p < 0.05, ** p < 0.01.

The glycoconjugate is based on the CRM_{197} carrier protein. Therefore, the immune response to the protein was evaluated as well. The production of anti-CRM_{197} IgG antibody was measured by ELISA. In addition to glycan specific antibodies, rabbits immunized with the glycoconjugate vaccine produced IgGs that recognize specifically CRM_{197} (Fig. 20). This is an expected effect, since after antigen processing by B cells and APCs, some carrier protein peptides are presented via

MHC II without the attached glycan (150). The levels of IgG antibodies against the protein increased already on day 14 after only one immunization (Fig. 20), while generation of IgG against the carbohydrate antigen is delayed till the second boost (Fig. 19 A, day 21). This is expected since MHC II receptors evolved to bind and present peptides with high affinity. Thus, the presentation of peptides without loaded glycans occurs significantly more often than peptides with a conjugated glycan. In addition, the amount of protein in the vaccine is higher in comparison to the amount of glycan, increasing the chances of peptidogenic antigen processing and presentation.

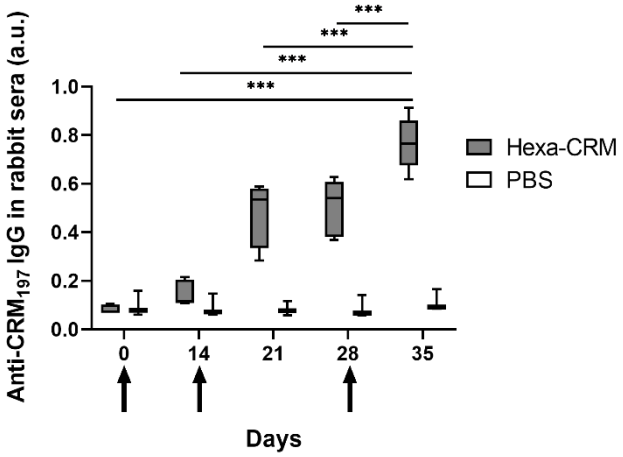

Figure 20. Rabbit anti-CRM_{197} antibody titer after glycoconjugate vaccination. Rabbits were immunized intramuscularly (i.m.) with either glycoconjugate vaccine containing one μg of the O2afg hexasaccharide synthetic antigen (Hexa-CRM) or PBS within the same vaccine formulation (PBS). The first immunization occurred at day 0 and was followed by two boosts at day 14 and day 28 (black arrows). Analysis of sera from rabbits immunized with glycoconjugate showed the production of IgG against the carrier protein CRM_{197} already on day 14. The levels increase after the first and the second boosts. The data represents the 95% CI distribution of five animals in the Hexa-CRM group and three animals in the PBS group. The antibody levels against the protein were analyzed by ELISA. a.u. – arbitrary units. Two-way ANOVA was used for statistical analysis. *** $p < 0.001$.

Native O2afg antigen induces a poor inflammatory immune response, impairing the generation of long-lasting immunity and a robust antibody response (201). With the conjugation of the synthetic antigen to a carrier protein, we successfully induced an immune response with high antibody titers, overcoming the lower immunogenicity of the glycan antigen. In summary, one

microgram of conjugated glycan induced a robust immune response in rabbits with production of specific IgGs and IgAs against the synthetic antigen. Since rabbits have proven to be a better animal model than mice, when translating vaccine response to humans (196), our vaccine candidate is likely to evoke a strong immune response in humans with the advantage of using a lower dose than marketed vaccines. This should reduce the costs of vaccine production significantly and should encourage future clinical trials.

3.5 Rabbit anti-O2afg IgG antibodies, generated after glycoconjugate vaccination, recognize exclusively the native O2afg antigen

The goal of immunization with synthetic glycoconjugates is to trigger the production of antibodies against the synthetic antigen, but more importantly, these antibodies also have to recognize the native antigen on the bacterial surface, to be able to protect the host against an infection, caused by the pathogen. To determine, whether the rabbit anti-O2afg IgG antibodies recognize the native O2afg antigen on the surface of *K. pneumoniae*, pooled polyclonal sera of rabbits, immunized with glycoconjugate from the time point day 35 (last time point after vaccine immunization regime), were incubated with *K. pneumoniae*, expressing the O2afg antigen. Bacteria cells bound by specific antibodies were quantified by flow cytometer using a fluorescent-labeled anti rabbit-IgG secondary antibody. A strain expressing unrelated *O*-antigen (O1) was used as negative control.

The results show that rabbit anti-O2afg IgG antibodies recognize the native antigen on the surface of the bacteria, while PBS control group IgGs do not bind to the bacterial surface. None of the sera contain antibodies that bind to unrelated O1 antigen (Fig. 21A). More importantly, anti-O2afg IgGs from rabbits that received the glycoconjugate vaccine did not cross-react with O1 *K. pneumoniae* serotype (Fig. 21 B), indicating that the vaccine induced exclusively IgG antibodies against the O2afg antigen. Both bacterial serotypes used in the assay shared a D-galactan-I antigen that is composed of → 3)-β-D-Gal*f*-(1 → 3)-α-D-Gal*p*-(1 → glycan units. However, no cross-reactivity with the *K. pneumoniae* O1 serotype was found (Fig. 21B),

highlighting the importance of the branching terminal α-D-Gal*p* as specificity determining element.

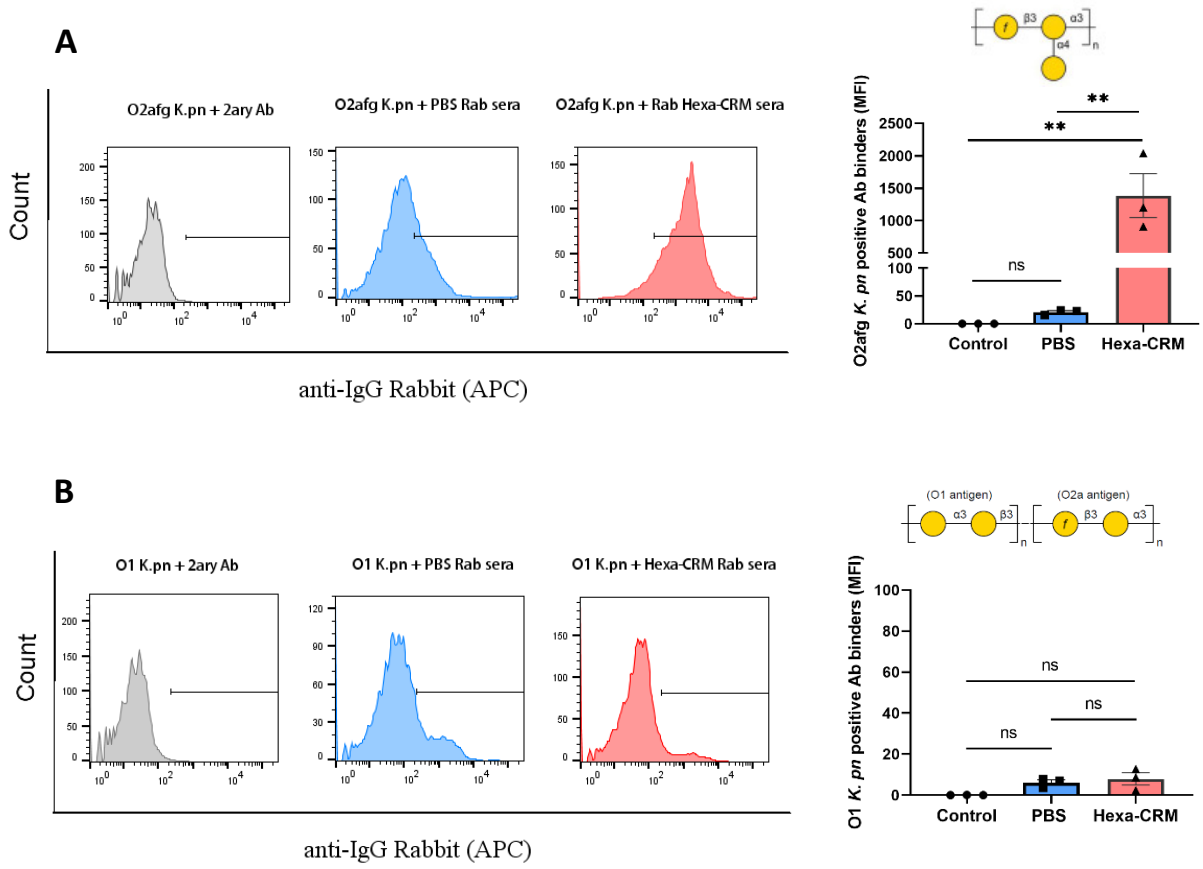

Figure 21. Rabbit anti-O2afg IgG binding assay measured by flow cytometry. *K. pneumoniae* O2afg (**A**) or O1 (**B**) were incubated with sera from day 35 (diluted 1:100) from rabbits immunized with either glycoconjugate (Hexa-CRM group, red) or PBS (PBS group, blue). A negative control containing bacteria with fluorescent secondary antibody (grey) was used as reference to establish the threshold for positive binding. The positive binding quantification shown in the right panel side (MFI) was based on the product of the percent of gated events that passed the fluorescence threshold and the median fluorescence of those events that passed the threshold. MFI – mean fluorescence intensity, K.pn – K. pneumoniae. The error bars represents the SD of three independent experiments. One-way ANOVA was used for statistical analysis. ** $p < 0.01$.

Antibodies that recognize the O2afg antigen are very rare in patients infected with CRKP, even though the incidence of O2afg serotype in CRKP group is higher than 80%. This effect is a result of a weak activation of the immune system by the low molecular weight of O2afg antigen, when compared to other serotypes, consequently a very low frequency of specific B cells against this

antigen is achieved (56). To date, there is no report of antibodies targeting specifically D-galactan-III (O2afg). Although, reported studies have successfully produced anti-O1 and anti-O2 (but not anti-O2afg) monoclonal antibodies (mAb) and polyclonal sera, using animal models infected with the respective *K. pneumoniae* serotypes (56, 202, 203). This is expected since the production of antibody *in vitro* depends on the isolation of specific B cells and the lower immunogenicity associated with lower B cell population frequency against O2afg antigen make the production of antibodies against this target very difficult.

In this thesis, I have reported for the first time the induction of anti-O2afg IgG antibodies that bind specifically to the O2afg antigen. The conjugation of the synthetic antigen to a carrier protein did solve the problem of the lower immunogenicity of the O2afg antigen *in vivo*. The glycoconjugate vaccine induced the production of antibodies against the synthetic structure and the antibodies are also able to recognize the native antigen selective and specifically. The lower molecular weight of O2afg antigens may not be sufficient to activate B cells in a T-independent manner during infection. Contrary, the glycoconjugate containing the synthetic O2afg antigen induced the activation of B cells in a T-dependent manner (155). Antibodies that target specifically CRKP are a key factor, because other groups of *K. pneumoniae* are residents of the human gut as commensal of the gut microbiome. Thus, the generated antibodies targeting specifically CRKP, contribute to the reduction of pathogenic bacterial strains, while not targeting the commensal strains in the gut.

3.6 O2afg glycoconjugate vaccine induces the production of anti-O2afg antibodies with opsonophagocytic killing activity

Although inducing specific antibodies after vaccine immunization is a crucial factor, the production of high antibodies titers does not guarantee a protection against infections. Opsonophagocytic killing activity (OPKA) is a parameter that establishes, if vaccine-induced antibodies are able to kill targeted microorganism, by enhancing complement protein deposition and subsequent opsonophagocytic killing by effector immune cells (204). This method has been widely used to evaluate the efficacy of vaccine candidates and estimates the potential killing

activity of antibodies from sera of vaccinated animals or humans (184). Serum dilution titers correlate well with the potency of vaccines to generate protection (205).

To evaluate if the antibodies produced after O2afg glycoconjugate immunizations have opsonophagocytic killing activity, pooled sera of immunized rabbits from the last time point after completion of the full immunization schedule (day 35) was incubated with O2afg *K. pneumoniae* in the presence of baby rabbit complement and differentiated HL-60 effector cells. Rabbit serum was pre-heated at 56 °C to inactivate the complement proteins naturally present in the serum samples without affecting the antibody proteins. Effector HL-60 cells were incubated with *N,N*-dimethylformamide (DMF), which is known to induce granulocytic differentiation with neutrophil-like cell characteristics that exert efficient phagocytosis in the presence of complement and bound antibodies onto bacteria (206). Whereas many protocols to evaluate OPKA activity exists for other strains, for example Pneumococci, no established protocols exist for *K. pneumoniae*. Guided by OPKA protocols developed for other bacteria, I have successfully established the OPKA using 1:400 multiplicity of infection (MOI), which defines the ratio between bacteria and effector cells, and only 2.5% baby rabbit complement (56, 183, 205). The reduced baby rabbit complement content compared to previous protocols with up to 10 % (v/v), indicates that O2afg strains are more susceptible to complement killing than other *O*-antigen serotypes and gram-positive bacteria. For the serum killing activity, a well-stablished protocol was followed (184) and the opsonic index, where 50% of bacteria are killed, was defined by a four-parameter linear regression extracted from four-fold serum dilution curves. Pooled sera from rabbits immunized with the PBS formulation vaccine was used as negative control and as a baseline reference.

The results show that the synthetic glycoconjugate vaccine elicit opsonic antibodies that kill O2afg *K. pneumoniae* (Fig. 22A-B). Antibodies from the group that received the glycoconjugate vaccine (Hexa-CRM) had an opsonic index of 1626.5 while the PBS group had a value of 4.5 (Fig. 22C). This represents a 99.6% significant increase in killing activity, confirming that the glycoconjugate induces the production of opsonic antibodies. Since *K. pneumoniae* are known to express thick capsular polysaccharides that can affect the interaction of antibodies with *O*-antigens, the effect of the capsule in the antibody binding interaction was evaluated using the

same serum and OPKA conditions with a mutant acapsular strain (*K. pneumoniae* K-). Glycoconjugate-induced antibodies were also able to kill the acapsular strain (Fig. 22D-E) with a 21 times higher opsonic index (Fig. 22F) when compared to the capsular strain (*K. pneumoniae* K+) (Fig. 22C). However, the absence of capsule in the bacteria have also increased the susceptibility to sera killing without the presence of specific antibodies, since the opsonic index in the PBS group increase 230-fold. Thus, the 99% increase in killing activity by antibodies induced with our glycoconjugate vaccine is remarkable, taking the shielding effect through the CPS into account. The excellent OPK activity, together with the advantage of lower antigen variability compared to CPS (32), supports the use of the O2afg antigen for vaccine development. This finding is even more encouraging if one compares the killing activity observed for anti-Pneumococci antibodies (184). Recently, a broad-spectrum glycoconjugate vaccine with four native O-antigens from *K. pneumoniae* conjugated to *P. aeruginosa* flagellin was reported (141). The vaccine elicited antibodies that induced opsonophagocytic uptake (OPA) and correlated with protection against *K. pneumoniae* infection. However, the study did not establish an OPKA or opsonic index parameters. A glycoconjugate vaccine using a synthetic CPS antigen against CRKP coupled to CRM_{197} was developed as well. The vaccine enhanced the production of antibodies with OPK activity with an opsonic index between 134.2, and 153.6 against capsulated strains (136). In comparison, the O2afg glycoconjugate vaccine induced antibodies with 90% more killing activity than the synthetic glycoconjugate vaccine targeting CPS, despite reduced antigen accessibility. Furthermore, native antibodies against O-antigens from patients infected with *K. pneumoniae* do not promote phagocytosis of the bacteria (118). Thus, glycoconjugate vaccines specifically induce antibodies with OPK activity.

Here, we have confirmed that our O2afg glycoconjugate vaccine induces the production of anti-O2afg antibodies harboring opsonophagocytic killing activity. The serum dilution necessary for 50% killing activity was above the pneumococcus marketed vaccine and other glycoconjugate vaccines targeting *K. pneumoniae*, even though the O2afg target antigen is thought to be partially shielded by CPS. The marketed Pneumococcus glycoconjugate vaccine has been successfully used in humans to prevent infections, despite that the induced antibodies are known to have a lower

opsonic index, compared to the designed O2afg glycoconjugate vaccine. Thus, our glycoconjugate vaccine candidatechas a great potential to fight CRKP infections.

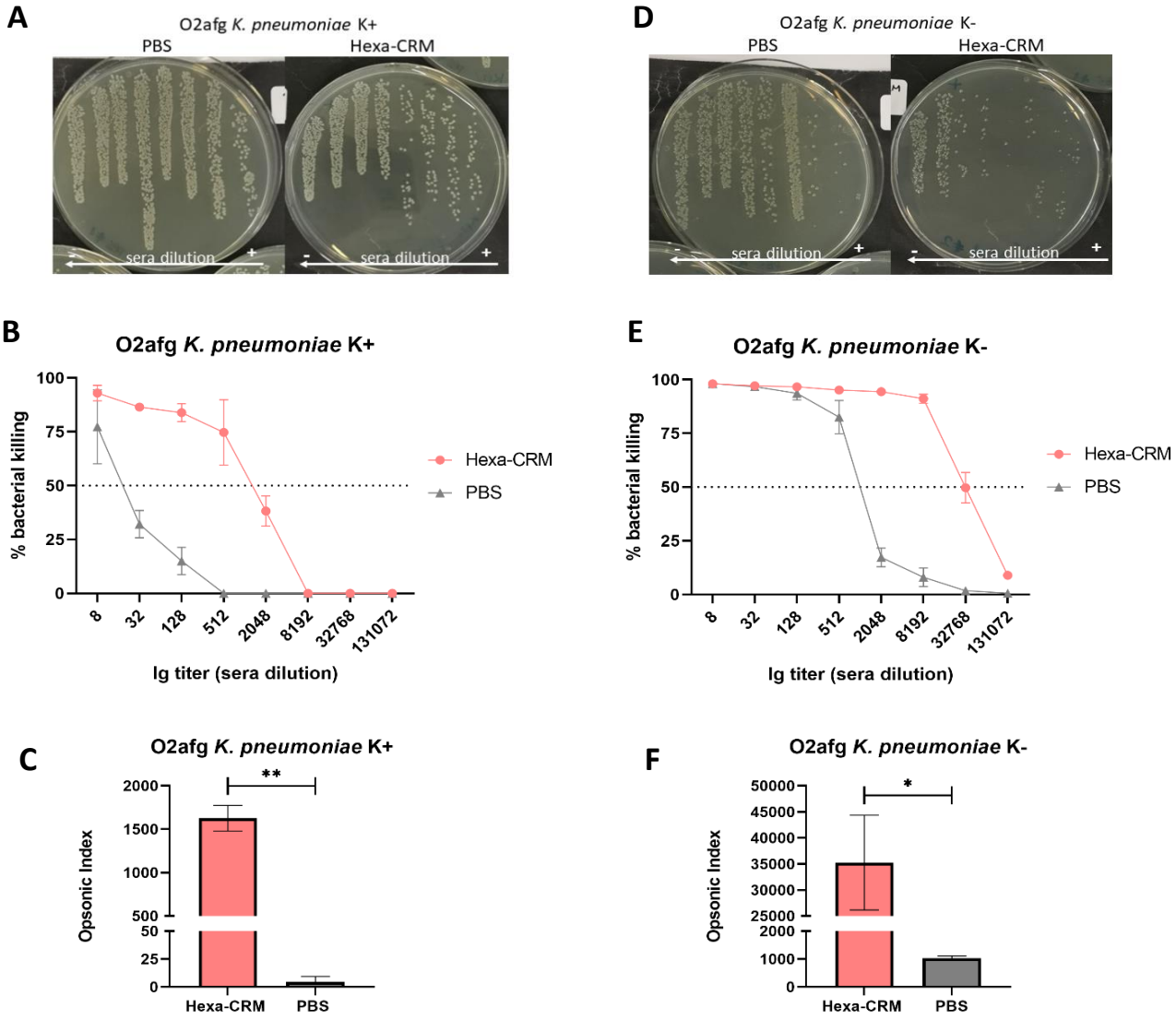

Figure 22. Opsonophagocytic killing activity of antibodies present in the sera of rabbits. The killing activity of antibodies present in the sera of rabbits vaccinated with the O2afg glycoconjugate (Hexa-CRM) and with PBS (PBS) was evaluated with *K. pneumoniae* expressing a capsule (**A**-**B**) and a mutant strain without capsule (**D**-**E**). The opsonic index represents the sera dilution for 50% of bacteria killing (**C** and **F**) and was based on a four-parameter linear regression of sera dilution curves from two independent assays done in duplicate. The percentage bacterial killing data (**B** and **E**) are mean ± SD of CFU reduction relative to negative control (sample lacking sera with complement and effector cells) of two independent assays done in duplicate. Unpaired t-test was used in the analysis. *p<0.05, **p<0.01.

3.7 Anti-O2afg antibodies enhance complement deposition on the cell surface of CRKP

One mechanism of *K. pneumoniae* to evade the host defense is to avoid complement activation and therefore reduce the recognition of the bacteria by immune cells. Adaptations like thick polysaccharide capsules and long O-antigen polysaccharide chains are correlated with lower amounts of C3b and MAC complement protein deposition on *K. pneumoniae* surfaces (28). High levels of C3b on the bacterial surface is a signal for phagocytic cells to promote uptake and kill the pathogen (88). To initiate C3b deposition, the complement system has to be activated. In the classical pathway the complement system is activated, once antibodies bind to the surface of bacteria (38). For example, high affinity IgG and IgM antibodies against CPS are correlated to increase C3b deposition on the surface of *K. pneumoniae* (31, 124, 207).

To evaluate the effect of anti-O2afg antibodies on C3b deposition on the cell surface of *K. pneumoniae*, pooled sera from rabbits immunized with the glycoconjugate or PBS vaccine from the last day after immunization schedule (days 35) were incubated with the bacteria in the presence of normal human sera (NHS) as a source of complement proteins. Human sera with inactivated complement (HIS) were used as negative control. Pooled rabbit sera were heated at 56°C for complement inactivation prior to use. After incubation, the presence of bound complement protein to the surface of the bacteria was detected with a fluorescent-labeled anti-C3c antibody, since it is part of C3-, C3b- and iC3b-complexes in humans (208). Therefore, the increase in C3b is correlated to an increase in C3c on the bacterial surface and are used as synonyms in this result section. Bacteria decorated with C3b have been subsequently quantified with flow cytometry. The results show that the glycoconjugate vaccine induced anti-O2afg antibodies increase complement C3b deposition on the surface of CRKP significantly in comparison to the PBS group (Fig. 23). Activation of the complement is very important for innate immune cell recognition. The increase in C3b deposition mediated by anti-O2afg antibodies reinforces the increase in opsonophagocytic killing by phagocyte cells in the Hexa-CRM group, because the opsonophagocytosis also depends on complement-bound proteins as signal for bacteria uptake and processing by phagocytes. Thus, I showed that anti-O2afg-antibodies mediated the increase in C3c protein deposition, and correlates to increased OPK activity. To date, there is no report of specific antibodies or polyclonal sera against O2afg antigen. However,

anti-CPS antibodies from mice targeting CRKP have demonstrated to enhance C3b deposition. Interestingly, it was observed that, depending on the IgG subclass, the complement activation can vary (207). In rabbits, only one IgG class is produced, rendering a subclass analysis unnecessary for this study. However, if mice or human vaccination trials are conducted it might be worthwhile to determine complement activation as function of the IgG-subclass while mice and humans have five and four IgG subclasses, respectively.

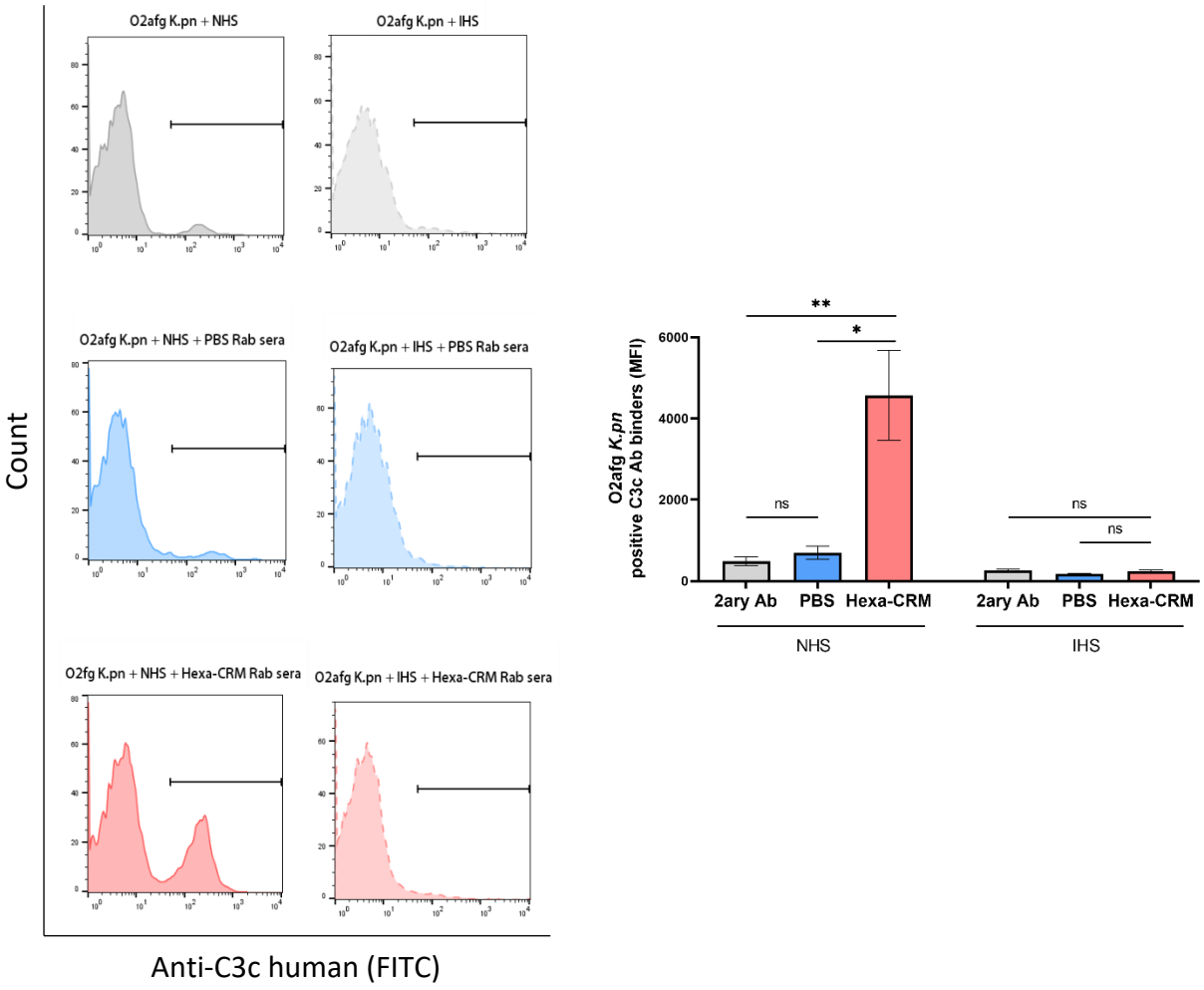

Figure 23. Complement C3c deposition on the surface of O2afg *K. pneumoniae* carbapenemase resistant strains. Sera from rabbits immunized with glycoconjugate (Hexa-CRM, red) or PBS (PBS, blue) vaccine, from day 35, were incubated with the bacteria in the presence of normal human sera (NHS) or inactivated human sera (HIS), as complement source. Positive binders were quantified by flow cytometer (left panel side) with fluorescence secondary antibody against C3c. Positive threshold was defined based on the bacteria incubated with secondary antibody without rabbit sera (grey). Mean fluorescence intensity (MFI) values ± SD, showed on the right panel side, are represented by the product of median intensity fluorescence and the percentage of cells that crossed the positive threshold of three independent experiments. Two-way ANOVA was used in the analysis. *p<0.05, **p<0.01.

Additionally to phagocytosis, the activation of C3 complement proteins can lead to a downstream activation pathway that results in MAC formation causing cell-lysis of gram-negative bacteria. The MAC is formed by a complex of different complement proteins, after C3 has been activated, and the C5b-9 a protein is present in the complex (88). In order to evaluate, whether anti-O2afg antibodies induce MAC formation in CRKP, the bacteria were incubated with pooled sera from rabbits immunized with the glycoconjugate or PBS vaccine from the last day after immunization schedule (days 35) in the presence of normal human sera (NHS) as a source of complement proteins. Human serum with inactivated complement (HIS) was used as negative control. Pooled rabbit sera were heated to 56°C for 30 minutes to inactivate the rabbit complement. After incubation, the presence of bound C5b-9 was detected with a fluorescent-labeled anti-C5b-9 antibody and the positive binders were quantified by flow cytometry. As expected, anti-O2afg antibodies do not induce MAC formation on the surface of CRKP (Fig. 24). CRKP are usually resistant to MAC formation, because the thick capsules and long O-antigen chains interfere with the complement C5b-9 protein complex attachment to the bacterial surface (91). Thus, the anti-O2afg antibodies can induce C3 complement activation, but cannot overcome the resistance of CRKP to the MAC complex. The main complement-mediated killing mechanism induced by anti-O2afg antibodies relies on OPK activity due to C3b deposition and not on complement-mediated lysis.

Anti-CPS antibodies targeting CRKP have been reported to induce MAC deposition on the bacteria surface. This effect varies between strains, depending on the specific CPS produced by the strain (31, 124, 207). Although studies showed that antibodies against CPS can enhance MAC deposition, the main reason for effective bacterial clearance is opsonophagocytic killing by phagocytotic cells or NETosis via neutrophil recognition through complement receptors after C3b deposition (31, 124, 207). Thus, anti-O2afg antibodies, produced after glycoconjugate vaccination with our O2afg vaccine, mediate a similar killing mechanism compared to anti-CPS antibodies when targeting CRKP. This strengthens the idea that the use of *O*-antigen as vaccine target against CRKP as viable alternative to CPS.

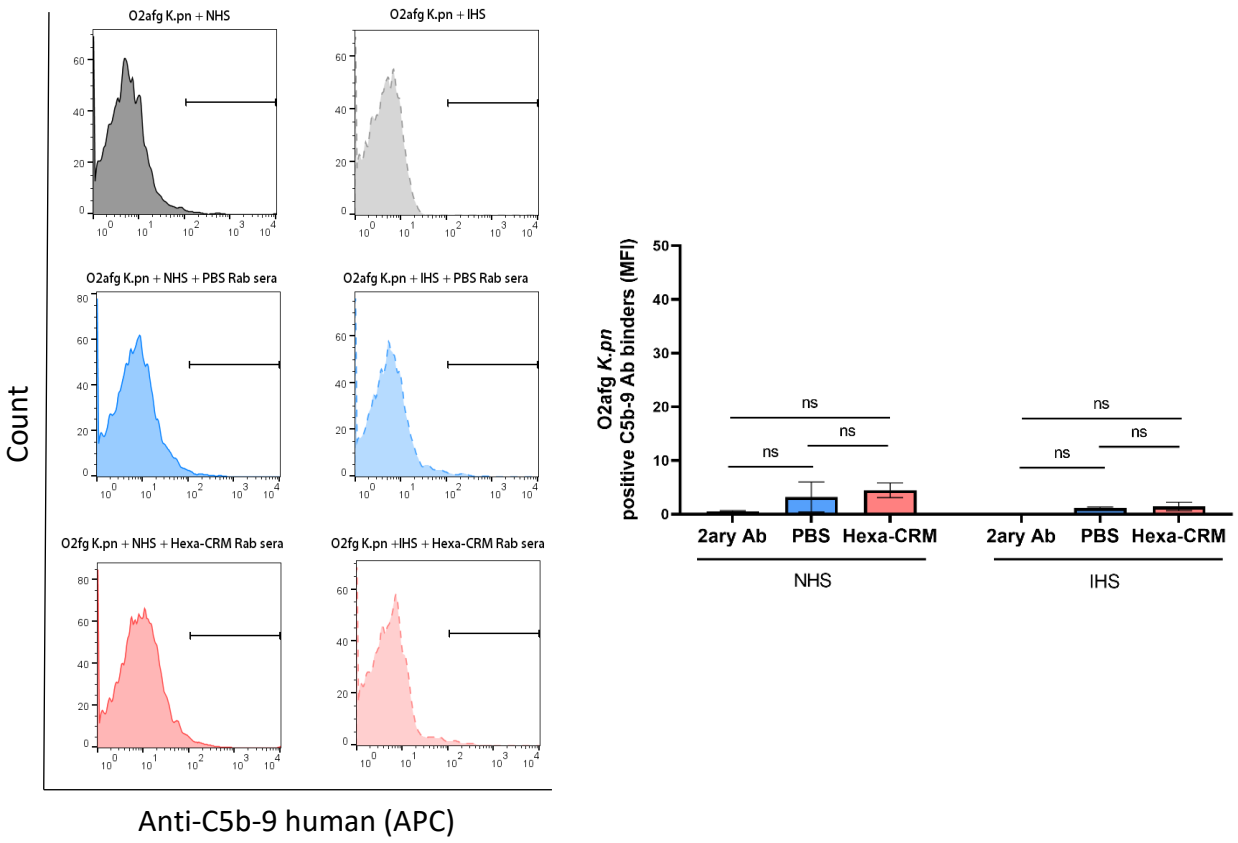

Figure 24. Complement C5b-9c deposition on the surface of O2afg *K. pneumoniae* carbapenemase resistant bacteria. Sera from rabbits immunized with glycoconjugate (Hexa-CRM, red) or PBS (PBS, blue) vaccine, from day 35, were incubated with the bacteria in the presence of normal human sera (NHS) or inactivated human sera (HIS), as complement source. Positive binders were quantified by flow cytometry (left panel side) with fluorescent labelled secondary antibody against C5b-9. The positive threshold was defined based on the bacteria incubated with secondary antibody without rabbit sera (grey). Mean fluorescence intensity (MFI) values ± SD, shown on the right panel side, are represented by the product of median intensity fluorescence and the percentage of cells that crossed the positive threshold of three independent experiments. Two-way ANOVA was used in the analysis. ns: non-significant

3.8 Establishment of parameters for passive immunization in mice in acute pneumonia infection model induced by CRKP

Different research groups have used polyclonal sera or purified monoclonal antibodies raised after *K. pneumoniae* infection for *in vivo* studies, either in a preventive (pre-infection) or therapeutically (post-infection) manner (127). Passive immunization is very important for

patients that encounter the pathogen and have insufficient time to mount an immune response, such as elderlies and children that are the main groups at risk for *K. pneumoniae* infection.

In vitro killing activity enhanced by antibodies often does not translate into *in vivo* activity. Because of this, the effect of anti-O2afg antibodies as a treatment for acute pneumonia induced by CRKP was evaluated in a mouse model. Parameters such as immune cell stimulation and bacterial clearance *in vivo* were determined after passive immunization of the mice with pooled polyclonal sera of vaccinated rabbits (complement depleted) post-infection with CRKP. Antibodies administered therapeutically (post-infection) represents the reality of the majority of hospitalized patients, who develop pneumonia after primary infections present in other body sites or after invasive procedures like surgeries (209, 210). The average half-life of IgG antibodies in sera is around 30 days (211), such that constant antibody infusions would be needed in order to be effective in a preventive (pre-infection) approach, thereby increasing treatment cost and management of patients at risk.

For the success of passive immunization, administered antibodies must reach the bloodstream of patients and subsequently all parts of the body to fight systemic *K. pneumoniae* infections. Thus, the optimal route of administration and dosage has to be established. Intraperitoneal (i.p.) administration of antibodies has been proven to cause a rapid transfer of antibodies to the bloodstream with the advantage of local activity in the peritoneal area, where *K. pneumoniae* usually resides in humans (212). In this study, rabbit polyclonal sera, containing specific antibodies were administered i.p. in uninfected mice. To establish the optimal dose, four groups with three mice each received either 1, 10 or 100 µL of pooled sera from day 35 of rabbits vaccinated with the glycoconjugate (Hexa-CRM) vaccine, or 100 µL of PBS as a negative control group. A blood sample was collected after 48h that is the time schedule for the designed acute pneumonia model. The presence of rabbit anti-O2afg antibodies in mice sera was detected using glycan microarrays. The results show that the dose of 100 µL of rabbit sera administered i.p. in mice was the only dose that led to detectable levels of anti-O2afg antibodies in the bloodstream after 48h (Fig. 25).

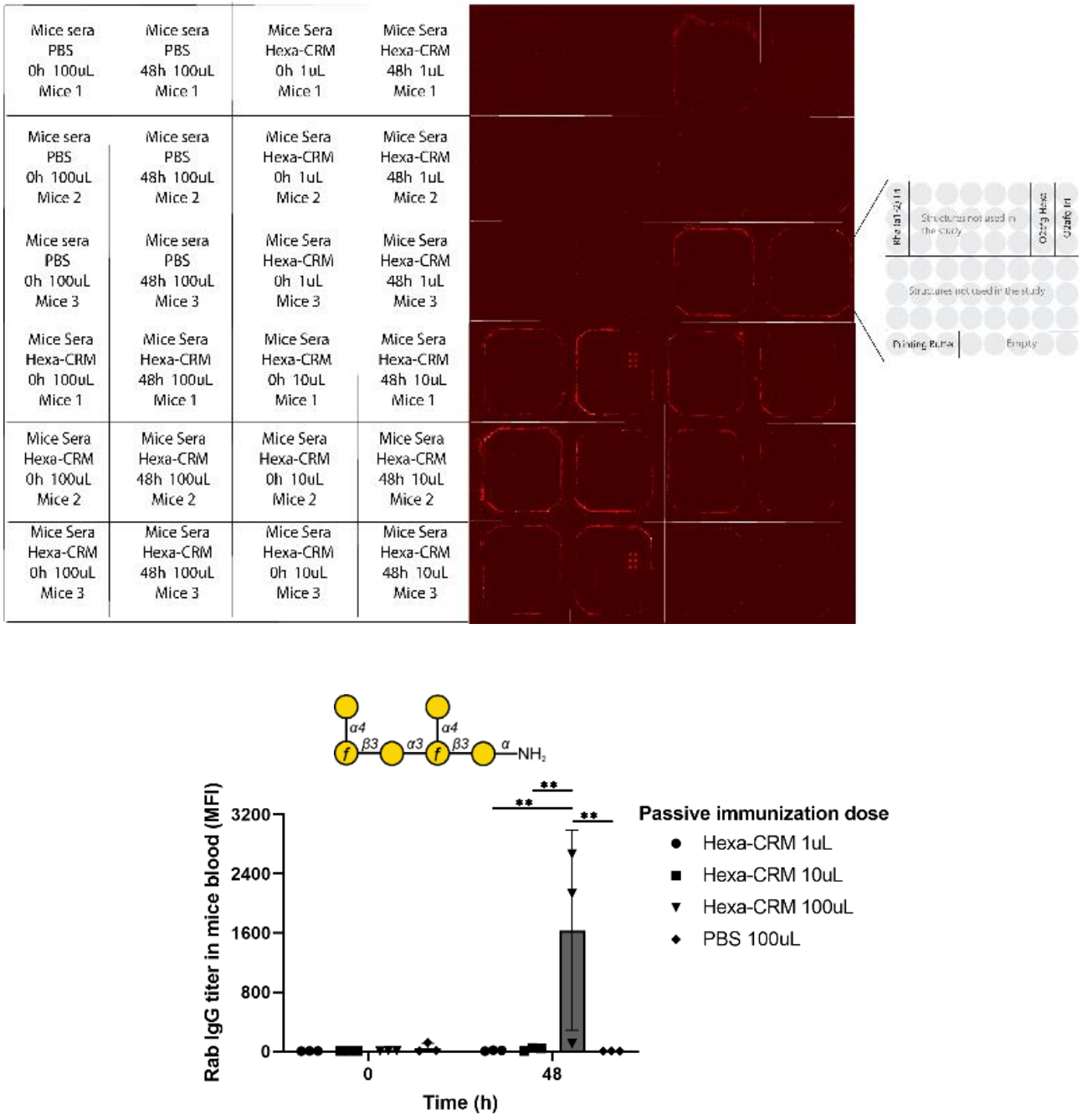

Figure 25. Anti-O2afg IgG rabbit antibodies present in mice sera 48h post passive immunization. Four groups with three mice each received either 1, 10 or 100 µL of pooled sera from day 35 of rabbits vaccinated with the glycoconjugate (Hexa-CRM) vaccine, or 100 µL of PBS as a negative control group. Antibody titer in mice sera were evaluated by glycan array (left panel) and the mean fluorescence intensity of bound antibodies to O2afg synthetic hexasaccharide from different groups were compared (graph on the right). The sera at time point 0 h were collected as control of pre-infection. Two-way ANOVA was used in the analysis. The data represents the mean of MFI values from triplicated printed spots of three mice per group ± 95% CI. **p < 0.01.

Bacteria strain, dosage, and administration route are other critical factors in a murine pneumonia model, since mice are less likely to develop sepsis or die from pneumonia in comparison to humans under the same dose regime (213, 214). The pneumology department at Charité – Universitätsmedizin Berlin has recently established a murine acute pneumoniae model using a CRKP non-lethal dosage of 1 x 10^8 CFU administered intranasally (data not published). Under these conditions, mice become ill but do not die 48h post-infection. The CRKP strain from the established model encodes carbapenemase genes and is an isolate from a patient of the clinic. Although the antimicrobial resistance profile of the bacteria was established, no information regarding the O-antigen serotype was available. Therefore, I have serotyped the CRKP strain by PCR, in order to determine the serotype and consequently its suitability as a model for this study. The CRKP strain used in the established model, encodes the gene responsible for O2afg serotype expression (Fig. 26) that allows for the usage of the murine mouse model in my study.

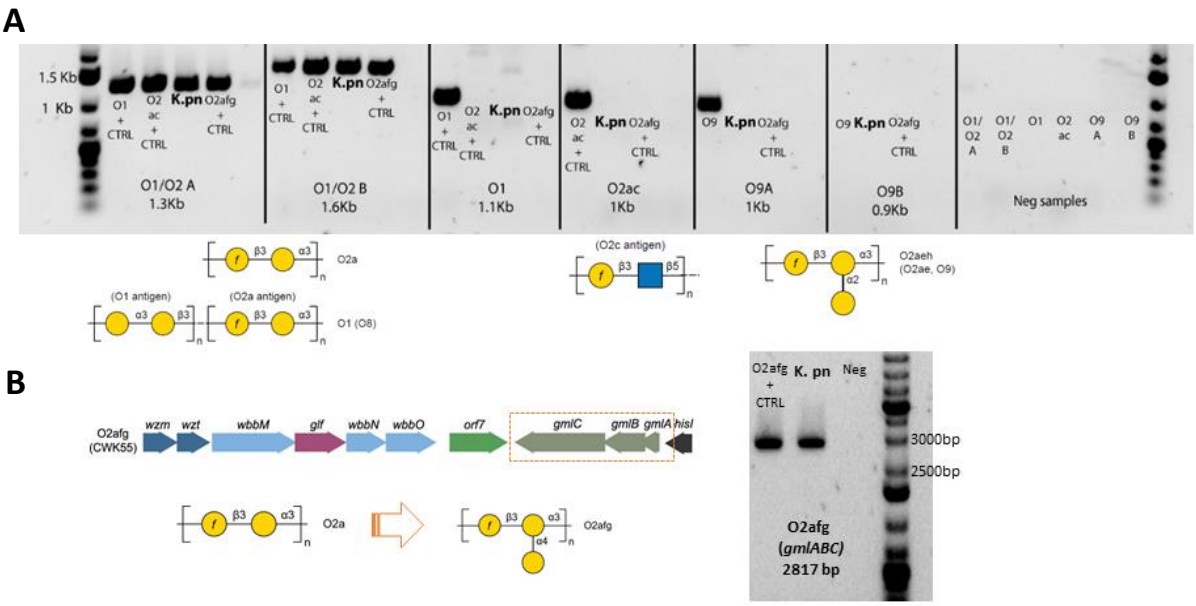

Figure 26. O-antigen serotyping of a CRKP strain (**K.pn**) from established murine pneumonia model. Primers sets amplifying O1/O2, O2ac, O9 (O2aeh) were used in order to identify if the strain (**K.pn**) belongs to the large group of O2 serotype but not to O2c or O2aeh (**A**). Amplification of the *gmlABC* operon responsible for addition of branching terminal α-D-Galp to the O2a serotype, and expression of O2afg serotype (**B**). The 1kb plus ladder was used as reference for DNA size. Samples were run at 1% agarose gel and stained with Sybr Safe DNA stain. Positive bacteria samples were used as reference for each prime set.

The following study design was used to evaluate the antibodies against the murine mouse model. Three groups with 12 mice each were divided as follows: 1- mice infected with PBS and passively immunized with Hexa-CRM rabbit sera, to verify the burden of inter-species antibodies in mice, 2- mice infected with CRKP and passively immunized with PBS rabbit sera (negative group), 3- mice infected with CRKP and passively immunized with Hexa-CRM rabbit sera (positive group). Body weight and temperature of the mice was monitored every 12h post-infection for 48h. Two days post infection, mice were sacrificed and the lungs were collected to evaluate the bacterial load. Lungs of five additional mice per group were used for histological evaluation, based on tissue damage, edema and cell infiltration quantification. Bronchoalveolar lavage fluid (BALF) was collected to measure the level of infiltrated proteins as an indication inflammation and subsequent lung tissue damage. Furthermore, BALF was used to quantify immune cells, enzymatic activity, and cytokines 48 hours post-infection. Finally, blood of mice was collected pre- and 48h post-infection in order to extract the serum and estimate the level of circulating IgG and IgA antibodies against O2afg after 48h, using glycan microarray analysis. Blood was used to count circulating blood cells by hemogram, and to measure the level of cytokines were quantified by FACS, as an indication of immune cells activity (section 2.5.4 Fig. 15).

3.9 Anti-O2afg rabbit IgG and IgA were present in the blood and BALF of infected mice

The presence of rabbit anti-O2afg IgG and IgA in blood and BALF of mice after 48h was confirmed by glycan microarray and the binding against the synthetic hexasaccharide structure was quantified between the groups. The data show that the Hexa-CRM polyclonal sera administered into infected mice (Kpn + CRM-Hexa) has higher levels of anti-O2afg circulating IgG than the uninfected mice (PBS + CRM-Hexa) (Fig. 27A). A higher and demanding recruitment of antibodies in the blood after bacterial infection may explain the higher levels of IgG in the blood of the infected group. The opposite occurred with IgA antibodies in the blood, with higher titers in the uninfected group and lower levels in the CRKP infected group (Fig. 27B). Lower levels of IgA in the blood of the infected group are compensated by an increase of IgA levels in the BALF (Fig. 27D). IgA is a mucosal antibody and might be redirected to the site of infection (Lung/BALF) lowering the levels of circulating IgA in the blood of this group. This is not observed for IgG, which

remains higher in the blood, compared to the titer in the BALF (Fig. 27C). This is in agreement with the fact that IgG do not cross the mucosal barrier such as IgA and the presence of IgG in the BALF may be a result of tissue damage caused by lung infection. Overall, the presence of antibodies in blood and BALF of mice confirm that i.p. immunizations lead to a rapid transfer of antibodies into the bloodstream and they remain in the circulation after 48 hours. Mice that received sera from rabbits immunized with the PBS control do not have any anti-O2afg specific IgG and IgA antibodies (Fig. 27).

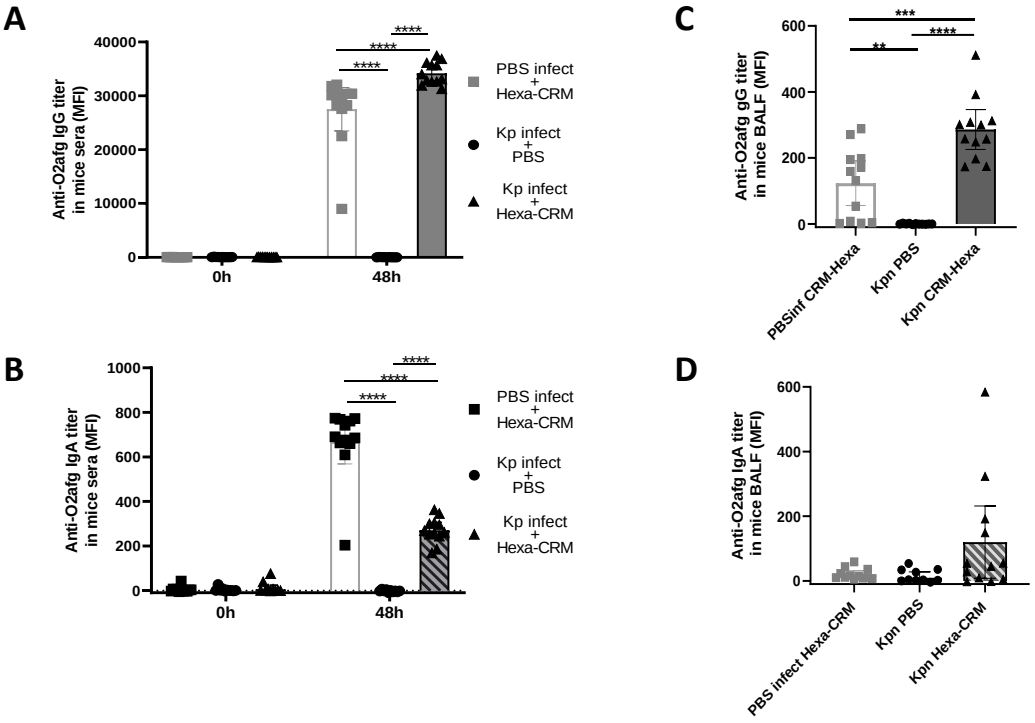

Figure 27. Anti-O2afg rabbit antibodies present in the sera and BALF of mice after intraperitoneal passive immunization. Mice were infected with CRKP (Kp infect) or PBS (PBS infect) and received either sera from rabbits vaccinated with the glycoconjugate vaccine (Hexa-CRM) of from rabbits vaccinated with PBS vaccine (PBS). Sera titers were obtained from the mean fluorescence intensity (MFI) of bound antibodies against the synthetic hexasaccharide antigen, measured by glycan microarray. **A** – Anti-O2afg rabbit IgG titer in blood of mice. **B** – Anti-O2afg rabbit IgA titer in blood of mice. **C** - Anti-O2afg rabbit IgG titer in BALF of mice. **D** - Anti-O2afg rabbit IgA titer in BALF of mice. The data represents the mean ± SD of 12 mice per group. Two-way ANOVA was used for the statistical analysis. **p < 0.01, *** p < 0.001, *** p < 0.0001. Kp – *K. pneumoniae*.

3.10 Anti-O2afg antibodies passively transferred into mice reduce burden in acute pneumonia model

In a first step, I evaluated whether the transfer of foreign antibodies might affect mice negatively. Changes in body temperature are a good marker to monitor any reaction of the mouse upon injection of the serum. The administration of rabbit antibodies into non-infected mice did not affect the body temperature, indicating that receiving foreign antibodies from different species does not cause a burden for the animal (Fig. 28A). This result encourages inter-species passive immunization, with rabbits and horses being great producers of polyclonal sera against infectious diseases (214).

Upon intranasal infection with CRKP, mice treated with anti-O2afg antibodies (Hexa-CRM) had significant higher body temperature twelve hours post-infection when compared to the infected group that received the mock (PBS). Hypothermia is a characteristic symptom of a prolonged severe infection, with poor prognosis of survival (215) Thus the improvement in body temperature caused by anti-O2afg antibodies should increase the chances of survival after CRKP infection. Independent of the treatment received, infected mice reached normal levels (36.5 – 38 °C) after 24 hours (Fig 28A). This is expected, because mice have better chances to fight gram-negative bacterial infections than humans, due to lower sensitivity to the endotoxin from LPS (216).

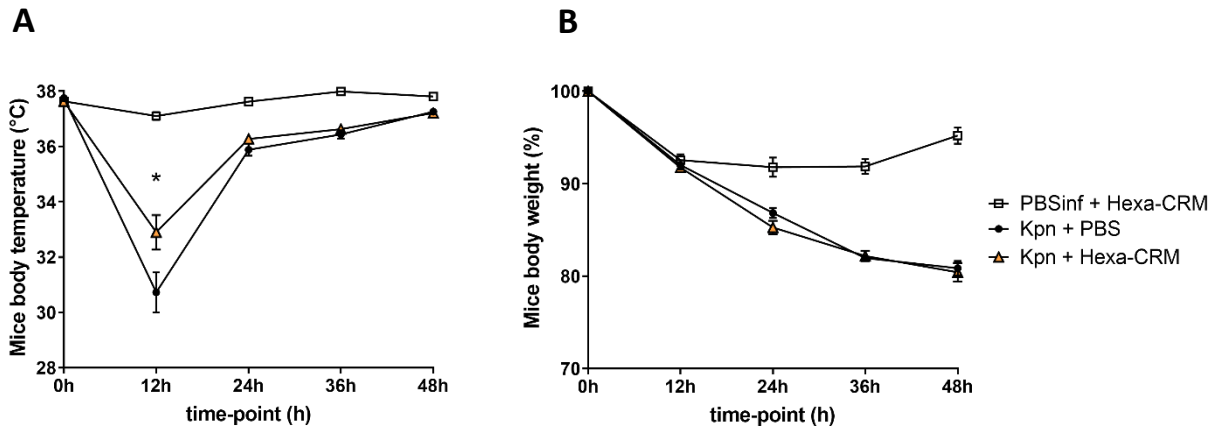

Figure 28. Anti-O2afg polyclonal sera improve body temperature in mice after infection with CRKP. Mice body temperature and body weight was inferred before infection (0h) and every twelve hours post-infection. Infected mice (Kpn) were passively immunized with polyclonal sera of rabbits immunized with either synthetic anti-O2afg vaccine (CRM-Hexa) or PBS vaccine (PBS) two hours post-infection with $1x10^8$ of O2afg-CRKP. Mice infected with PBS (PBSinf) but receiving Hexa-CRM sera was used as a negative control. **A** – Mouse body temperature. **B** – Mouse body weight. The data represents the mean ± SE of 12 mice per group. Unpaired t-test was used for the statistical analysis. *$p < 0.05$.

Another important parameter associated with higher survival rates is the body weight after infection where higher body weight increases the chances of survival (215). Here, passive immunization with anti-O2afg polyclonal sera (Hexa-CRM) did not increase the body weight of mice when compared to the group that did not receive specific antibodies (PBS) (Fig. 28B). Unfortunately, the time window of 48 hours is relatively short to evaluate the increase in body mass after lung infection with *K. pneumoniae* that occurs normally 3-4 days post-infection (217). The, body weight changes in both groups are within 80% of the initial weight, which is in accordance with other studies that have used *K. pneumoniae* to infect mice intranasally (217, 218). The lack of improvement in body weight does not necessarily rule out the treatment effect, but rather has to be attributed to the short time window used in this study. Even non-infected mice showed a 10% reduction in body weight that is in accordance with published results (< 10%) as a normal effect after narcosis and handling procedures (217, 218).

Pneumonia and sepsis are the major cause of death in patients infected with CRKP (219). Sepsis is often a result of severe lung infection where the mucosal barrier is broken, enabling the bacteria to enter the bloodstream (1). Thus, the reduction of bacteria CFU in lungs is a very important factor to reduce inflammation and the progress of pneumonia into sepsis. To quantify the bacterial load, mice were sacrificed after 48 hours and the CFU, as marker for the bacterial load, quantified in the lungs, BALF, and blood. Mice that received anti-O2afg polyclonal sera had a reduction of more than one-log in bacteria CFU in the lungs (Fig. 29A) and BALF (Fig. 29B) when compared to the infected group that received polyclonal sera without anti-O2afg antibodies. This suggests that the presence of anti-O2afg antibodies is the cause of the bacterial clearance in the lungs and BALF. The presence of bacteria in BALF is an indication that the mucosal barrier has been breached, allowing the bacterial to invade the bloodstream, Therefore, the observed reduction of CFU in BALF is a very important factor to limit the spread of CRKP in the body and avoid a sepsis.

Although CRKP was found in the BALF, analysis of blood of infected mice showed that the bacteria were not present in the bloodstream in all groups (Fig. 29C), indicating that the inflammation caused by CRKP in the lungs was not sufficient to cause the invasion of the bacteria into the blood circulation. Often, lung sepsis models using *K. pneumoniae* rely on lethal doses or intravenous administration of the bacteria (220). Unfortunately, these models do not represent the reality since *K. pneumoniae* sepsis in humans evolves from bacterial colonization of different organs (lung, bladder, and intestine), inflammation after bacterial growth those results in the access of the bacteria to the bloodstream. Therefore, the lung murine pneumonia model used in this study reflects better the course of infection occurring in humans, but the bacterial dose used in this study was not sufficient to induce sepsis. The production of toxins such as pneumolysin and hydrogen peroxide facilitates bacterial invasion into the bloodstream due to cytotoxic damage of the lung epithelial barrier (221). However, *K. pneumoniae* does not produce such toxins and the lack of these proteins in the lung airways during infection results in lower septicemia rates, supporting our data where no bacteria were found in the blood of mice infected with CRKP.

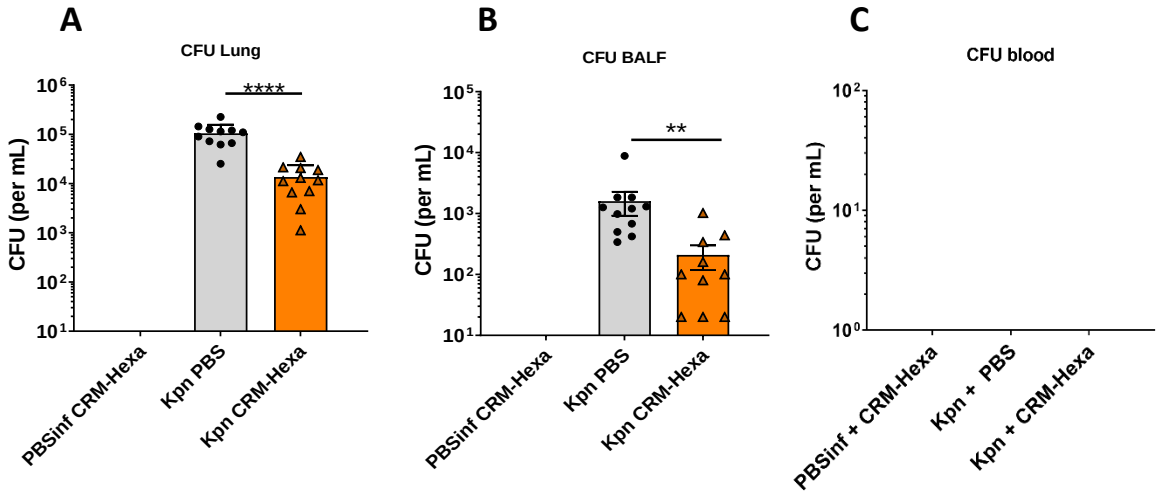

Figure 29. Anti-O2afg polyclonal sera reduce CFU in the lung and BALF of mice infected with CRKP. Lung, BALF and blood was collected 48 hours post-infection. Infected mice (Kpn) were passively immunized with polyclonal sera of rabbits immunized with either synthetic anti-O2afg vaccine (CRM-Hexa) or PBS vaccine (PBS) two hours post-infection with $1x10^8$ of O2afg-CRKP. Mice infected with PBS (PBSinf) but receiving Hexa-CRM polyclonal rabbit sera was used as a negative control. **A** – CFU of CRKP in lungs. **B** – CFU of CRKP in BALF. **C** – CFU of CRKP in blood. The data represents the mean ± SE of 12 mice per group. Mann-Whitney was used for the statistical analysis. ** $p < 0.01$ ****$p < 0.0001$. One mouse was excluded from Kpn infected PBS group due to death after narcosis and one mouse outlier was excluded from the Kpn infected Hexa-CRM group.

Improved CRKP lung clearance by anti-O2afg antibodies may be due to the increase in phagocytosis, as a result of the increased concentration of IgGs in the lung (Fig. 27). A similar effect was reported in a recent study that evaluated the protection of anti-O2 and anti-O1 monoclonal antibodies passively transferred into mice in a pneumonia model. The antibodies reduced bacteria CFU in mouse lungs with a one to two-log decrease when compared to the control. Moreover, the reduction was sufficient to improve mouse survival after *K. pneumoniae* infection (126). Another research group proved that passively transferred anti-O1 monoclonal antibodies killed *K. pneumoniae* in the lungs of mice with a one-log reduction rate. This was sufficient to increase the survival rate of mice infected with a lethal bacterial dose considerably (202). In our study, anti-O2afg antibodies present in rabbit polyclonal serum reduced the bacteria CFU in the lungs of infected mice within the range observed for the reported monoclonal antibody studies, targeting different *K. pneumoniae O*-antigens. Interestingly, both studies showed that the antibody injections were sufficient to protect mice after a lethal dose of *K.*

pneumoniae. Therefore, it is likely that anti-O2afg antibodies, generated after vaccination with our glycoconjugate vaccine, will increase the survival rate of animals infected with CRKP as well. Further studies using a lethal dose of CRKP in a mouse model are needed to evaluate this hypothesis.

3.11 Anti-O2afg antibodies increase the number of immune cells and cytokine production while reducing cell infiltration in BALF of mice with acute pneumonia

Immune cells from the native and adaptive immune system are the first line of defense against bacterial infections (24). Thus, enhancing the recruitment of immune cells to the site of infection is a crucial factor to fight *K. pneumoniae* lung infections. To evaluate whether anti-O2afg antibodies increased the recruitment of immune cells, blood and BALF of mice passively immunized with rabbit polyclonal serum, after lung infection with CRKP, was used to quantify immune cells 48 hours post-infection. The blood cell count was determined by a hemogram, while cells in BALF were evaluated by FACS. Infected mice treated with anti-O2afg polyclonal sera showed significantly higher levels of neutrophils, monocytes, lymphocytes and eosinophils in blood when compared to the infected group that received PBS polyclonal sera (Fig. 30A). We conclude that the higher immune cell recruitment is caused by the presence of anti-O2afg antibodies in this group, since it was the only difference between the infected groups. The opposite was observed in the BALF, with significant higher levels of neutrophils, inflammatory monocytes, and eosinophils in mice that received PBS polyclonal sera (Fig. 30B). Excessive infiltration of neutrophils, eosinophils, as well as the presence of inflammatory macrophages and eosinophils into BAL has been associated with chronic inflammation and edema in lungs leading to acute lung injury (ALI), dysfunction of airways, and death (222). Considering that anti-O2afg antibodies reduce the infiltration of neutrophils, macrophages and eosinophils in the BALF, the likelihood of ALI should be significantly reduced.

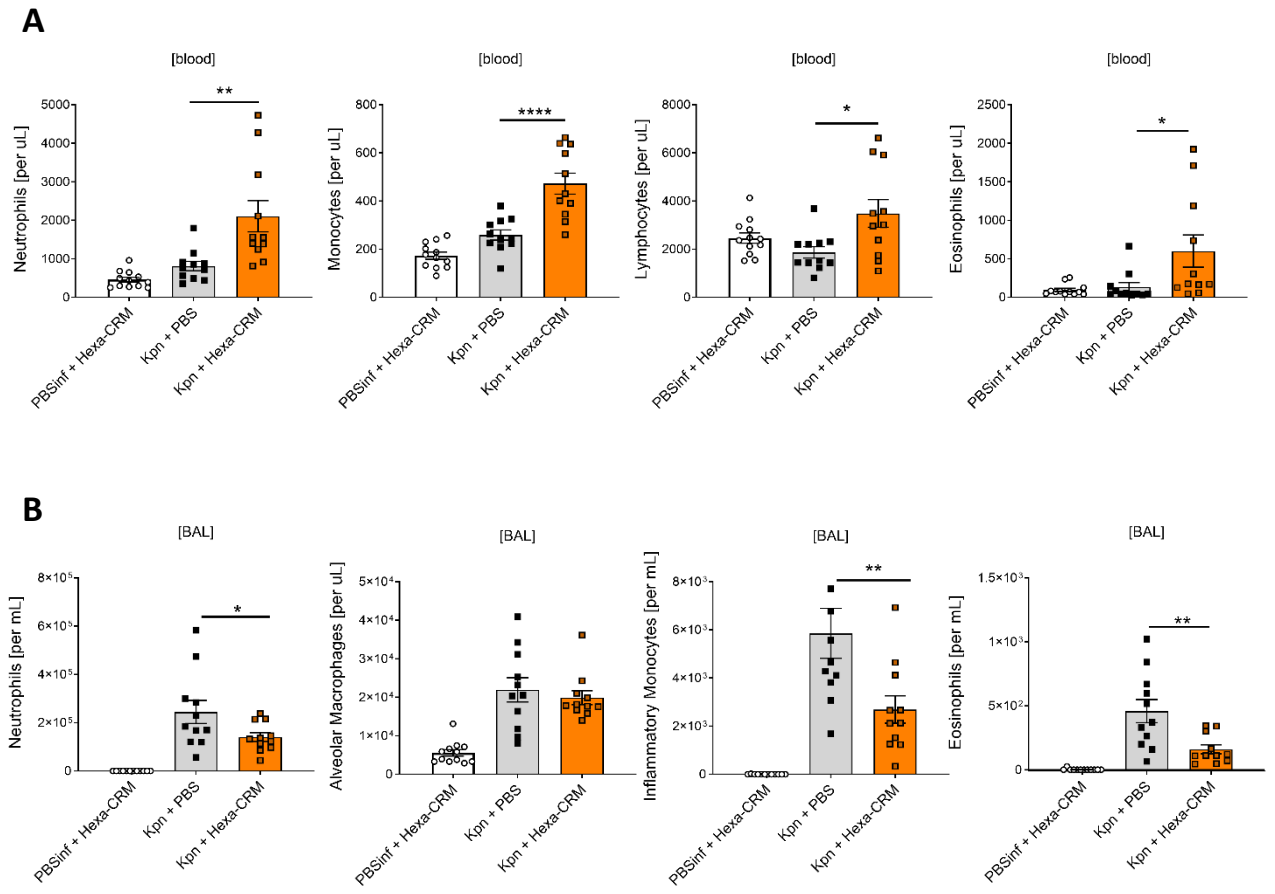

Figure 30. Anti-O2afg polyclonal induce immune cells in blood and reduce cell infiltration in BALF of mice infected with CRKP. Blood and BALF was collected 48 hours after infection. Infected mice (Kpn) were passively immunized with polyclonal sera of rabbits immunized with either synthetic anti-O2afg vaccine (CRM-Hexa) or PBS vaccine (PBS) two hours post-infection with $1x10^8$ of O2afg-CRKP. Mice infected with PBS (PBSinf) but receiving Hexa-CRM polyclonal rabbit sera was used as a negative control. **A** – Neutrophils, monocytes, lymphocytes, and eosinophils levels in blood. **B** – Neutrophils, alveolar macrophages, inflammatory monocytes, and eosinophils levels in BALF. The data represents the mean ± SE of 12 mice per group. One-way ANOVA was used for the statistical analysis. * $p < 0.05$ ** $p < 0.01$ ****$p < 0.0001$. One mouse was excluded from Kpn infected PBS group due to death after narcosis and one mouse outlier was excluded from the Kpn infected Hexa-CRM group.

Apart of immune cell stimulation, the production of pro-inflammatory cytokines and chemokines is crucial to mediate *K. pneumoniae* clearance in infected lungs, since these molecules have an effect cell activation that leads to immunostimulatory reactions (22). Levels of cytokines and chemokines, associated with bacterial lung infections, were quantified in the blood and BALF of mice 48 h post-infection. Mice that received anti-O2afg polyclonal serum showed significantly

higher blood level concentrations of Th1 pro-inflammatory cytokines IL-6, IL-12 and IFN- γ (Fig. 31A). Especially IFN-γ is a critical mediator to control gram-negative pulmonary pneumonia and mice with reduced expression of IFN-γ have lower survival rates after infection with *K. pneumoniae* (223). The induction of IFN-γ by anti-O2afg antibodies could be an important factor to fight CRKP infections. The increased levels of IL-6 and IL-12 indicate higher numbers of activated phagocytic cells (APCs) that is dependent on efficient bacterial uptake, processing, and presentation via MHC II that once stimulated by T cells, results in the production of the cytokines (224). The increased activation of phagocytic cells is supported by the results *in vitro,* which confirmed that anti-O2afg antibodies induced complement deposition and opsonophagocytosis (Fig. 22), both activities that increase bacterial uptake and processing by phagocytic cells. The presence of activated phagocytic cells is also supported by the increased number of monocytes in the blood (Fig. 30A), which can differentiate into macrophages or dendritic cells, after pathogen stimulation. IL-12 augments the production of IFN-γ by T cells and NK cells to improve bacterial clearance in the lung (224). The high level of IFN-γ and IL-12 might explain the higher levels of lymphocytes in blood of Hexa-CRM serum mice, because T cells and NK T cells belong to the lymphocyte family. The results also showed that mice passively immunized with anti-O2afg polyclonal sera had significantly higher expression of CXCL1 chemokine in blood when compared to the control group (Fig. 31A), correlating with the higher number of neutrophils at the same site (Fig. 30A). CXCL1 activates and recruits neutrophils to the site of infection and triggers the release of proteases and ROS for microbial killing (225) and subsequently protection from severe pneumonia (220). No difference in the expression of IL-1β, IL-17a, TNF-α pro-inflammatory cytokines and CCL2 chemokine was observed in blood of infected mice that received different polyclonal sera treatments (Fig. 30A). Usually, the reduction of these pro-inflammatory cytokines has been associated with increased survival rates of mice infected with CRKP (31, 207).

In BALF, IL-6 and IL-12 levels were also significantly higher in the group of mice that received anti-O2afg polyclonal sera when compared to mice treated with PBS polyclonal sera (Fig. 31B) and might be a result of higher levels of these cytokines in the blood of this group (Fig 31A). As mentioned above, IL-12 and IL-6 are expressed by activated phagocytic cells that are specialized in killing pathogens. Thus, higher phagocytic killing activity induced by anti-O2afg antibodies

explains the reduction of bacteria CFU in BALF in this group (Fig. 29). Interestingly, the levels of Th17 pro-inflammatory IL-17a was significantly reduced in BALF of mice treated with anti-O2afg polyclonal sera when compared to mice treated with PBS polyclonal sera (Fig. 31B). Although IL-17 plays an important role in bacterial clearance, the overexpression of IL-17 is correlated to lung injury because it promotes chemokine secretion by the respiratory epithelium that attracts neutrophils and target inflammatory monocytes to the site of infection (226). This explains the higher levels of neutrophils and inflammatory monocytes in the BALF of mice treated with PBS (Fig. 29B) since this group expressed the highest levels of IL-17 in this study (Fig 31B). The effect of higher infiltration of neutrophils and inflammatory monocytes is correlated to severe tissue damage in the lungs. In humans, neutrophil infiltration has been associated to acute and chronic airway inflammation (227). Thus, the reduction of IL17-a caused by anti-O2afg antibodies in BALF of infected mice may contribute to lower lung inflammation, permeability, and consequently lower lung edema, without lowering the effect of *K. pneumoniae* clearance in the lungs, since anti-O2afg antibodies reduced bacterial CFU in BALF and lungs of infected mice (Fig. 29) under lower IL-17a expression (Fig. 31B). Furthermore, inter-species passive transfer of antibodies in uninfected mice did not enhance the expression of pro-inflammatory cytokines (Fig. 31).

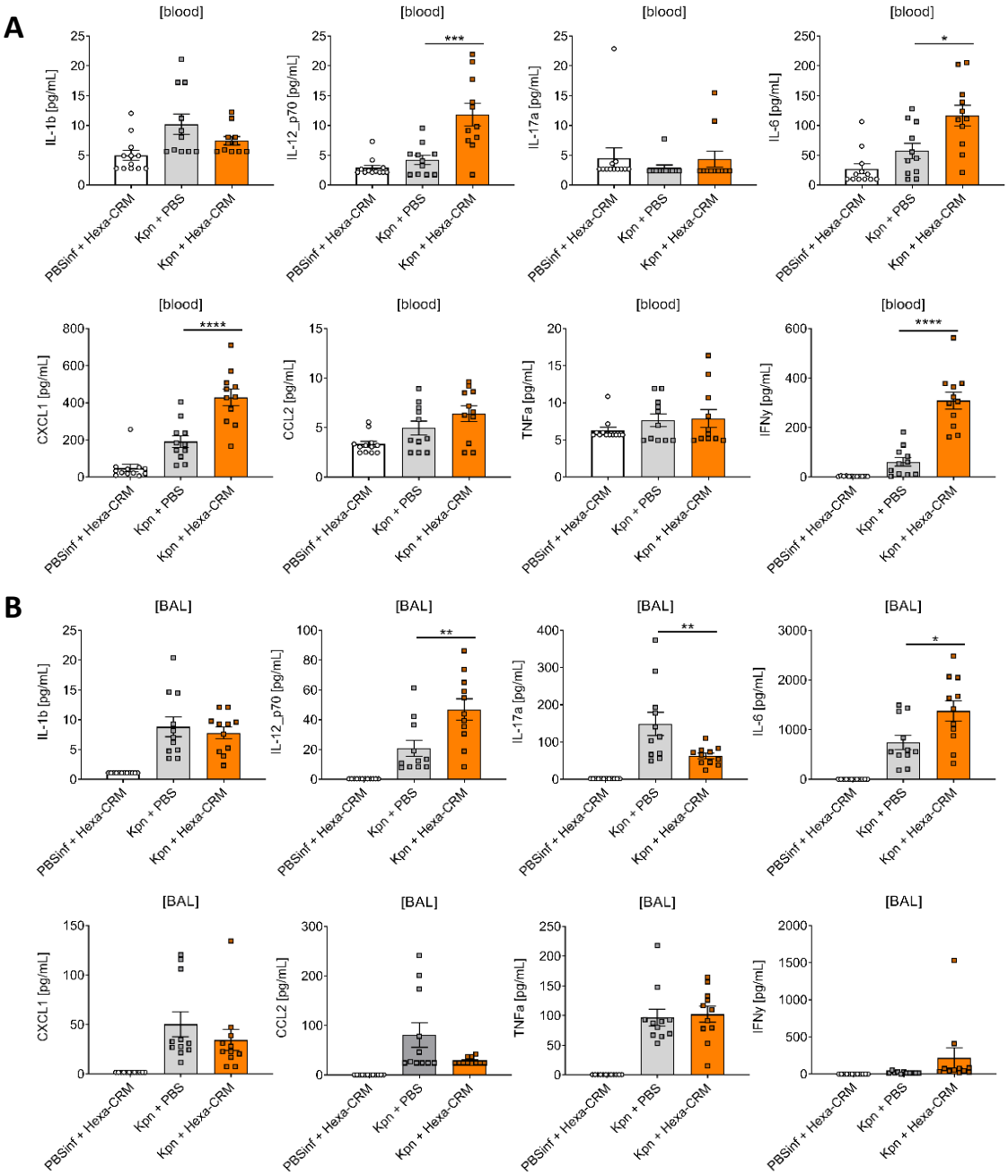

Figure 31. Pro-inflammatory cytokine and chemokine quantification in blood and BALF of infected mice with CRKP 48 hours post-infection. Infected mice (Kpn) were passively immunized with polyclonal sera of rabbits immunized with either synthetic anti-O2afg vaccine (CRM-Hexa) or PBS vaccine (PBS) two hours post-infection with $1x10^8$ of O2afg-CRKP. Mice infected with PBS (PBSinf) but receiving Hexa-CRM polyclonal rabbit sera was used as a negative control. **A** – Levels of IL-1β, IL-12, IL-17, IL-6, CXCL1, CCL2, TNFα, IFNγ in blood. **B** – Levels of IL-1β, IL-12, IL-17, IL-6, CXCL1, CCL2, TNFα, IFNγ in BALF. The data represents the mean ± SE of 12 mice per group. One-way ANOVA was used for the statistical analysis. * $p < 0.05$ ** $p < 0.01$ ***$p < 0.001$. One mouse was excluded from Kpn infected PBS group due to death after narcosis and one muse outlier was excluded from the Kpn infected Hexa-CRM group.

3.12 Anti-O2afg antibodies reduce lung permeability, cytotoxic enzyme expression, and perivascular edema in lungs of mice with acute pneumonia

Recruitment of immune cells and the production of cytokines are important factors to fight bacterial pneumonia. However, the presence of pro-inflammatory cytokines on the site of infection may lead to increased lung inflammation and tissue damage (228, 229). Lung tissue injury can be enhanced by the production of cytotoxic enzymes by immune cells at the site of infection. Myeloperoxidase (MPO) is a cytotoxic enzyme with antimicrobial activity, produced in neutrophil granulocytes. It is also associated with increased lung permeability due to acute pulmonary inflammation and epithelial lung tissue injury (230). In order to estimate the protective effect of anti-O2afg antibodies in infected lungs, MPO levels and lung permeability was measured, 48 hours post-infection, after passive immunization with either rabbit antibodies vaccinated with O2afg glycoconjugate vaccine or PBS vaccine. MPO activity was measured by the breakdown of the TMB substrate by the enzyme, present in the BALF of mice. Lung permeability was quantified by measuring the total protein content in BALF.

Anti-O2afg antibodies significantly reduced the levels of MPO expression in the lungs of infected mice, compared to the group that received PBS polyclonal sera (Fig. 32A). This finding is supported by the lower number of neutrophils, the MPO producing cells, in BALF of O2afg serum treated mice. Although MPO cause tissue injuries in the lungs, and exerts clearance of *K. pneumoniae* in infected mice (231), it is not always sufficient to clear the infection. This observation is supported by the higher CFU in BALF of PBS polyclonal serum treated mice (Fig. 29B). Interestingly, lung permeability was significantly reduced in the group that received anti-O2afg antibodies in comparison to the group that did not receive specific anti-O2afg antibodies (Fig. 32B) revealing that lower MPO expression might be associated with lower tissue injury and therefore lower protein infiltration in the BALF of infected mice in this group. Taken together, the main protective mechanism of our O2afg antibodies might be enhanced OPK activity due to complement activation, since elevated levels of IL-6 and IL-12 represents higher number of activated phagocytes. In addition, the lower levels of MPO might reduce cellular damage of the lung epithelial layer, thereby preventing the spread of the infection to the body.

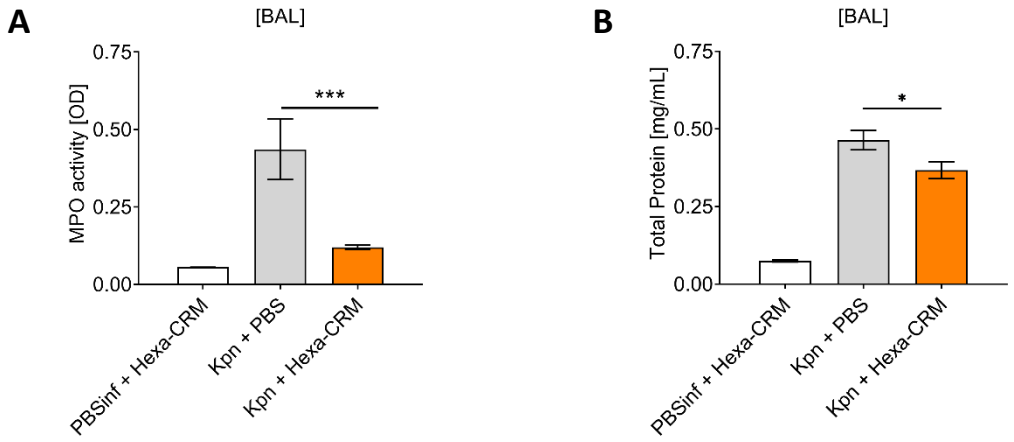

Figure 32. Myeloperoxidase (MPO) activity and lung permeability in BALF of mice infected with CRKP 48 hours post-infection. Infected mice (Kpn) were passively immunized with polyclonal sera of rabbits immunized with either synthetic anti-O2afg vaccine (CRM-Hexa) or PBS vaccine (PBS) two hours post-infection with $1x10^8$ of O2afg-CRKP. Mice infected with PBS (PBSinf) but receiving Hexa-CRM polyclonal rabbit sera was used as a negative control. **A** – Levels of MPO measure by enzyme activity against TMB substrate. **B** –Lung permeability measure by the amount of total protein infiltrated in mice BALF. The data represents the mean ± SE of 12 mice per group. One-way ANOVA was used for the statistical analysis. * $p < 0.05$ ** $p < 0.01$ ***$p < 0.001$. One mouse was excluded from Kpn infected PBS group due to death after narcosis and one mice outlier was excluded from the Kpn infected Hexa-CRM group.

Anti-CPS antibodies, targeting CRKP, have been associated with increased neutrophil activity measured by the production of ROS and NEtosis *in vitro,* but MPO activity or lung injury was not evaluated in infected mice (124, 207). There is also a lack of data about neutrophil activity and lung permeability using O-antigen antibodies against CRKP pneumonia. A biosynthetic vaccine targeting O2 *K. pneumoniae* has generated anti-O2 antibodies that reduce bacterial CFU in lungs of infected mice, but the effect on neutrophil activity was not measured (203). Anti-O1 and anti-O2 monoclonal antibodies also have been protective against CRKP pneumonia in passively immunized mice by reducing CFU in the lungs, thereby increasing survival rates. However, neutrophil recruitment or activity as well as lung permeability was not measured by these studies (126, 202).

Histopathology of CRKP infected lungs of mice passively immunized with either anti-O2afg or PBS polyclonal sera were also evaluated in order to visualize if the reduction in MPO and lung permeability caused by anti-O2afg antibodies corresponded to lower tissue damage in the

infected lungs, since both parameters are associated with acute lung tissue injury (228). Histopathology is a reliable method for the evaluation of morphological changes in animal lung infection experiments for many years and relies on qualitative diagnoses of microscopically observed morphological and cellular changes in the lung tissue (232). A semiquantitative scoring system is used to compare lesion between treated and control groups and is scored by certified pathologists. This system has been accepted by regulatory agencies as gold standard of tissue alteration in animal experiments (233). In this study, lungs of mice from different treatment groups were given to pathologist certified from the European College of Veterinary Pathologists (ECVP) board and they were blinded about the study groups. The degree of edema formation was assessed semiquantitatively (0 = no effect, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe) from three microscopically evenly distributed sections per lung. High levels of edema formation are correlated to high epithelial tissue injury and cell infiltration in lungs (228). Levels of inflammation and total cells were also evaluated. Comparison between groups of scored data was performed by non-parametrical statistical analysis since score values are ordinal and not continuous data (234).

Lungs of infected mice were diagnosed with a moderate bronchopneumonia with increased lesion in the lung periphery and a minimal extended spread of inflammation to the lung periphery was identified in all infected animals (Fig. 33 A). These parameters have been reported in lungs of mice infected with *K. pneumoniae* and are associated with acute lung infection (ALI) (228). In ALI, high levels of inflammation as well as pulmonary edema and higher cell infiltration is present in infected lungs. Lungs of infected mice show increased inflammation and developed edema when compared to the uninfected group control, confirming that mice developed ALI (Fig. 33). However, no difference in the number of total cells was found between the groups (Fig. 33B). There was no reduction in inflammation scores or alveolar edema in lungs of infected mice treated with anti-O2afg polyclonal sera when compared to infected mice treated with PBS polyclonal sera (Fig. 33C-D), despite the observed reduction in CFU in the lungs and BALF this group (Fig. 29). Reduction of CFU in lungs does not always correlate with a rapid decrease in lung inflammation, because immune cells can still be activated by alternative pathways rather than direct stimulation through the microorganism, or high level of pro-inflammatory cytokines on

site. Studies have shown that mice that recover from *K. pneumoniae* pneumonia manage to reduce the levels of cytotoxic enzymes and pro-inflammatory cytokines three days post-infection (235). Thus, the lack of effect in the inflammation may also be accounted for by the reduced time window used in this study.

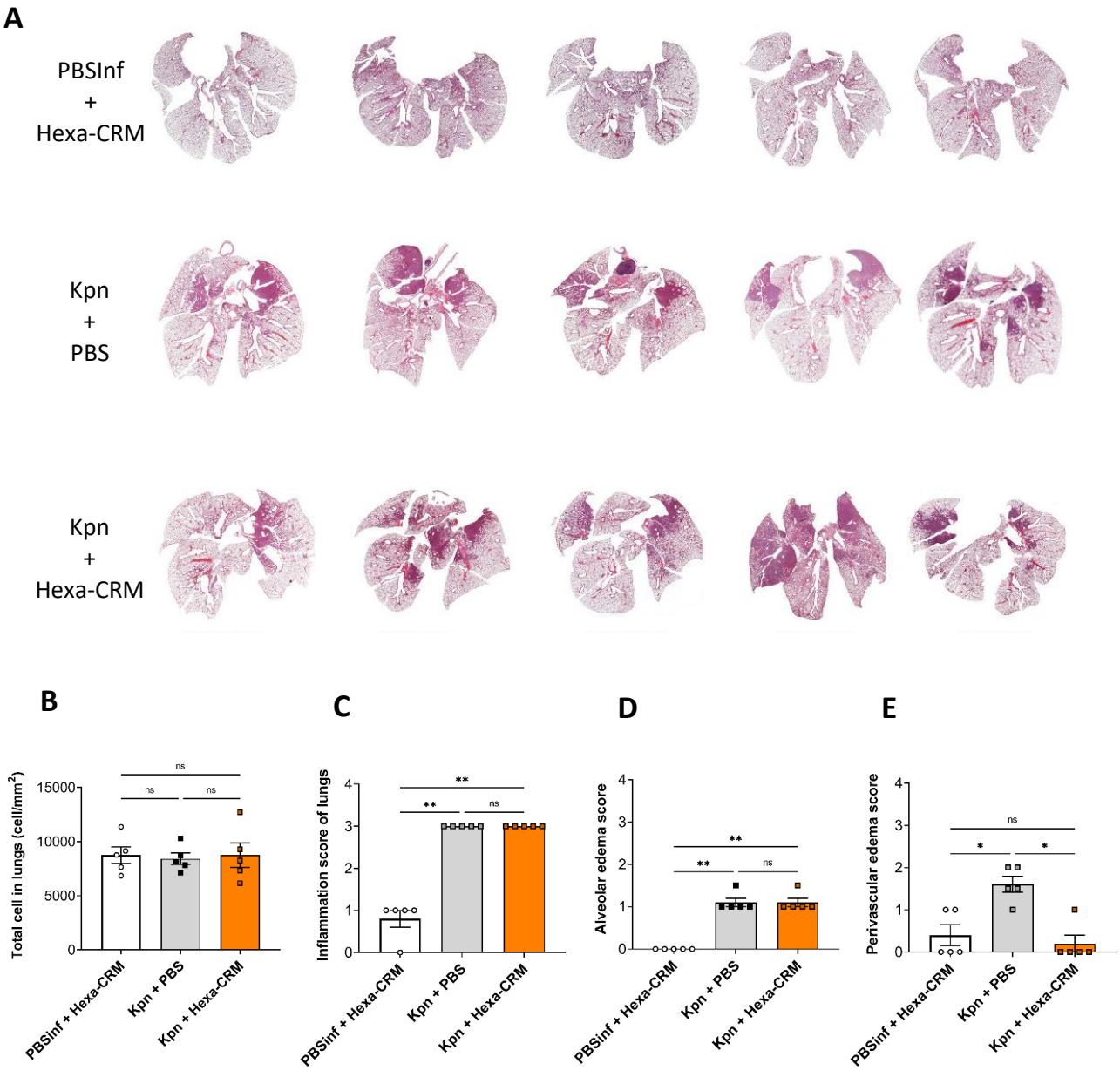

Figure 33. Histology and lungs parameters of mice infected with 1 x 10^8 CFU of *K. pneumoniae* carbapenem-resistant strain. **A** - Lung sections stained with HE from mice non-infected (PBSInf), mice infected and treated with polyclonal sera of rabbits immunized with O2afg vaccine (Kpn+Hexa-CRM), and mice infected and treated with polyclonal of rabbits vaccinated with PBS (Kpn+PBS), 48 hours post-infection. B – Total cells in lungs. **C** –Inflammation score of lungs. **D** – Alveolar edema of lungs. **E** – Perivascular edema of lungs. The data represents the mean ± SE of 5 mice per group. Kruskal–Wallis was used for the statistical analysis. * $p < 0.05$ ** $p < 0.01$.

Interestingly, perivascular edema of infected mice that received anti-O2afg antibodies were significantly reduced to the same level as uninfected mice, when compared to mice treated with PBS polyclonal sera (Fig. 33E). Development of edema in ALI is a very common phenomenon and is a result of the increase in capillary permeability due to epithelial damage causing fluid to move into perivascular spaces (236, 237). Epithelial injury in lungs of mice infected with *K. pneumoniae* that have developed ALI has been associated with an increased number of neutrophils and higher myeloperoxidase activity (235). Thus, the lower levels of neutrophils and MPO in BALF of mice that received anti-O2afg antibodies may explain the lower level of perivascular edema in this group. Studies have shown that the decrease in neutrophils and MPO in BALF of mice reduced mortality in acute respiratory distress syndrome induced by the influenza virus, as well as death of mice with cystic fibrosis (238, 239). Hence, the improved effect in lung and BALF parameters caused by anti-O2afg antibodies may contribute to increased survival rates of mice infected with CRKP. Further studies with a lethal dose of CRKP are needed to confirm this hypothesis.

In summary, anti-O2afg antibodies have been proven to reduce MPO levels and lung permeability in BALF of mice infected with CRKP. This has contributed to a reduction in perivascular edema in infected mice, while no effect in lung inflammation or on alveolar edema was found. Although many studies reported anti-CPS and anti-LPS antibodies enhancing neutrophil activation and MPO activity *in vitro*, it does not always translate into an elevated level *in vivo*. My work supports an alternative hypothesis that the reduced levels of MPO and neutrophils in BALF contribute to lower severity in lungs of mice infected with CRKP due to a reduction in lung permeability and edema in acute pneumonia murine model.

4. Conclusion and Outlook

Bacterial infection is the cause of millions of deaths worldwide affecting mainly children and the elderly. Antimicrobial resistant bacteria that do not respond to currently available treatment, such as *K. pneumoniae* resistant to carbapenems (CRKP), aggravates this situation and may surpass cancer mortality rates. Many scientists have focused on the development of alternative therapies to combat CRKP, based on antibody therapy and vaccine production, mainly targeting capsular polysaccharide. However, the increased variability of CPS serotypes in this group is associated with a lower epidemiological correlation of clinical isolates. This rules out the use of CPS to target CRKP, due to complexity in vaccine formulation to achieve high epidemiological coverage. Antigens have been proposed as a target alternative because only four serotypes encompass more than 90% of *K. pneumoniae* clinical isolates, contributing to a high epidemiological coverage with a lower vaccine formulation effort. Interestingly, the O2afg serotype represents more than 80% bacterial isolates that belong to the CRKP group. Vaccines targeting CRKP, using either inactivated bacteria or isolated O-antigens, have been reported. However, both vaccine approaches contain impurities, such as endotoxins that are very toxic for humans even in the picomolar ranges. We overcame this problem by using a designed synthetic hexasaccharide that mimics the native O2afg, as a vaccine antigen. The synthetic antigen was successfully conjugated to the CRM_{197}, as a carrier protein, and adsorbed into alum, creating a semi-synthetic glycoconjugate vaccine against CRKP.

To date there are no reported monoclonal antibodies or vaccines targeting O2afg antigens due to poor B cell activation induced by this serotype. Consequently, memory B cells and high affinity antibody titers against this serotype are rare. In this work, a dose of one μg of the designed synthetic glycoconjugate sufficed to induce a memory response and evoke the production of IgG and IgA, in rabbits, overcoming the problem of low antigen immunogenicity. The generated antibodies not only recognized the synthetic antigen but also target the native antigen of the bacteria in a specific manner. Furthermore, the glycoconjugate vaccine induced opsonic antibodies that contributed to complement protein deposition onto the bacterial surface and induced effective clearance of the pathogen by phagocytic cells. Importantly, the vaccine dose

used in this study is lower than in any marketed glycoconjugate vaccines, thus reducing the costs of vaccine production and encourage future clinical trials.

K. pneumoniae is one of the most common causes of hospital-acquired pneumonia, especially among individuals with critical illness in intensive care units. Recently, the pathogen has been associated with SARS-CoV-2 pulmonary co-infections contributing to chronic obstructive pulmonary diseases, a severe COVID-19 condition with high mortality rates. In this thesis, I have proven the protective effect of anti-O2afg antibodies against acute pneumonia in mice passively immunized with polyclonal sera of rabbits vaccinated with O2afg glycoconjugate. Anti-O2afg antibodies proved to reduce the number of bacteria in lungs of infected mice with CRKP and increase the recruitment of immune cells and cytokines. Furthermore, anti-O2afg antibodies reduce the production of MPO and levels of neutrophils in the BALF of infected mice, which contributed to a reduced perivascular edema in infected lungs.

In summary, we have developed a semi-synthetic glycoconjugate vaccine lead targeting specifically O2afg-CRKP for the first time. The vaccine lead elicits a robust immune response and the generated antibodies have been proven to reduce the burden of acute pneumonia *in vivo*. This may allow to extend our concept to other bacterial pathogens with multiple resistance against antimicrobials and create an alternative route to not only response to an infection, but also to prevent it in the first place.

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