Identifying Novel Pneumococcal Pneumonia Vaccine Candidates

Thesis submitted in accordance with the requirements of the Liverpool School of Tropical Medicine for the degree of Doctor in Philosophy by

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Declaration

I, Katerina Stamatina Cheliotis, declare that this thesis is the result of my own work and effort. In some instances, work was made possible by collaboration with colleagues and external collaborators. The attribution of work and responsibilities related to the project are detailed in full in Contributions. The research of this thesis was carried out at the Liverpool School of Tropical Medicine. The contents of this thesis have not been presented, nor are currently being presented, wholly or in part, for any other degree or qualification.

Dedication

I dedicate this work to all the women who have fought for a woman's right to education and to all women and girls with big dreams, may we continue the fight so that they may follow them.

Contributions

This work has been made possible thanks to the contributions of many people outlined below. I would also like to extend a special thanks to all the EHPC volunteers. Individual acknowledgments are given at the end of relevant chapters.

Activity	Responsibility
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EHPC-New Strains (ISRCTN68323432; 20815) study team	Victoria Connor, Caz Hales, Helen Hill, Angie Hyder-Wright, Hugh Adler, Seher Zaidi, Rachel Robinson, Cath Lowe, Sherin Pojar, Esther German, Elena Mitsi, Elissavet Nikolaou, Carla Solórzano, and Daniela Ferreira
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B-cell ELISpot technical support	Dr Carla Solórzano
Design of nanoparticle co-culture assay protocol	Dr Carla Solórzano, Dr Britta Urban
Development and supply of nanoparticle-based vaccines	Prof Imran Saleem, Dr Nashwa Osman (Formulation Drug Delivery Research Group, Liverpool John Moores University, Liverpool, UK)

Abstract

Identifying novel pneumococcal pneumonia vaccine candidates

The burden of pneumococcal pneumonia remains high worldwide. The disease disproportionately affects infants, the elderly and the immunocompromised. Nasopharyngeal colonisation with *S. pneumoniae* is the pre-requisite for disease but also, paradoxically, an immunising event. Current pneumococcal vaccines offer limited protection against pneumonia and do not offer serotype-independent coverage. Serotype replacement is particularly concerning. Identification of serotype-independent correlates of protection against *S. pneumoniae* infection will accelerate development of vaccines with broad coverage. Protein vaccines are a particularly promising way to confer serotype-independent protection.

The experimental human pneumococcal challenge (EHPC) model enables us to investigate correlates of protection against experimental colonisation with *S. pneumoniae* and study protein-directed immune responses to nasopharyngeal colonisation with the bacteria. A multiplex Luminex assay was developed and used to measure serum IgG against 75 conserved pneumococcal antigens pre- and post-intranasal inoculation with two serotypes of pneumococcus (6B and 15B) in EHPC volunteers. Peripheral blood mononuclear cells taken pre- and post-challenge with serotype 6B pneumococcus were used to evaluate correlation between protein-specific IgG^+ memory B-cell responses and protection against experimental colonisation as well as memory B-cell responses to pneumococcal challenge by ELISpot. Memory B-cell responses to six selected proteins and the novel PnuBioVax vaccine were investigated. The results of this work showed that colonisation with serotype 6B pneumococcus induces significant systemic protein-mediated antibody and memory B-cell responses. No single protein antigen correlated with protection against experimental colonisation with *S. pneumoniae*. However, whilst not significant, there was a trend that PnuBioVax-specific memory B-cells conferred protection against experimental pneumococcal colonisation. Five novel nanoparticle-based vaccines were also investigated to determine their effects on dendritic cell maturation and support of T-cell activation. One of these nanoparticles was subsequently loaded with pneumococcal protein PspA to investigate antigen-specific response. Further work is needed to ascertain the immune response to these novel vaccines, though they are a promising method of serotype-independent vaccine delivery.

It is likely that for a novel pneumococcal protein vaccine to confer serotype-independent protection against pneumococcal pneumonia, a combination of protein antigens will need to be included in vaccine formulations. Mucosal vaccination in particular will likely be needed to induce robust immunity.

Publications, presentations, secondments, awards

Publications arising during the course of this PhD, not presented within this thesis:

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FMedSci, on behalf of the **Oxford COVID Vaccine Trial Group***. (2021) 'Safety and efficacy of the ChAdOx1 nCoV-19 vaccine (AZD1222) against SARS-CoV-2: an interim analysis of four randomised controlled trials in Brazil, South Africa, and the UK'. *The Lancet* 307, 10269.

***members are listed in Appendix 1 (pp 21-44).**

V. Connor, H. Hill, E. Mitsi, E. Nikolaou, C. Solórzano, S. Pojar, **K. S. Cheliotis**, S.P. Jochems, E. German, C. Hales, A. Hyder-Wright, H. Adler, S. Zaidi, J. Reine, S. Gordon, B. Morton, H. Burhan, A. M. Collins, *J. Rylance and *D.M. Ferreira. 'Controlled Human Infection with *Streptococcus pneumoniae* is Not Protective against Re-challenge with Heterologous Serotype'. [in preparation]

Poster Presentations

2021 - Oral poster at the British Academy of Lung Research Summer Conference 2021 (Exeter, England, UK). Poster title: A novel multiplex assay using Luminex xMAP technology to screen for pneumococcal vaccine candidates.

Oral Presentations

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2022 - Talk at the 15th European Meeting on the Molecular Biology of the Pneumococcus (Europneumo2022) (Liverpool, UK). Title: Influence of sex, season and environmental air quality on experimental human pneumococcal carriage acquisition: a retrospective cohort analysis.

Secondments

27th March – 27th June 2021: secondment to SARS-CoV-2 clinical trials

- SAFER: SARS-CoV-2 Acquisition in Frontline Health Care Workers – Evaluation to Inform Response
- FASTER: Facilitating A SARS CoV-2 Test for Rapid triage
- COVID-19 Oxford Vaccine Trial

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Acronyms and abbreviations

ABC	ATP-binding cassette
AMR	Antimicrobial resistance
APC	Antigen-presenting cell
B-cell	B lymphocyte
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BCR	B-cell receptor
BLP	Bacterium-like particle
BSA	Bovine serum albumin
CCR2	C-C Motif Chemokine Receptor 2
CD40	Cluster of differentiation 40
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CPS	Capsular polysaccharide
CT	Cholera toxin
CV	Coefficient of variation
DC	Dendritic cell
ECL	Electrochemiluminescence
EHPC	Experimental human pneumococcal challenge
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immune absorbent spot
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GC	Germinal center
HALDR	Human leukocyte antigen D related isotype
HIV	Human immunodeficiency virus
iBALT	Inducible bronchoalveolar lymphoid tissue
IFN	Interferon

Ig	Immunoglobulin
IL	Interleukin
IPD	Invasive pneumococcal disease
IQR	Interquartile range
LAIV	Live Attenuated Influenza Vaccine
LPS	Lipopolysaccharide
LytA/C	Autolysin A/C
MAIT cell	Mucosal associated invariant T-cell
MCP-1	Monocyte chemoattractant protein-1
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
Mos	Months
MSD	Meso Scale Diagnostics
NaCl	Sodium chloride
NALT	Nasopharynx-associated lymphoid tissue
NaNA/B	Neuraminidase A/B
NET	Neutrophil extracellular trap
NICE	National Institute for Health and Care Excellence
NK cell	Natural killer cell
PAMP	Pathogen-associated molecular pattern
PavA	Pneumococcal adhesion and virulence A
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBV	PnuBioVax
PcpA	Pneumococcal choline binding protein A
PCR	Polymerase chain reaction
PcsB	Putative peptidoglycan hydrolase
PCV	Pneumococcal conjugate vaccine
PdB	Pneumolysin toxoid B, pneumolysin derivative with a Trp-433→Phe mutation
PE	Phycoerythrin

PerCP	Peridinin-Chlorophyll-Protein
PhtD/E	Polyhistidine triad protein D/E
PiaA	Pneumococcal iron acquisition A
PiuA	Pneumococcal iron uptake A
PLGA	Poly(lactic-co-glycolic acid)
Ply	Pneumolysin
PNS	Penicillin–neomycin-streptomycin
PPV	Pneumococcal polysaccharide vaccine
PRR	Pattern recognition receptor
PsaA	Pneumococcal surface adhesin A
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
PVDF	Polyvinylidene difluoride
qPCR	Quantitative PCR
RPMI	Roswell Park Memorial Institute
RrgA/B	Pilus-associated adhesin
SA-PE	Streptavidin R-phycoerythrin
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
Spn	*Streptococcus pneumoniae*, pneumococcus
Stkp	Serine threonine protein kinase
T4	TIGR4
T-cell	T lymphocyte
TCR	T-cell receptor
TIV	Trivalent Influenza Vaccine
Tfh	Follicular helper T-cell
TGFβ	Transforming growth factor β
Th cell	Helper T-cell
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor necrosis factor

Treg	Regulatory T-cell
T_{RM}	Tissue-resident memory T-cell
URT	Upper respiratory tract
WCA	Whole cell antigen
WCV	Whole-cell vaccine
Wks	Weeks
WHO	World Health Organization
Yrs	Years

Chapter 1

General Introduction

Pneumonia is the leading infectious cause of death in infants worldwide (Wahl *et al.*, 2018; McAllister *et al.*, 2019). 90% of all deaths caused by pneumonia occur in the African subcontinent and South Asia, where the burden of disease is highest (WHO, 2013). Children who are malnourished and living in rural areas of the global south are most likely to be affected by the disease. *Streptococcus pneumoniae* is the principal cause of morbidity and mortality of lower respiratory infections, including pneumonia, globally and contributes to more deaths than all other aetiologies combined (Troeger *et al.*, 2018). Since the commencement of this work in October 2018, approximately 4,164,780 deaths will have ensued as a direct result of pneumococcal pneumonia, of which around 1,193,602 will have occurred in children under 5 years of age. It is likely this number is an underestimate, especially given the SARS-CoV-2 pandemic, due to undiagnosed cases, particularly in resource-poor settings, and overwhelming pressures on health care systems worldwide. Pneumococcal disease is preventable through improved hygiene and immunisation strategies.

There are currently three pneumococcal vaccines licensed for use: the polysaccharide vaccine PPV23 (licensed in 1983; Merck) and the pneumococcal conjugate vaccines PCV10 (licensed in 2009; GlaxoSmithKline) and PCV13 (licensed in 2010; Pfizer). PPV23 comprises purified capsular polysaccharides from 23 serotypes of *Streptococcus pneumoniae*. PCV10 contains capsular polysaccharides from 10 pneumococcal serotypes conjugated to three binding proteins: non-typeable *Haemophilus influenzae* protein D (NTHi protein D), diphtheria toxoid, and tetanus toxoid and PCV13 includes capsular polysaccharides from 13 serotypes conjugated to a nontoxic variant of diphtheria toxin known as CRM197. The conjugation of polysaccharides to carrier proteins ensures PCVs induce a robust anti-capsular immune response in both adults and children.

1.1 The biology of *Streptococcus pneumoniae*

Streptococcus pneumoniae (pneumococcus, Spn) is an extracellular, gram-positive pathogen. Of the order Lactobacillales, it is a facultative, anaerobic bacterium belonging to the *Streptococcus* genus and is alpha-hemolytic under anaerobic conditions. Often found in diplococci, the bacteria are non-motile. Pneumococcus is a commensal bacterium, commonly found as part of the natural flora of the human nasopharynx.

The pathogenesis of pneumococcus is depicted in Figure 1. Pneumococcus colonises the mucosal surfaces of the upper respiratory tract (URT) to establish carriage of the bacteria in the nasopharynx. The bacteria can be shed in nasal secretions or airborne droplets, leading to transmission of the bacteria. Local spread of the bacteria can lead to otitis media, aspiration of the bacteria from the nasopharyngeal niche into the lung can lead to pneumonia and spread of the bacteria through the epithelium to sterile sites, most commonly occurring as a complication of pneumonia, can lead to bacteraemia and meningitis.

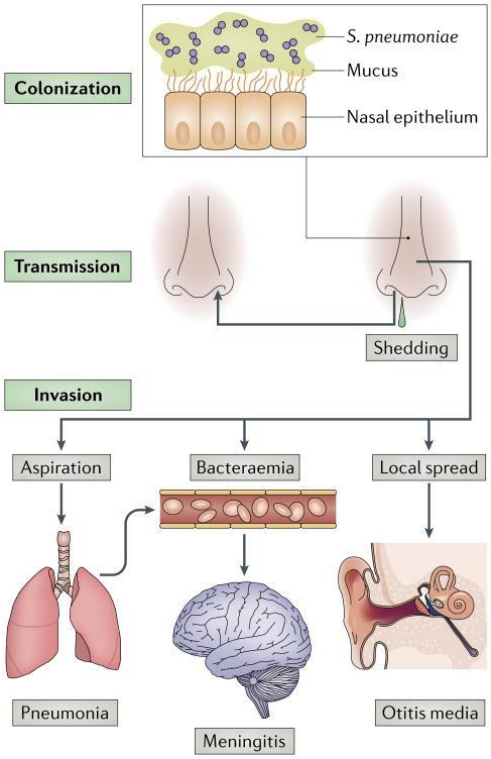


Figure 1: The life cycle of *Streptococcus pneumoniae* and the pathogenesis of pneumococcal disease. *Streptococcus pneumoniae* colonises the mucosa of the upper respiratory tract (URT). This carriage is the prerequisite for both transmission to other individuals and invasive disease in the carrier. Carriers can shed *S. pneumoniae* in nasal secretions and thereby transmit the bacterium. Dissemination beyond its niche along the nasal epithelium, either by aspiration, bacteraemia or local spread, can lead to invasive diseases, such as pneumonia, meningitis, and otitis media. Image source: Weiser, Ferreira and Paton, 2018.

1.1.1 Epidemiology of pneumococcal carriage

Pneumococcus transiently colonises the mucosal surfaces of the upper respiratory tract, predominantly the nasopharynx (Weiser, Ferreira and Paton, 2018). Generally, colonisation with the bacteria is asymptomatic or very mildly symptomatic (for example, rhinorrhea) and is termed carriage (Bogaert, De Groot and Hermans, 2004). Carriage of the bacteria is highest in childhood and declines into adulthood, with carriage rates estimated to be between 40-95% in children and less than 10% in adults (Regev-Yochay *et al.*, 2004; Goldblatt *et al.*, 2005; Le Polain De Waroux *et al.*, 2014; Usuf *et al.*, 2014;

Esposito *et al.*, 2016). Adults in contact with small children are likely to have higher incidence of pneumococcal colonisation (Hussain *et al.*, 2005). Incidence of carriage is higher in low and middle-income countries as compared to high-income countries (Adegbola *et al.*, 2014). Generally, carriage rates peak in children under 5 years of age (Usuf *et al.*, 2014). In high-income countries, peak carriage occurs at 2-3 years of age, whereas in low-income countries, peak carriage occurs much earlier, before 1 year of age (Ferreira, Jambo and Gordon, 2011). High prevalence of pneumococcal carriage and density are associated with frequent disease in the young (Weiser, 2010). Paradoxically however, in the elderly, low carriage rates are associated with increased episodes of pneumococcal disease and mortality (Ridda *et al.,* 2010). It is likely that this paradox is not entirely true and occurs because standard nasopharyngeal swabbing methods are insufficient to detect carriage in adults and the elderly. The colonisation niche may be altered in the elderly, occupying the oropharynx rather than the nasopharynx. The use of molecular methods and saliva sampling instead of nasopharyngeal swabs could resolve under-detection in elderly people and would likely result in a generally higher prevalence of carriage in this age-group (Suzuki *et al.*, 2006; Krone *et al.*, 2014; van Deursen *et al.*, 2016; Adler *et al.*, 2017). Immunosenescence and age-related changes in immunity are significant contributors to the increased risk of pneumococcal pneumonia in the elderly (Krone *et al.*, 2014; Grant *et al.*, 2021). Duration and density of carriage also decline with age (Hill *et al.*, 2010; Roca *et al.*, 2011; Turner *et al.*, 2012). Furthermore, carriage duration differs depending on serotype and non-vaccine serotypes have been significantly associated with increased rate of acquisition and shorter duration of carriage (Hill *et al.*, 2008, 2010).

A longitudinal study in The Gambia found that over 80% of children acquired pneumococcus within the first month of their life and also experience multiple carriage episodes within a year (Hill *et al.*, 2008). A study conducted in Thailand also reported a median of seven pneumococcal acquisitions during the first 24 months of life (Turner *et al.*, 2012). Simultaneous colonisation with multiple pneumococcal serotypes in young children in low and middle-income countries is also a common phenomenon (Kamng'ona *et al.*, 2015; Satzke *et al.*, 2015). Duration of carriage in children under 5 years of age varies depending on serotype and the duration of carriage significantly declines with each increasing year of age from birth to 5 years (Högberg *et al.*, 2007).

Importantly, pneumococcal carriage is an immunising event and has been shown to confer 100% protection against homologous re-colonisation in an experimental human pneumococcal carriage (EHPC) model of infection up to 11 months after clearance of the first carriage episode (Ferreira, *et al.*, 2013).

Delayed re-acquisition and reduced carriage duration of serotypes 14 and 19F has been observed following previous colonisation with the homologous serotype (Hill *et al.*, 2008; Turner *et al.*, 2012).

In past work, the most common serotypes found to colonise the population in the African subcontinent were 6A, 6B, 14, 19F, 23F (Hill *et al.*, 2008; Usuf *et al.*, 2014). A study of colonisation prior to the introduction of conjugate pneumococcal vaccines in school-aged children in Russia also found these serotypes to be the most prevalent (Vorobieva *et al.*, 2020). The introduction of conjugate pneumococcal vaccines has led to a decrease in prevalence of vaccine type serotypes but an increase in prevalence of non-vaccine serotypes, including antibiotic-resistant strains, with a rise in incidence of carriage and invasive pneumococcal disease being caused by non-vaccine serotypes around the world (Gonzalez *et al.*, 2006; Usuf *et al.*, 2014; Janoir *et al.*, 2016; Nakano *et al.*, 2016; Ladhani *et al.*, 2018; Ouldali *et al.*, 2021). It has been shown that polysaccharide capsule structure predicts serotype prevalence and more prevalent serotypes are those most resistant to non-opsonic killing by human neutrophils (Weinberger *et al.*, 2009).

1.1.2 Colonisation and transmission

Nasopharyngeal carriage of the bacteria is a prerequisite of disease and a source of transmission. Pneumococci successfully colonise the nasopharynx utilising various mechanisms. Figure 2 shows the main ways in which the bacteria successfully colonise the host: adherence to host cells and tissues, subversion of mucosal innate and adaptive immunity, and evasion of mucociliary clearance (Weiser, Ferreira and Paton, 2018).

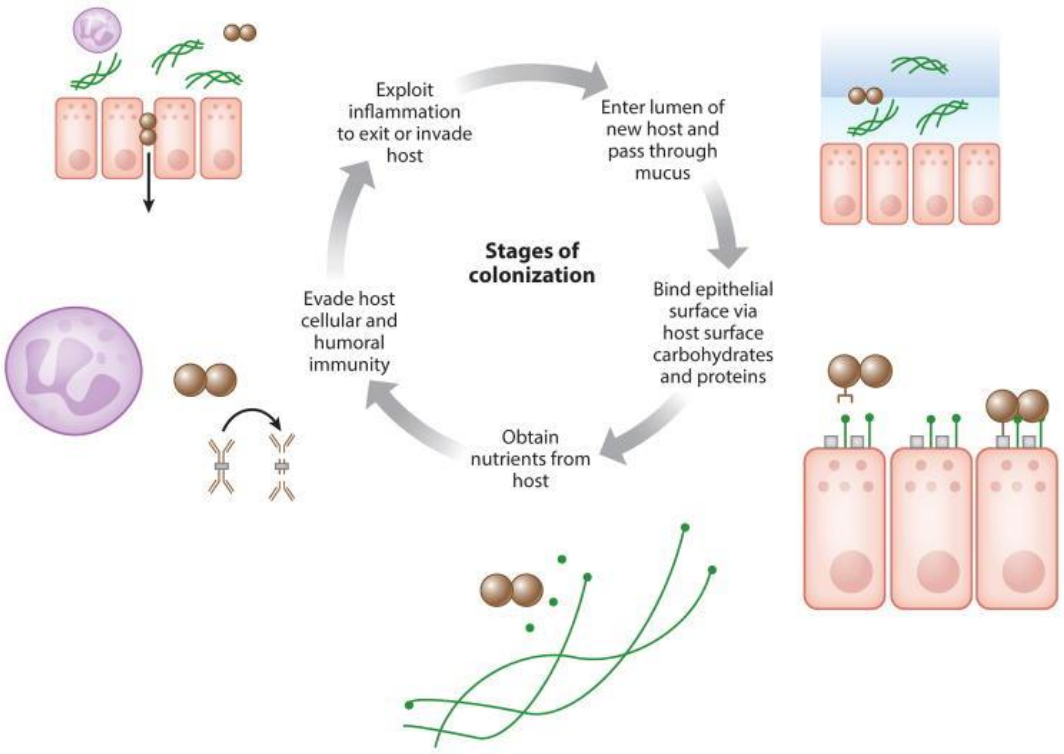


Figure 2: The essential stages of colonisation of the upper respiratory tract presented as a cycle. Colonising bacteria (brown) enter the nasopharyngeal lumen and pass through the mucous layer (blue), in part facilitated by their capsule. Next, bacteria reach the epithelial surface and bind loosely and tightly to host surface carbohydrates and proteins, respectively. Bacteria obtain nutrients by exploiting host inflammation (through, for example, digestion of sialylated mucins) and evading nutritional immunity. These nutrients allow for microbial replication; persistence also involves evasion of host immune responses, both humoral (through IgA1 protease) and cellular. Opportunistic pathogens exploit these responses to drive transmission by exiting the host, and the same factors that allow for increased colonisation and transmission predispose to invasion of host barriers, potentially a strategy for persistence. Image source: Siegel and Weiser, 2015.

Using an experimental human model of infection, it has been shown that colonisation can take up to 24 hours to establish following nasal challenge (Nikolaou *et al.*, 2021). The bacteria are transmitted when they are shed in nasal or oral secretions of colonised individuals (Mitsi *et al.,* 2019). Transmission is believed to occur through inhalation of airborne droplets, direct person-to-person contact and contact with contaminated surfaces. Generally, adults acquire pneumococcus when they are in contact with children. Environments in which adults are in closed settings including daycare centers, nursing homes, military camps, prisons and shelters for the homeless are known to facilitate transmission of the bacteria (Musher, 2003). Recently, hand-to-nose transmission of pneumococcus resulting in successful colonisation events was demonstrated using an experimental human model of infection (Connor *et al.*, 2018). Aerosol transmission of the bacteria was first described in 2010 in a ferret co-infection model with influenza A (McCullers *et al.*, 2010). In pairs of ferrets infected (donor) and uninfected (contact) with *S.*

pneumoniae, aerosol transmission of the bacteria over 3.5 meters occurred when both donor and contact ferrets were infected with influenza. In the absence of infection with influenza in the contact ferrets, no aerosol transmission of the bacteria was observed.

Shedding of the bacteria from the colonised host increases with inflammation of the upper respiratory tract, which may be caused by concomitant viral infection (Regev-Yochay *et al.*, 2004). Moreover, infection of the nasopharynx with influenza virus has been demonstrated to promote nasopharyngeal pneumococcal acquisition, increase colonisation density of pneumococcus and alter host immune responses, thereby reducing control of the bacteria (Wadowsky *et al.*, 1995; Grijalva *et al.*, 2014; Mina, McCullers and Klugman, 2014; Siegel, Roche and Weiser, 2014; Jochems *et al.*, 2018). Studies conducted by Morens and colleagues and many others have found that during past influenza pandemics, secondary bacterial pneumonia has been the leading cause of death (Morens, Taubenberger and Fauci, 2008). Moreover, using a human co-infection model of *S. pneumoniae* and Live Attenuated Influenza Vaccine (LAIV), re-stimulation of nasal cells with serotype 6B pneumococcus *in vitro* following homologous experimental pneumococcal challenge *in vivo* showed increased immune response, which was impaired by antecedent LAIV administered directly to the nasal mucosa (Jochems *et al.*, 2018; Rylance *et al.*, 2019). Antecedent vaccination with LAIV promoted transient increases in pneumococcal acquisition and density in the nasopharynx, whereas bacterial density did not change when immunisation followed bacterial acquisition. Consequently, the timing of vaccination with LAIV may alter host immune responses to pneumococcus and influence transmission of the bacteria. Similarly, increased nasopharyngeal colonisation density of pneumococcus has been observed within a month after vaccination with LAIV in healthy children carrying the bacterial species at the time of vaccination as compared to a control group (Thors *et al.*, 2016). These results indicate that in the immediate period following vaccination with LAIV, the nasopharynx may become increasingly more susceptible to pneumococcus colonisation and persistence. This theoretically could have an impact on transmission of pneumococcus on a population level, affecting susceptible individuals in close contact of vaccinated children. Interestingly, it was recently shown that experimentally induced pneumococcal colonisation in humans dampens LAIV induced immune responses in the nose and lungs (Carniel *et al.*, 2021). This is an important finding as pneumococcal colonisation at the time of LAIV immunisation could hamper the development of a robust adaptive immune response to the virus.

Significantly increased pneumococcal transmission in infants is associated with cooler temperatures and drier climates (Numminen *et al.*, 2015). It is well established that climatic and

environmental conditions correlate with incidence of pneumococcal acquisition and invasive disease. Prevalence is highest during winter months and in temperate climates, which could be attributed to climatic factors, concurrent viral infections, indoor crowding and air pollution (Kim *et al.*, 1996; Dowell *et al.*, 2003; White *et al.*, 2009; Vodonos *et al.*, 2016; Sahuquillo-Arce *et al.*, 2017).

1.1.2.1 Pneumococcal capsule involvement in colonisation

Arguably, the most important virulence factor of pneumococcus and a major component of pneumococcal colonisation is the capsule. The capsule is predominantly made up of negatively charged polysaccharides – although in some cases, such as serotype 1, zwitterionic polysaccharides – which inhibit entrapment of the bacteria in nasopharyngeal mucus by repelling sialic-rich mucopolysaccharides and thus prevent mucus-mediated clearance (Nelson *et al.*, 2007). Moreover, the capsule inhibits opsonophagocytosis by impairing binding of C3b to the pneumococcal cell-wall or blocking access to bound C3b on the cell-wall following activation of the host alternative complement pathway (Winkelstein, Abramovitz and Tomasz, 1980). Resistance to non-opsonic killing of pneumococcus by human neutrophils has been directly linked to the degree of encapsulation as survival increases with capsule size (Weinberger *et al.*, 2009). It is the vast number (>100) of pneumococcal capsular serotypes, which poses the biggest challenge to the control of the pathogen around the world. The prevalence of serotypes varies amongst populations, and different serotypes predominate in invasive and non-invasive disease. Antigenically similar subgroups of serotypes are grouped by number and distinguished by a letter (for example, serotype subgroup 6 has four members, A-D). More heavily encapsulated serotypes tend to be more prevalent in the population. Moreover, it was observed that polysaccharide structure within the capsule is associated with degree of encapsulation, prevalence and resistance to non-opsonic killing, suggesting a direct relationship between polysaccharide biochemistry and the success of a serotype colonising the nasopharynx (Weinberger *et al.*, 2009).

In 1994, Weiser and colleagues reported on major differences in opaque and transparent pneumococcal phenotypes (Weiser *et al.*, 1994). Spontaneous switching between opaque (thicker capsule) and transparent (thinner capsule) phases was observed, with serotype independent variation. Most notably, the transparent morphology demonstrated efficient and stable colonisation of the murine nasopharynx whereas no significant colonisation by the opaque phenotype was observed. A lack of significant difference in incidence of bacteraemia in the murine model following intraperitoneal inoculation with the transparent phase suggested that differences in colony morphology may play a role in the interaction of the bacteria with the host. Subsequent work has found that the opaque and

transparent phases are distinct in their metabolism and cell surface features, with phenotype playing a key role in ability to colonise and virulence (Li and Zhang, 2019). Whilst the opaque morphology is a poor coloniser of the nasopharynx, the phenotype predominates in invasive disease (Trappetti *et al.*, 2011). Conversely, transparent morphology, in which a thinner polysaccharide capsule is expressed, promotes nasopharyngeal colonisation, potentially due to the exposure of surface proteins (Kadioglu *et al.*, 2008; Pichichero, 2017). Phenotypic variation of the polysaccharide capsule varies during initiation of pneumococcal infection (Hammerschmidt *et al.*, 2005) and thus phase variation in colony morphology of the pathogen is one mechanism by which the polysaccharide capsule plays a role in pathogenesis. Phase variation is driven by recombination of *hsdS* genes in the type-I restriction-modification system locus of the pneumococcal genome, leading to alternative methylation and gene expression patterns, including of the capsule locus (Croucher *et al.*, 2014; Li *et al.*, 2016; De Ste Croix *et al.*, 2020).

Some serotypes, for example serotypes 1, 4, 7F and 14, have been shown to be associated with higher risk of invasive disease in children and are less persistent colonisers of the nasopharynx (Brueggemann *et al.*, 2004; Sandgren *et al.*, 2004). Others commonly colonise the nasopharynx of healthy carriers for longer periods of time as well as being associated with high invasive disease potential, such as serotypes 6A, 6B, 14, 19F and 23F (Brueggemann *et al.*, 2004). A study in a cohort of children in the US found that the relative invasiveness of different capsular serotypes in children is inversely correlated with duration of carriage and significantly correlated with increased likelihood of invasive disease (Sleeman *et al.*, 2006). Capsular serotypes 4, 14, 7F, 9V, and 18C were associated with rates of >20 invasive pneumococcal disease cases/100,000 acquisitions, whereas capsular serotypes 23F, 6A, 19F, 16F, 6B, and 15B/C were associated with <10 invasive pneumococcal disease cases/100,000 acquisitions.

Furthermore, it has been shown in murine models of colonisation that unencapsulated variants of pneumococcus, while capable of colonising, do so at reduced density and duration as compared to their encapsulated parent strains (Nelson *et al.*, 2007; Cohen *et al.*, 2012). However, a 2016 review by Keller and colleagues found that unencapsulated pneumococcal strains are increasing in prevalence amongst the global population, possibly due to effects on the pneumococcal niche by pneumococcal vaccines. Non-encapsulated strains have been shown to cause disease and notably, significantly higher rates of genetic recombination occur in sporadic lineages as compared to classical lineages, increasing potential for acquisition of antibiotic resistance and virulence factors. Unencapsulated pneumococcal strains are also able to colonise the nasopharynx and cause disease, but prevalence is higher in non-invasive disease (such as otitis media (8%) and conjunctivitis (85%)) (Keller, Robinson and McDaniel, 2016).

1.1.2.2 The role of pneumococcal surface proteins and enzymes in colonisation

There are three main protein groups which adorn the pneumococcal surface: lipoproteins, LPXTG proteins and choline-binding proteins (Pérez-Dorado *et al.*, 2012). There are also a small number (approximately six) of non-classical surface proteins, which lack leader peptide and membrane-anchoring motifs. Directly beneath the polysaccharide capsule is the bacterial cell wall, made up of an upper layer of teichoic acids and lower layer of peptidoglycan, beneath which is the phospholipid membrane. The lipoproteins possess an *N*-acyl diacylglyceryl group at the N-terminal through which they are anchored to the cell membrane. Notably, a number of these lipoproteins are ATP-binding cassette transporters and thus play an essential role in maintaining the survival of the commensal pneumococcus (Tettelin *et al.*, 2001). More than a dozen degradative enzymes and pilus proteins are covalently anchored by an LPXTG bridge to the cell wall peptidoglycan. Choline-binding proteins are characterized by a biologically active module and a choline-binding module, which is made up of repeats of approximately 20 amino acids (Pérez-Dorado *et al.*, 2012). These proteins are anchored to the cell wall through non-covalent binding between teichoic acid residues and the choline-binding domain. Figure 3 provides an overview of the role of pneumococcal proteins in adherence to host mucosal surfaces, subversion of the host immune response and colonisation.

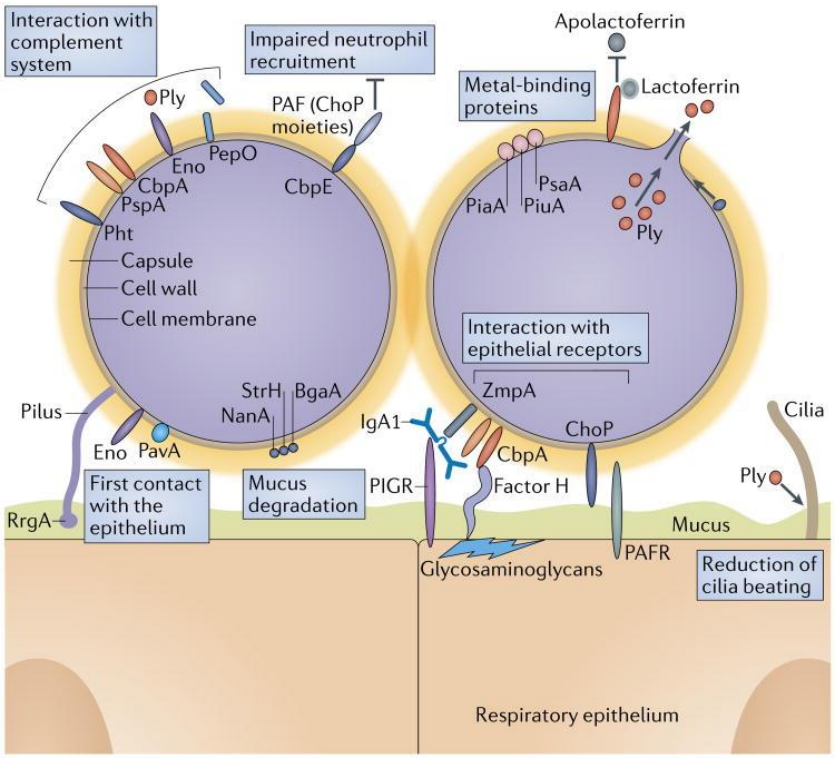


Figure 3: Molecular mechanisms of pneumococcal colonisation of host surfaces. Key functions that enable *Streptococcus pneumoniae* colonisation are: establishing the first contact with the epithelium and epithelial receptors, interaction with the complement system, mucus degradation, metal binding, impairment of neutrophil activity and the pro-inflammatory effects of the toxin pneumolysin (Ply). The pneumococcal enzymes Neuraminidase A (NanA), β-galactosidase (BgaA) and β-N-acetylglucosaminidase (StrH) degrade mucus and thereby inhibit mucociliary clearance. Furthermore, the LytA (autolysin)-facilitated release of Ply damages the epithelium and reduces ciliary beating. Negatively charged capsular polysaccharide (CPS) inhibits bacterial mucus entrapment. CPS and several pneumococcal proteins, including pneumococcal surface protein A (PspA), choline-binding protein A (CbpA), enolase (Eno) and pneumococcal histidine triad protein (Pht), directly and indirectly block complement deposition. PspA also binds to lactoferrin to acquire iron and blocks the antimicrobial effect of apolactoferrin. Endopeptidase (PepO), which is released from the pneumococcal surface, binds to C1q, and thereby depletes complement components. Pneumococcal CbpE impairs neutrophil recruitment by degrading platelet-activating factor (PAF), a host-derived inflammatory phospholipid. CbpA interacts with factor H interactions to facilitate adherence and subsequent internalization of *S. pneumoniae* via cell glycosaminoglycans. CbpA also binds to polymeric immunoglobulin receptor (PIGR) to promote adherence. The zinc metalloprotease ZmpA (also known as immunoglobulin A1 protease) subverts mucosal humoral immunity by cleaving IgA1. Phosphorylcholine (ChoP) on teichoic acid mimics host PAF and allows binding to its receptor. Piliated strains express an ancillary pilus subunit tip adhesin called RrgA. Other *S. pneumoniae* adhesins include enolase (Eno) and adherence and virulence protein A (PavA). PAFR, platelet-activating factor receptor. Image source: Weiser, Ferreira and Paton, 2018.

Once in the host, pneumococcus evades the agglutinating activity of IgA1 on the mucosal surfaces of the URT by producing IgA1 protease (zinc metalloprotease ZmpA), which cleaves IgA1 at its hinge region. IgA1 is the most abundant immunoglobulin on mucosal surfaces. In addition, surface-associated exoglycosidases expressed by the pneumococcus (neuroaminidase A, β-*N*-acetylglucosaminidase, β-galactosidase, neuroaminidase B and a second β-galactosidase in many serotypes) aid in inhibiting

mucociliary clearance by removing terminal sugars present on many human glycoconjugates, thereby degrading mucus, and further potentially revealing host receptors to which the bacteria can adhere, promoting colonisation. The main way in which pneumococcus escapes clearance by mucociliary flow to facilitate colonisation is adherence to host cells and surfaces. Pneumococcus is a pathogen known to bind to host cell glycoconjugates, including *N*-acetylglucosamine-β-(1,4)-galactose.

Phosphorylcholine (ChoP), a functional group derived from phosphocholine, is a component of the cell wall teichoic acid and aids in the adherence of the bacteria to mucosal surfaces by binding platelet-activating factor receptors (PAFR), which are widely distributed on epithelial surfaces of the human nasopharynx. The protein PspC (also known as choline-binding protein A or CbpA) is non-covalently anchored to ChoP and binds the human secretory component of polymeric immunoglobulin receptors and secretory forms of immunoglobulin. PspC also binds both C3 and host immune complement inhibitor, factor H at two contact sites, which has been shown to facilitate bacterial adherence to mucosal epithelium (Dave *et al.*, 2001; Hammerschmidt *et al.*, 2007). Binding of PspC to factor H promotes cleavage of C3b on the bacterial cell surface and thus inhibits alternative complement-mediated clearance of the bacteria (Pathak *et al.*, 2018).

Pneumococcal surface protein A (PspA) is a serologically highly variable and ubiquitous surface-exposed choline-binding protein (Crain *et al.*, 1990) and has been shown to play a role in pneumococcal virulence in various ways. PspA has five domains, including (i) a signal peptide, (ii) an α-helical and charged domain, (iii) a proline-rich region domain, (iv) a choline-binding domain, which is required for the attachment of PspA to the pneumococcal cell surface via interaction with choline in membrane-associated lipoteichoic acid, and (v) a C-terminal 17-amino-acid tail (Hollingshead, Becker and Briles, 2000). PspA can be grouped into three families that, in turn, can be subdivided into six different clades based on the sequences of the alpha helical region. PspA binds human lactoferrin, a protein released in large quantities during inflammation. It is postulated that the binding of PspA to lactoferrin may be a way in which PspA inhibits recruitment of the alternative pathway and thus deposition of C3 onto the pneumococci (Tu *et al.*, 1999; Håkansson *et al.*, 2001; Ren *et al.*, 2004). Absence of PspA has been shown to result in enhanced deposition of C1q and thus classical-pathway mediated deposition of C3 on pneumococci (Li *et al.*, 2007). Moreover, the binding may facilitate adherence to the host epithelium. PspA may also compete with C-reactive protein (CRP) for binding sites on phosphocholine, thereby inhibiting CRP-mediated complement deposition (Mukerji *et al.*, 2018).

PspC is a paralog of PspA and the absence of both PspC and PspA results in a major increase in C1q-independent C3 deposition through the alternative pathway, although lack of PspC alone does not result in such an increase of C3 deposition (Li *et al.*, 2007). Clearance of pneumococcus in murine models is accelerated in the absence of PspC and PspA (Balachandran *et al.*, 2002). Furthermore, knock-out of PspA or PspC results in reduced C4b-binding protein deposition on bacterial surfaces and thus an increase in C4b and iC4b deposition, with greatest effect when both PspC and PspA are absent (Haleem *et al.*, 2019). Absence of PspC and PspA in a murine model of infection also resulted in loss of bacterial pathogenicity (Haleem *et al.*, 2019). In this way, it is believed PspC and PspA enable the pneumococcus to evade host complement attack.

Pneumolysin is another major virulence factor of the pneumococcus, well studied for its cytotoxic and haemolytic activity (Paton *et al.*, 1993). A cholesterol-dependent cytolysin, pneumolysin binds cholesterol on host cell membranes and induces pore formation, causing cell lysis. In a mouse model, it was demonstrated that pneumolysin facilitated intra-alveolar replication of pneumococci, penetration of bacteria from alveoli into the interstitium of the lung, and dissemination of pneumococci into the bloodstream during experimental pneumonia (Rubins *et al.*, 1995). Inflammatory cell influx in response to pneumococcal infection is significantly delayed and dampened in the absence of pneumolysin; the rate of neutrophil influx in particular is reduced and less intense (Kadioglu *et al.*, 2000). Pneumolysin promotes 12-Lipoxygenase-dependent neutrophil recruitment across pulmonary epitheliums in a pore-dependent manner (Adams *et al.*, 2020). The protein has also been implicated in biofilm formation, an important part of the colonisation process, independent of its haemolytic activity (Shak *et al.*, 2013). The release of pneumolysin is facilitated by the major pneumococcal autolysin LytA, which has also been implicated in thinning of the capsule during the invasion process (Kietzman *et al.*, 2016). Notably, the binding of pneumolysin to mannose receptor C type 1 (MRC-1) promotes pneumococcal invasion of MRC-1-proficient immune cells, such as alveolar macrophages, thereby inhibiting inflammatory response and neutrophil infiltration, enhancing pneumococcal survival in the airways (Subramanian *et al.*, 2018). Pneumolysin has also been implicated in the increased pathogenicity of serotype 1 pneumococcus (Jacques *et al.*, 2020).

Adhesin proteins surface-located pneumococcal adherence and virulence protein A (PavA), PavB and enolase (Eno) bind extracellular matrix proteins fibronectin and plasminogen, facilitating pneumococcal adherence to host cells and tissues (Bergmann *et al.*, 2001; Holmes *et al.*, 2001; Jensch *et al.*, 2010).

Table 1 provides a summary of well characterised pneumococcal surface proteins and their functions.

Table 1: Summary of well characterised surface proteins of *S. pneumoniae* (Bergmann and Hammerschmidt, 2006; Kadioglu et al., 2008).

Lipoproteins	**Full name**	**Role**
PiaA	Pneumococcal iron acquisition A	Iron transport
PiuA	Pneumococcal iron uptake A	Iron transport
PpmA	Putative proteinase maturation protein A	Adherence to epithelial cells
PsaA	Pneumococcal surface adhesin A	Adhesion to host cells, Manganese transport
SlrA	Streptococcal lipoprotein rotamase A	Modulates virulence factors
Choline binding proteins		
CbpD	Choline binding protein D	Role in competence-induced cell lysis, responds to CSP
CbpE	Choline binding protein E	Phosphorylcholine esterase, removes phosphorylcholine from cell wall
LytA	Autolysin A	Cell wall hydrolysis role in autolysis
PspA	Pneumococcal surface adhesin A	Binds apolactoferrin, Inhibits complement deposition
PspC	Pneumococcal surface adhesin C	Binds complement factor H, Role in adhesion to host tissue
LPxTG protein		
HtrA	High-temperature requirement A	Heat-shock induced serine proteases, resistance to oxidative stress
Hyal	Hyaluronidase	Breakdown of host extracellular matrix
IgA1ase	IgA1 protease	Cleaves IgA1
NanA	Neuraminidase A	Cleavage of terminal sialic acid residues on host cell glycolipids and glycoproteins
PrtA		Serine protease
Non-classical		
Eno	Enolase	Binds to plasminogen, promotes degradation of extracellular matrix
PavA	Pneumococcal adhesion and virulence A	Binds fibronectin
PhtD	Pneumococcal histidine triad protein D	Zinc binding protein
PhtE	Pneumococcal histidine triad protein E	Metal binding protein, adherence to host tissue
PilusA	Pneumococcal pilus	Adhesion to host cells

1.2 Clinical presentations of pneumococcal disease

Beyond asymptomatic nasopharyngeal carriage of the bacteria, pneumococci may cause localised or invasive disease (Figure 4). Local spread of the bacteria can lead to otitis media or sinusitis. Aspiration of the bacteria into the distal pulmonary alveoli without effective clearance by the immune response in the lungs results in pneumonia, an inflammation of the alveoli causing build-up of fluid or pus within the alveoli. If the infection breaks through mucosal surfaces into sterile sites, including the bloodstream, the pneumonia may become bacteremic. Spread of the bacteria from the alveoli to the pleura or pericardium may also cause empyema. Invasive pneumococcal disease, including septicemia and meningitis, can also occur independently of lung infection when the bacteria penetrates epithelial or endothelial surfaces into the bloodstream or tissues. In the case of meningitis, the bacteria also cross the blood brain barrier. Septicemia may further lead to empyema, peritonitis, arthritis/osteomyelitis or meningitis (Bogaert, De Groot and Hermans, 2004). The severity of pneumococcal disease is inversely related to incidence rate (Figure 4).

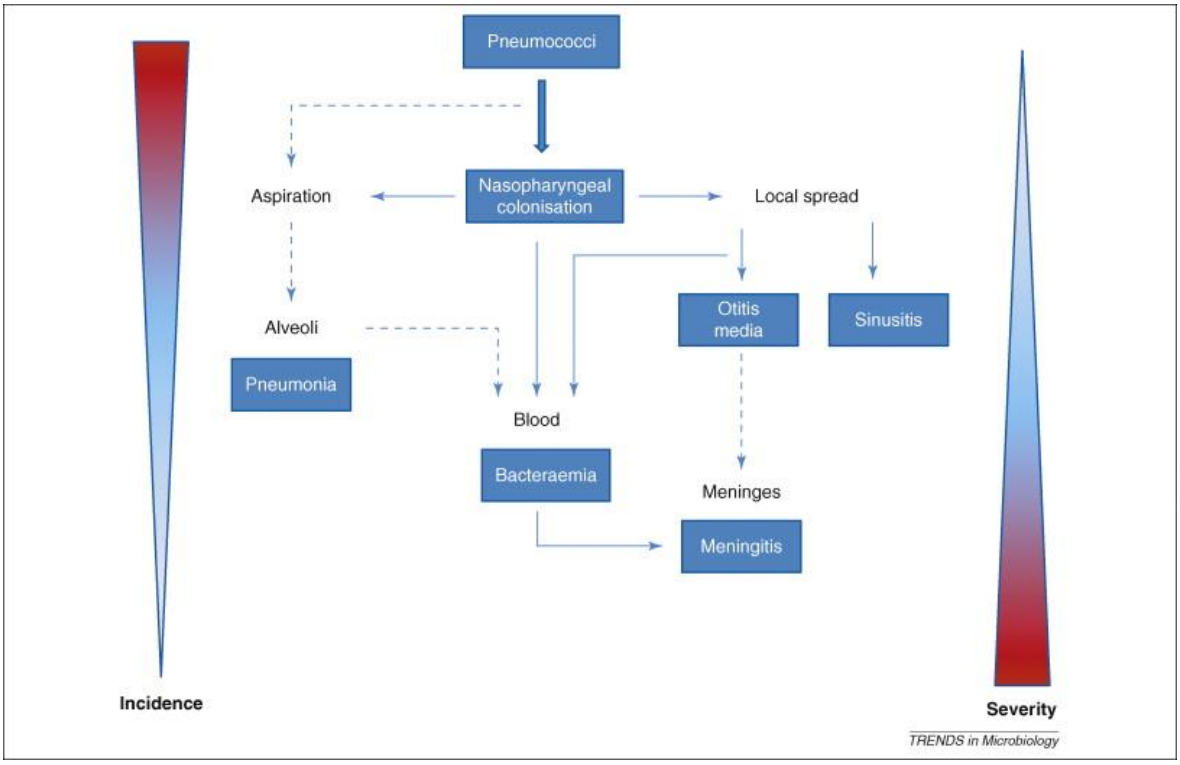


Figure 4: Diseases caused by Streptococcus pneumoniae. Pneumococci colonise the nasopharynx, evade host immunity and spread to the middle ear, sinus, lower respiratory tract, blood, and meninges. Pneumococci cause otitis media in the middle ear, sinusitis in the sinus, pneumonia in the lower respiratory tract, bacteraemia in blood and meningitis in the meninges. The incidences of different types of pneumococcal infection are inversely related to the severity of disease: otitis media is the most common but the least severe. Image source: Jambo et al., 2010.

Colonising pneumococci are microaspirated from the nasopharynx into the lungs (Mitsi *et al.*, 2020). Recently, it has been shown for the first time that nasopharyngeal colonisation density with pneumococcus positively correlates with severe pneumonia in children (Carr *et al.*, 2021).

1.3 The burden of pneumococcal pneumonia

Most childhood pneumonia deaths occur in children under 2 years of age. There is an undeniably strong correlation between country income and pneumonia mortality rate (Figure 5) (Wahl *et al.*, 2018; Watkins and Sridhar, 2018; McAllister *et al.*, 2019; Bernadeta Dadonaite and Max Roser, 2019). In 2017, over 50% of all 808, 694 deaths in children under 5 years of age attributed to pneumonia occurred in just 5 countries: India, Nigeria, Pakistan, the Democratic Republic of Congo, and Ethiopia (WHO, 2019; Bernadeta Dadonaite and Max Roser, 2019). Global pneumonia mortality rates in 2017 were highest in people aged 70 and over (261 people in this age group out of every 100,000 died because of pneumonia), killing 1.13 million people of this age (Bernadeta Dadonaite and Max Roser, 2019). 75% of all pneumonia deaths occur in adults over 70 and children under 5 (Forum of International Respiratory Societies (FIRS), 2020).

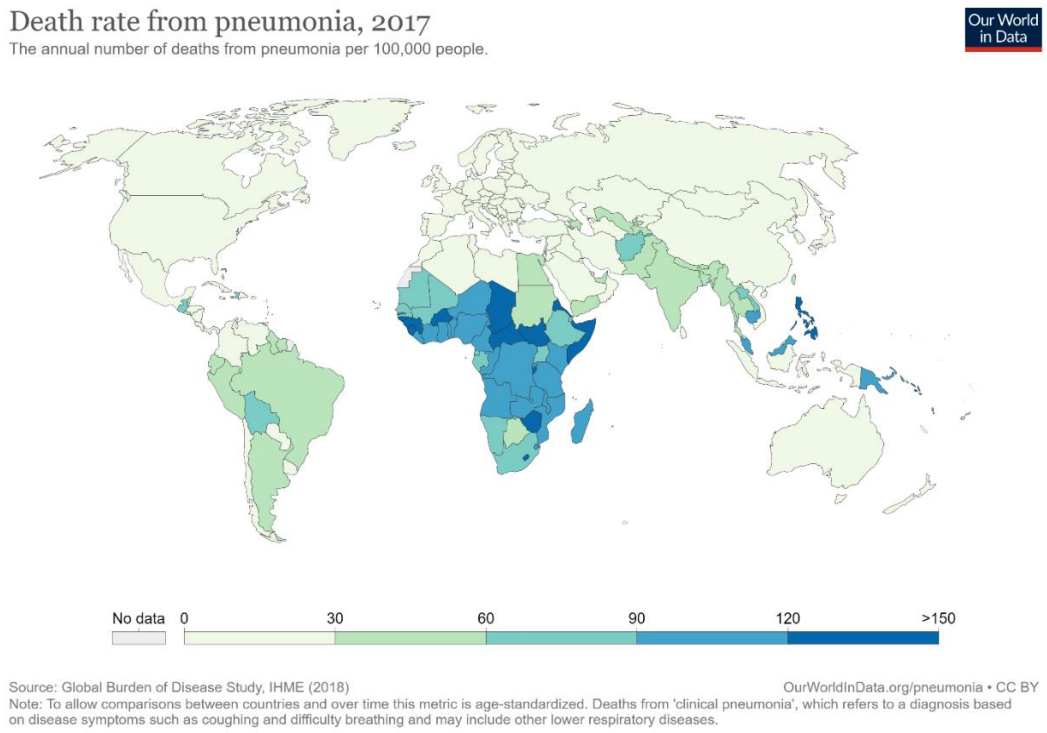


Figure 5: Death rates from pneumonia in 2017. Image source: IHME, 2017.

Nevertheless, community-acquired pneumonia continues to be a major source of morbidity and mortality in high income countries (Ferreira-Coimbra, Sarda and Rello, 2020); at least 25% of all cases of community-acquired pneumonia are caused by pneumococcus (Said *et al.*, 2013). The burden of the disease is high across the UK, Europe and North America with community-acquired pneumonia a leading cause of admission to hospital and intensive care units (Kaplan *et al.*, 2002; Trotter *et al.*, 2008; File and Marrie, 2010; Welte, Torres and Nathwani, 2012; Millett *et al.*, 2013; Walden *et al.*, 2014; Quan *et al.*, 2016). Prior to the SARS-CoV-2 pandemic, in England and Wales, pneumonia was the sixth-leading cause of death (Daniel *et al.*, 2016). It is estimated that prior to the pandemic, pneumonia affected 8 in 1000 adults in the UK each year and 29,000 deaths occurred annually because of the disease. 5-15% of patients admitted to hospital with community-acquired pneumonia die within 30 days of admission (Chalmers *et al.*, 2017). Pneumonia is the leading infectious cause of death across Europe and 90% of all pneumonia deaths in Europe occur in the over 65 age group (Welte, Torres and Nathwani, 2012; Torres *et al.*, 2013). Moreover, a study conducted in the Netherlands found that in patients admitted to hospital with community-acquired pneumonia, there was a six-fold increased rate of mortality and a 16% lower quality of life in the year following discharge from hospital compared to non-diseased individuals (Mangen *et al.*, 2017).

Aside from age, risk groups for community-acquired pneumonia include smokers, or those exposed to second-hand smoke in the home, and heavy drinkers. Malnutrition and wasting are also significantly associated with greater risk of acquiring pneumonia. Individuals with co-morbidities, such as chronic respiratory diseases, diabetes, viral co-infection, weakened immune system, chronic heart disease, neurological disorders and chronic renal disease are particularly at risk from community-acquired pneumonia (Millet *et al.,* 2013; Pereira *et al.,* 2013; Torres *et al.,* 2013).

It has been well documented that the most common infectious cause of community acquired pneumonia is *Streptococcus pneumoniae* (Welte, Torres and Nathwani, 2012; Walden *et al.*, 2014; WHO, 2019). Furthermore, pneumococcal pneumonia has been shown to be associated with a three-fold higher risk of mortality than non-pneumococcal pneumonia (Welte, Torres and Nathwani, 2012). The Global Burden of Disease collaboration estimated that, in 2016, pneumococcal pneumonia resulted in a total of 1,189,937 deaths across all ages, including 341,029 children under 5 and 494,340 people aged 70 years and older. The disease accounted for 197,050,000 episodes (Troeger *et al.*, 2018). Of the 10 million reported cases of childhood pneumococcal pneumonia in 2015, global mortality rate was approximately 36%, with a 76% mortality rate in Africa (Wahl *et al.*, 2018).

Of all pneumococcal deaths in HIV-uninfected children in 2015, pneumonia accounted for 81%, meningitis accounted for 12%, and non-pneumonia/non-meningitis accounted for 7%. A systematic review and meta-analysis of 35 studies conducted in middle and high income countries found that around 25% of all pneumococcal pneumonias were bacteremic (Said *et al.*, 2013).

The mean cost of pneumococcal pneumonia per episode in the US reaches $4,725 in adults with no additional risk conditions and up to $6,534 and $9,168 in at-risk and high-risk individuals, respectively (Zhang, Petigara and Yang, 2018). It is estimated that associated pneumonia costs in Europe are approximately €10 billion per year, mainly due to hospitalisation and lost working days (Torres *et al.*, 2013).

1.3.1 Antimicrobial resistance (AMR)

1,270,000 deaths were caused by and 4,950,000 deaths were associated with antimicrobial resistance in 2019 (Murray *et al.*, 2022). 122,000 deaths were caused by antibiotic-resistant *S. pneumoniae* (15.9% of all deaths attributed to AMR), with the highest burden in the African subcontinent. The first line of treatment against pneumococcal disease is administration of amoxicillin, a member of the Penicillin class of antibiotics, or doxycycline, clarithromycin or erythromycin if amoxicillin is inappropriate (for example in the case of penicillin allergy) (NICE, 2019). Antibiotic resistant pneumococci are considered a “serious threat” by the CDC and a priority 3 pathogen by the WHO.

A study using an agent-based modelling simulation of the impact of antibiotic resistance on the treatment of pneumococcal disease in children under 5 in Ethiopia found that increasing PCV coverage over 5 years would significantly reduce resistance against first-line antibiotics (Chen *et al.*, 2019). Importantly, the sensitivity rates of pneumococci isolated from middle ear fluid to multiple antibiotics, including penicillin, have significantly increased from the pre-PCV period (2002-2008) to the post-PCV period (2010-2019) in northern Israel (Paker *et al.*, 2022). Furthermore, over the 10 years since implementation of any PCV product, there have been significant absolute reductions in resistance and non-susceptibility to penicillin, sulfamethoxazole, 3rd generation cephalosporins, macrolides and tetracycline in paediatric pneumococcal isolates (Andrejko *et al.*, 2021). Rates of antibiotic non-susceptible invasive pneumococcal disease caused by serotypes included in PCV13 but not PCV7 also declined in children and adults in the US 3 years post-PCV13 implementation (97% reduction in children under 5 years and 64% reduction in adults over 65 years) (Tomczyk *et al.*, 2016).

Whilst the development of novel antibiotics has stalled over the last 3 decades, vaccine development has advanced, thereby preventing bacterial infections and the use/misuse of antibiotics driving the emergence of antimicrobial resistance (Buchy *et al.*, 2020). Prevalence of antibiotic non-susceptible or resistant non-vaccine serotypes could begin to increase with higher incidence of disease caused by non-vaccine serotypes and subsequent antibiotic therapy (Keller, Robinson and McDaniel, 2016; Tomczyk *et al.*, 2016). However, the implementation of vaccines with even modest effectiveness against serotypes with high levels of resistance could significantly reduce their burden (Van Effelterre *et al.*, 2010). It has been shown that vaccines generally are less likely to induce resistance than antimicrobials (Buchy *et al.*, 2020). To combat the emergence of further resistant strains in the future, vaccines with broad coverage that prevent pneumococcal disease and the need for subsequent antibiotic use are thus essential.

1.4 *S. pneumoniae* and SARS-CoV-2

The relationship between pneumococcus and SARS-CoV-2 has been investigated. Whilst data suggests a low proportion of patients with COVID-19 experience bacterial co-infection, including with pneumococcus (Lansbury *et al.*, 2020), it is unclear whether this lack of incidence of pneumococcal disease in COVID-19 patients is due to reduced transmission following non-pharmacological interventions introduced during the SARS-CoV-2 pandemic, such as social distancing, increased use of masks and improved hygiene procedures, or liberal use of antibiotics within hospital settings for severely ill patients (74.5% of COVID-19 patients admitted to intensive care units in the UK receive antibiotics (Cox *et al.*, 2020)). Large declines in invasive pneumococcal disease (IPD) were observed following the SARS-CoV-2 pandemic lockdown in the UK and COVID-19/IPD cases were rare, though associated with a higher case fatality rate (Amin-Chowdhury *et al.*, 2021). Contradictorily, there has been reporting of a significant rate of bacterial pneumonia (diagnosed by culture of blind bronchoalveolar lavage), mostly late-onset ventilator-associated, in critically ill patients with COVID-19 in an intensive care unit (Dudoignon *et al.*, 2021). A recent observational study in the US suggested that there was a 30% reduction in COVID-19 disease, hospitalisation and deaths in older adults vaccinated with PVC13 (Lewnard *et al.*, 2021). A study was conducted examining the relationship between pneumococcus and SARS-CoV-2 in two distinct cohorts of a) healthcare workers with asymptomatic or mildly symptomatic SARS-CoV-2 infection identified by systematic screening and b) patients with moderate to severe disease who presented to hospital (Mitsi *et al.*, 2021). Findings indicated that colonisation with pneumococcus is associated with diminished anti-viral immune response, reducing mucosal IgA levels among individuals with mild or

asymptomatic infection and cellular memory B-cell populations and $CD4^+$ T-cell responses in infected patients. Whilst further investigation is necessary to ascertain the relationship between pneumococcus and SARS-CoV-2, it is reasonable to hypothesis that pneumococcus may hamper anti-viral response and facilitate immune escape of respiratory viruses.

1.5 Innate immunity to *Streptococcus pneumoniae*

1.5.1 The complement system

The complement system can be divided into three pathways: the classical, mannose-binding lectin (MBL) and alternative. The role of the complement system is to facilitate the recognition and opsonisation of invading pathogens. It links the innate and adaptive immune responses by promoting antigen recognition and improving antibody efficacy. The complement system consists of >30 proteins in the plasma, epithelial lining fluid and cell surfaces. On identification of an invading pathogen, the system is activated via a hierarchical protease cascade. All three complement pathways converge in the cleavage and activation of C3 to C3b, an opsonin deposited on the surface of pathogens along with its degradation product iC3b. Targeted lysis of pathogen surfaces is also achieved by the pore-forming membrane attack complex (MAC). Studies in mice suggest that the classical pathway is most relevant to clearance of pneumococcus (Brown *et al.*, 2002). In addition, patients with an inherited deficiency of the classical complement pathway component C2 have increased incidence of pneumococcal disease and phagocytosis of the bacteria is impaired, suggesting the importance of the classical pathway in control of pneumococcal infection (Yuste *et al.*, 2008). It has also been demonstrated that, in human sera, the classical pathway is vital for C3b-iC3b deposition onto pneumococcus and has a more important role than the alternative pathway for phagocytosis (Yuste *et al.*, 2008). Pneumococci evade complement attack in several ways. The capsule impairs binding of C3b to the pneumococcal cell-wall or blocks access to bound C3b on the cell-wall following activation of the host alternative complement pathway (Winkelstein, Abramovitz and Tomasz, 1980). It has also been shown that capsular serotype has a significant impact on resistance to complement and increased resistance to complement is associated with a higher concentration of polysaccharide-specific antibodies needed for opsonophagocytic killing (Melin *et al.*, 2010). Binding of PspC to factor H promotes cleavage of C3b on the bacterial cell surface and thus inhibits alternative complement-mediated clearance of the bacteria (Pathak *et al.*, 2018). PspA may also compete with C-reactive protein (CRP) for binding sites on phosphocholine, thereby inhibiting CRP-mediated complement deposition (Mukerji *et al.*, 2018). Furthermore, PspC and PspA sequester binding of host C4 protein on the bacterial surface and thus prevent C4b and iC4b deposition (Haleem *et al.*, 2019).

Endopeptidase (PepO), which is released from the pneumococcal surface, binds to C1q and thereby depletes complement components (Weiser, Ferreira and Paton, 2018).

1.5.2 Pattern recognition

Upon contact with the epithelia of the respiratory tract, invading pathogens are first recognized by pattern recognition receptors (PRRs), which can be subdivided into different classes including, Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and the C-type lectin receptors (CLRs). PRRs recognize highly conserved pathogen-associated molecular patterns (PAMPs), bacterial virulence factors, and danger-associated molecular patterns (DAMPs), which are endogenous molecules released after tissue damage (Koppe, Suttorp and Opitz, 2012). Upon recognition of the pathogen by PRRs, an inflammatory response is triggered.

1.5.2.1 Toll-Like receptors

There are 10 known TLRs, of which 3 are relevant to host defense against pneumococcus: TLR-2, TLR-4, TLR-9. TLR signaling involves five adapter proteins: MyD88 (myeloid differentiation primary-response gene 88), MAL (MyD88-adaptor-like protein), TRIF (TIR-domain-containing adaptor protein inducing interferon-β (IFNβ)) and TRAM (TRIF-related adaptor molecule) are recruited to TIR (Toll/interleukin-1 (IL-1) receptor) domains to initiate signalling (O'Neill and Bowie, 2007). Genetic variation in the TLR signaling pathway has been associated with increased risk of invasive pneumococcal disease in children (Tomlinson *et al.*, 2014).

TLR-2 resides on the plasma-membrane and amongst its many ligands, binds to bacterial lipoproteins (Tomlinson *et al.*, 2014) and pneumococcal lipoteichoic acid (Schröder *et al.*, 2003). Reduced clearance of pneumococcus has been observed in TLR-$2^{-/-}$ murine models of colonisation (Knapp *et al.*, 2004; Van Rossum, Lysenko and Weiser, 2005). However, it has also been shown that, while TLR-2 plays a role in the early inflammatory response to pneumococcal pneumonia in mice, clearance of serotype 3 pneumococci is indistinguishable between wild-type and TLR-$2^{-/-}$ mice and mortality unchanged (Knapp *et al.*, 2004). In mice, lipoproteins may enhance immunogenicity of pneumococcal Th17 antigens through activation of TLR-2 receptors (Moffitt *et al.*, 2014). TLR-4 receptors are also transmembrane receptors and recognize lipopolysaccharide (Medzhitov, Preston-Hurlburt and Janeway, 1997) as well as pneumolysin (Srivastava *et al.*, 2005). Work in murine models suggests that innate immune response to pneumococcus and protection against colonisation and pneumococcal disease is dependent on the TLR-4-mediated enhancement of pneumolysin-induced apoptosis (Malley *et al.*, 2003; Srivastava *et al.*, 2005). However, contrasting observations have been made suggesting that clearance of pneumococcus from the murine

nasopharynx is TLR-4 independent (Van Rossum, Lysenko and Weiser, 2005), which may be due to different experimental approaches and the strain of pneumococcus used. Moreover, TLR-4 plays only a minor role in pneumonia, systemic infection and meningitis which may be explained by the redundancy between TLRs (Paterson and Orihuela, 2010). TLR-9 is expressed within endosomal compartments and thus may modulate post-phagocytosis response. The receptor binds unmethylated cytosine-phosphate-guanosine (CpG) motifs of bacterial DNA (Ishii and Akira, 2006). TLR-9 has been implicated in the clearance of TIGR4 pneumococcus from the lower respiratory tract, but not the nasopharynx, of mice challenged with the bacteria and TLR-$9^{-/-}$ mice display enhanced susceptibility to pneumococcal pneumonia (Albiger *et al.*, 2007). However, local inflammatory response in mice is likely TLR-9 independent and bacterial clearance is believed to be mediated by resident alveolar macrophages (Albiger *et al.*, 2007).

1.5.2.2 NOD-like receptors

Intracellular Nucleotide-binding Oligomerization Domain (NOD) 1 and 2 receptors reside in the cytosol and recognize bacterial cell wall components containing D-glutamyl-meso-diaminopimelic acid and muramyl dipeptide, respectively (Paterson and Orihuela, 2010). NOD1 has been shown to play a role in innate response during pneumococcal sepsis in a murine model as *Nod1*$^{-/-}$ mice show increased susceptibility to early pneumococcal sepsis due to the role of NOD1 recognition of the intestinal microbiota in priming the innate immune system (Clarke *et al.*, 2010). Pneumococcus has also been shown to stimulate NOD2 receptors *in vitro* (Opitz *et al.*, 2004) and induce caspase-1 production (Shoma *et al.*, 2008). However, *in vivo,* caspase-$1^{-/-}$ mice have shown no enhanced susceptibility to pneumococcal infection (Albiger *et al.*, 2007).

1.5.2.3 Scavenger proteins

Additional PRRs, which may play a role in the host response against *S. pneumoniae* include scavenger proteins and SIGN-R1 present on macrophages. Receptors scavenger receptor A (SRA) (Arredouani *et al.*, 2006), macrophage receptor with collagenous structure (MARCO) (Arredouani *et al.*, 2004), and mannose receptor (MRC-1) (Macedo-Ramos *et al.*, 2011) have been identified as non-opsonic receptors for *S. pneumoniae* in the lung and contributors to immune defense against pneumococcus and inhaled particles. MARCO$^{-/-}$ mice were shown to have significantly impaired clearance of pneumococcus from the nasopharynx, however the same was not observed for SRA and MR knockout mice (Dorrington *et al.*, 2013). In addition, MARCO is required for TLR-2 and NOD2-dependent NF-κB activation and signalling ultimately involved in the clearance of *S. pneumoniae* from the murine nasopharynx (Dorrington *et al.*, 2013).

1.5.3 Inflammatory response

A delicate balance exists between the beneficial and detrimental effects of the inflammatory response to *S. pneumoniae.* Whilst increased inflammation contributes to pneumococcal clearance, it can also lead to increased tissue damage and susceptibility to invasive disease or pneumonia. For example, the promotion of IFN-γ secretion by natural killer (NK) cells in response to exogenous IL-12 has been shown to protect mice from pulmonary pneumonia due to increased neutrophil recruitment to the lungs (Sun *et al.*, 2007). On the other hand, elevated NK-cell activity can amplify further inflammation in the lungs and systemically, leading to tissue damage and enhancing likelihood of bacteraemia and subsequent poor outcome (Kerr *et al.*, 2005). Increased IL-6 response is known to facilitate pneumococcal clearance through pro-inflammatory activity. Yet the cytokine is also responsible for down-regulating the cytokine network during pneumococcal pneumonia, contributing to host defense (Van Der Poll *et al.*, 1997). TNFα is another pro-inflammatory cytokine well established to play a key role in protection against *S. pneumoniae* infection in the acute phase by enhancing neutrophil influx to infection sites (Takashima *et al.*, 1997; O'Brien *et al.*, 1999; Wellmer *et al.*, 2001). However, in well-functioning elderly adults, high baseline levels of circulating TNFα and IL-6 associate with increased risk of hospitalisation with community-acquired pneumonia in smokers and those with existing medical conditions (Yende *et al.*, 2005). Moreover, in mice, pneumococcal bacteraemia has been associated with TNFα and IL-6 release in blood (Bergeron *et al.*, 1998).

In mice, pneumococcus stimulates a pneumolysin-dependent, type I interferon (IFN) response in airway epithelial and dendritic cells, which promotes pneumococcal clearance (Parker *et al.*, 2011). However, co-infection with influenza creates a synergistic type I IFN response that impairs MCP-1 secretion and subsequent macrophage recruitment, leading to increased pneumococcal colonising density and susceptibility to invasive disease (Nakamura, Davis and Weiser, 2011). It has been shown that lung epithelial cells are stimulated to express IFN-stimulated genes by IFNα/β secreted by macrophages (Koppe, Suttorp and Opitz, 2012). IFN secretion by macrophages further promotes RANTES production, an essential factor for protective adaptive pneumococcal immunity (Palaniappan *et al.*, 2006), by macrophages and lung epithelial cells (Koppe, Suttorp and Opitz, 2012).

1.5.4 Phagocytes

Phagocytes play a key role in the immediate host response to invasion by internalizing the foreign pathogen into a phagosome, which fuses with lysosomes to destroy the engulfed pathogen.

1.5.4.1 Neutrophils

Neutrophils are abundant at the human nasal mucosal epithelium and sub-epithelium, even in the absence of infection (Jochems *et al.*, 2019). Colonisation with pneumococcus has been shown to enhance neutrophil degranulation, which promotes pneumococcal clearance, thus suggesting an important role in the initial control of carriage (Jochems *et al.*, 2018). Moreover, neutrophil activity has been found to play a vital role in two distinct profiles of bacterial clearance in an experimental model of colonisation (Nikolaou *et al.*, 2021). Resident mucosal neutrophil activity mediated immediate (within 1-hour post-challenge) clearance of pneumococcus in the nose (defined by the absence of pneumococcal DNA in saliva 1-hour post exposure) and increased levels of neutrophils in the nose prior to challenge were protective against pneumococcal colonisation. Delayed clearance following rapid movement of the bacteria to the saliva following exposure (defined by the presence of pneumococcal DNA in saliva 1-hour post exposure) was mediated by an inflammatory response and increased neutrophil activity 24-hours post bacterial encounter (Nikolaou *et al.*, 2021).

Neutrophil recruitment to the lungs of mice was shown to elicit protection against *S. pneumoniae* infection (Sun *et al.*, 2007) and antibody-depletion of neutrophils has also led to increased bacterial burden in the alveolar spaces of mice 24 hours post intranasal challenge with *S. pneumoniae* (Sun and Metzger, 2008). However, dysregulation of neutrophil influx and activity in the lung, can lead to toxic inflammation and lung injury, characteristic of pneumonia, and subsequent poor outcome (Dockrell, Whyte and Mitchell, 2012; Bordon *et al.*, 2013). Migration of neutrophils into the alveolar space can enhance bacterial invasion into the blood, bacteraemia and mortality (Marks *et al.*, 2007).

Neutrophil extracellular traps (NETs) are released by activated neutrophils to trap and kill pathogens (Brinkmann *et al.*, 2004). However, the expression of a surface endonuclease, endA, by pneumococcus facilitates degradation of the DNA scaffold of NETs and thus escape by the bacteria (Beiter *et al.*, 2006). NET formation in response to pneumococcal infection in a mouse model has been shown to strongly positively correlate with capsule thickness and proportionate with disease severity. Thicker capsules appear to offer protection against neutrophil-mediated killing (Moorthy *et al.*, 2016). NET formation frequency also increases in secondary pneumococcal pneumonia following primary influenza infection (Moorthy *et al.*, 2016), however these NETs are associated with severe pulmonary injury rather than any antibacterial activity (Moorthy *et al.*, 2013).

1.4.4.2 Monocytes

Classical monocytes proliferate in the bone marrow and migrate along a chemokine pathway in a CCR2-dependent manner to the site of infection (Serbina and Pamer, 2006; Davis, Nakamura and Weiser, 2011). In response to infection, monocytes proliferate, phagocytose pathogens, present antigens via MHC class II complexes and secrete chemokines promoting recruitment of immune cells. In the murine lung, classical monocytes are able to remain undifferentiated or differentiate in pulmonary macrophages or dendritic cells (Landsman and Jung, 2007; Landsman, Varol and Jung, 2007; Chiu and Bharat, 2016). In the human nose however, limited differentiation into macrophages occurs and classical monocytes predominate (Jochems *et al.*, 2019). TLR-2-dependent recruitment of monocytes/macrophages in the upper airway lumen of a murine model resulted in pneumococcal engulfment by those cells, which were key effectors in clearing primary pneumococcal colonisation (Zhang, Clarke and Weiser, 2009). Findings from a co-culture model of human monocytes and $CD4^+$ T-cells suggested that live pneumococci trigger a Th1-biased response via monocyte production of IL-12p40, whereas heat-killed pneumococci trigger a Th17 response through TLR-2 signaling (Olliver *et al.*, 2011).

In a human model of pneumococcal infection, establishment of pneumococcal carriage led to a significant recruitment of monocytes to the nasal mucosa as early as 2 days post-inoculation with serotype 6B, peaking at 9 days post-inoculation and monocyte function associated with pneumococcal clearance (Jochems *et al.*, 2018). Monocyte numbers remained elevated up to 29 days post-inoculation. Importantly, pneumococcal density is positively associated with monocyte recruitment, with no increase in monocyte numbers when pneumococcal density is low. Furthermore, monocyte recruitment was shown to increase in response to increased concentration of MCP-1 (monocyte chemoattractant protein-1).

Digestion of pneumococci by phagocytes in mice results in the secretion of MCP-1 and additional recruitment of monocytes via an auto-feedback loop (Davis, Nakamura and Weiser, 2011). C-C-chemokine receptor type 2 is responsible for promoting monocyte migration to infected sites in response to chemo-attractants; its deficiency on the surface of monocytes perturbs the recruitment of TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) expressing cells into the lung and thus damage of lung tissue and bacterial load is reduced.

1.5.4.3 Alveolar macrophages

Alveolar macrophages are the major line of cellular defense against lung infection and are the predominant cell type recovered in bronchoalveolar lavage (95%). The alveolar macrophage response to pneumococcal infection is complex (Aberdein *et al.*, 2013). Activation of TGFβ or IL-10 receptor signalling

or interaction with mannose receptor, signal-regulatory protein-α, triggering receptor expressed by myeloid cells 2 (TREM2), or CD200 receptor suppresses alveolar macrophage function in humans (Hussell and Bell, 2014). Whereas, stimulation of alveolar macrophages via TLR-2, TLR-4 or TLR-9 inhibits alveolar macrophage IL-10 receptor signal transduction, which ordinarily keeps alveolar macrophages in a suppressed state in response to constitutive epithelial-cell produced IL-10 (Fernandez *et al.*, 2004).

In the distal airway, pneumococci are ingested and killed by alveolar macrophages. However, when the bacterial load becomes too high for alveolar macrophages to destroy the bacteria without help, cellular interactions result in the secretion of chemokines and a pro-inflammatory feedback loop leading to the recruitment of polymorphonuclear cells to take over the antibacterial response. Alveolar macrophages secrete moderate to high levels of proinflammatory cytokines including IL-1β, TNFα, IL-6 and IL-8 (Losa García *et al.*, 1999). Alveolar macrophages also play a vital role in the control of inflammation, however. The anti-inflammatory role of alveolar macrophages is a vital part of the murine host response to pneumococcal pneumonia, potentially due to the role of alveolar macrophages in eliminating apoptotic polymorphonuclear cells that build up as result of acute phase response to pneumococcal infection (a process termed efferocytosis) (Knapp *et al.*, 2012). Interestingly, clearance of pneumococci from the murine lung in this study was not affected by alveolar macrophage depletion. A murine model of pneumococcal pneumonia showed that macrophage migration inhibitory factor (MIF), an innate immune mediator, was detrimental to survival and was associated with increased lung pathology, inflammation and pneumonia, despite playing a pivotal role in pneumococcal clearance from the nasopharynx (Weiser *et al.*, 2015). Yet, treatment of animals with a small-molecule inhibitor of MIF improved survival by reducing inflammation and improving bacterial control. A study of murine bone-marrow-derived-macrophages showed that 1A/1B-light chain 3-associated phagocytosis (LAP) of bacteria declined with mouse aging and an increase in pro-inflammatory cytokine production accompanied the deficiency in LAP and bacterial killing (Inomata *et al.*, 2020), potentially providing one explanation for age-related susceptibility to lung infection.

In a human model of infection, microaspiration of pneumococcus in the nasopharynx during colonisation into the lungs primed alveolar macrophages to increase uptake of bacterial pathogens, including *S. pneumoniae,* up to 3 months post-inoculation with pneumococcus (Mitsi *et al.*, 2020).

A key mechanism by which alveolar macrophages kill pneumococcus is through production of reactive nitrogen species and nitric oxide (NO) (Orman, Shenep and English, 1998); specifically, the cell wall and pneumolysin stimulate NO-mediated killing (Braun *et al.*, 1999). However, in a mouse model, NO

was associated with increased bacterial load during pneumococcal bacteraemia and decreased survival despite being an essential part the antibacterial response during pneumonia (Kerr *et al.*, 2004). Pneumococci have developed defenses against alveolar-macrophage mediated killing: protein PspC reduces NO production (Peppoloni *et al.*, 2006), AdhC is an S-nitrosoglutathione reductase (Stroeher *et al.*, 2007), and ClpP has been implicated in resistance to reactive nitrogen species (Park *et al.*, 2010). NO accumulation within alveolar macrophages switches the phenotype from an active state to an apoptotic state, a key component of the host defense (Marriott *et al.*, 2004).

1.5.4.4 Splenic macrophages

Work conducted by Marco Oggioni and colleagues has shown that the major source of bacteremia during pneumonia is not the lungs, but the spleen and specifically, splenic macrophages (Carreno *et al.*, 2021). Bacterial load in the spleen, but not the lungs, correlates with bacteremia. Previously, it was shown that in mice, pneumococcal sepsis is preceded by intracellular replication of the bacteria within splenic macrophages (Ercoli *et al.*, 2018). In the human spleen, pneumococci are detectable in the splenic macrophages, where they form foci of increasing bacterial numbers over time (Carreno *et al.*, 2021).

1.6 Adaptive immunity to *Streptococcus pneumoniae*

1.6.1 Humoral immunity to *S. pneumoniae*

Pneumococcal carriage is an immunising event and induces production of systemic anti-capsular and anti-protein IgG (Goldblatt *et al.*, 2005). Anti-capsular IgG acquired by natural carriage can reduce the risk of re-acquisition of carriage with a homologous serotype, but this is highly serotype-dependent; re-colonisation events with some serotypes can be frequent (Goldblatt *et al.*, 2005; Hill *et al.*, 2008; Weinberger *et al.*, 2008). In healthy adults, 100% protection for up to 1 year against re-colonisation with a homologous strain has been demonstrated using a controlled model of infection in which increased mucosal and systemic anti-protein and anti-capsular IgG was induced by colonisation (Ferreira, 2013). Furthermore, passive transfer of post-carriage sera from adults into mice conferred 70% protection against invasive pneumococcal pneumonia by a heterologous strain. In contrast, recent colonisation in healthy adults does not confer protection against colonisation with a heterologous strain (Pennington *et al.*, 2016). Vaccine-induced humoral response to the pneumococcal capsule protects against pneumococcal carriage acquisition and disease caused by vaccine serotypes (Shinefield and Black, 2000; Ota *et al.*, 2012; Collins *et al.*, 2015; Nagel *et al.*, 2015; Bar-Zeev *et al.*, 2021). Agglutination by vaccine-induced anti-capsular IgG is associated with protection against experimental carriage in humans (Mitsi *et al.*, 2016) and may provide a mechanism by which vaccines reduce colonisation events or colonising

density (Collins *et al.*, 2015; German *et al.*, 2019). However, analysis of datasets from the USA, Finland and Israel found that age-dependent declines in incidence of invasive pneumococcal disease in childhood were independent of anti-capsular antibody response and may be more likely due to serotype-independent forms of protection such as anti-protein response or maturation of non-specific immune responses (Lipsitch *et al.*, 2005). Anti-protein antigen antibodies have been shown to be protective against pneumococcal pneumonia and invasive disease in mice, whilst there is little or unclear evidence to suggest protection conferred by anti-capsular polysaccharide antibodies in mice or humans regarding pneumococcal pneumonia or invasive disease in unvaccinated individuals (Ramos-Sevillano, Ercoli and Brown, 2019). In a human model of infection performed in the USA, pre-existing IgG levels against pneumococcal protein PspA isolated from a serotype 23F clinical isolate inversely correlated with susceptibility to experimental colonisation by serotype 23F (McCool *et al.*, 2002). Protection was not conferred by pre-existing anti-capsular antibodies, which was consistent with findings that there is no correlation between baseline anti-serotype 6B capsular antibodies and susceptibility to experimental colonisation with the same serotype (Pennington *et al.*, 2016).

Notably, whilst anti-capsular antibody titres > 0.35µg/ml are generally considered to confer protection against invasive disease following PCV vaccination (Jódar *et al.*, 2003; Cohen *et al.*, 2017), although this varies based on serotype, titres above 4.0µg/ml may be necessary to confer protection against colonisation (Millar *et al.*, 2007; Dagan *et al.*, 2016). However, actual anti-capsular IgG levels needed to confer protection against carriage remains unclear (Ojal *et al.*, 2017). There is no definition of a protective immune response for novel protein-based vaccines, regarding carriage acquisition or disease.

Although IgA is the predominant antibody on mucosal surfaces, *S. pneumoniae* is able to circumvent this defense mechanism through the production of protease against IgA1 (Janoff *et al.*, 2014), the predominant IgA subgroup in the upper (90%) and lower (70%) airways (Burnett, 1986). Thus, humoral immunity to pneumococcus at mucosal sites must be additionally mediated by other Ig subclasses, including IgG. Systemic IgG is able to access mucosal sites (Nurkka *et al.*, 2001; Roche *et al.*, 2015) and blocks acquisition and transmission of pneumococcus in murine models, most likely through its agglutinating activity (Roche *et al.*, 2015). It is not fully elucidated whether mucosal IgG is produced locally or diffuses from the peripheral circulation, although it has been shown that increases in salivary IgG correlate with increased serum IgG, suggesting that salivary IgG is derived from serum (Nurkka *et al.*, 2001).

1.6.2 B-cell mediated immunity to *S. pneumoniae*

The process of B-cell-mediated immunity is excellently reviewed by Akkaya, Kwak and Pierce, 2019 and Kurosaki, Kometani and Ise, 2015. An overview is given in Figure 6. In the secondary lymphoid organs, naïve $CD4^+$ helper T-cells are primed upon encounter with antigens presented on MCH class II molecules of dendritic cells leading to antigen-specific T-cell proliferation. Antigen-specific T-cells then migrate to the border of T-cell zones, the T-cell-B-cell interface, as pre-follicular helper T-cells. In the first phase of the primary B-cell response, naïve B-cells exit circulation and enter B-cell follicles in secondary lymphoid organs. Here, they encounter antigens, presented by follicular dendritic cells. Antigens are internalized by B-cells and subsequently processed and presented on MCH class II molecules. B-cells then migrate to the border of T-cell zones. Antigen-presenting B-cells then interact with primed antigen-specific T-cells, which have encountered the same antigen. Upon interaction with B-cells, T-cells differentiate into mature follicular helper T-cells. Following interaction with helper T-cells, naïve B cells may undergo three fates: (i) differentiation into short-lived plasma cells to produce pathogen-specific antibodies of both class switched and unswitched isotypes with generally low affinity, (ii) differentiation into germinal centre (GC) B-cells that will subsequently enter the GC in the second phase of the primary B-cell response or (iii) differentiate into memory B-cells in the follicle independent of the GC (in this instance, B-cells might undergo class switching but generally have low affinity). Of the various signals elicited by T-cells, CD40 signalling is able to induce B-cell differentiation into a memory phenotype (Taylor, Pape and Jenkins, 2012). However, in addition to the CD40 signal, cytokine signalling is required to induce B-cell differentiation into GC B-cells. Specifically, IL-21 signalling results in upregulation of B cell lymphoma 6 (BCL-6), which is required for GC formation and maintenance (Dent *et al.*, 1997; Linterman *et al.*, 2010; Zotos *et al.*, 2010).

In the second phase of the primary B-cell response, newly differentiated GC B-cells form GCs and undergo proliferation and somatic hypermutation in the GC dark zone before exiting to the GC light zone, where they undergo affinity maturation. In the GC light zone, GC B-cells encounter antigens presented on follicular dendritic cells. B-cells again internalize and process antigens, which are then presented on MHC class II molecules to follicular helper T-cells. Upon encounter with follicular helper T-cells, GC B-cells will either (i) differentiate into class-switched and high affinity memory B-cells, which ultimately reside in secondary lymphoid organs or tissues, (ii) differentiate into class-switched and high affinity long-lived plasma cells, which migrate to the bone marrow to secrete antibodies with high affinity, contributing to serological memory or (iii) re-enter the GC dark zone to undergo the GC reaction again, ultimately giving rise to long-lived plasma cells or memory B-cells.

In the secondary B-cell response, or re-call response, memory B-cells respond to antigen in an anamnestic manner and differentiate into long-lived plasma cells or GC B-cells that undergo the GC reaction, i.e. rounds of population expansion, somatic hypermutation and selection. The GC reaction thereby enables replenishing of the memory B-cell pool.

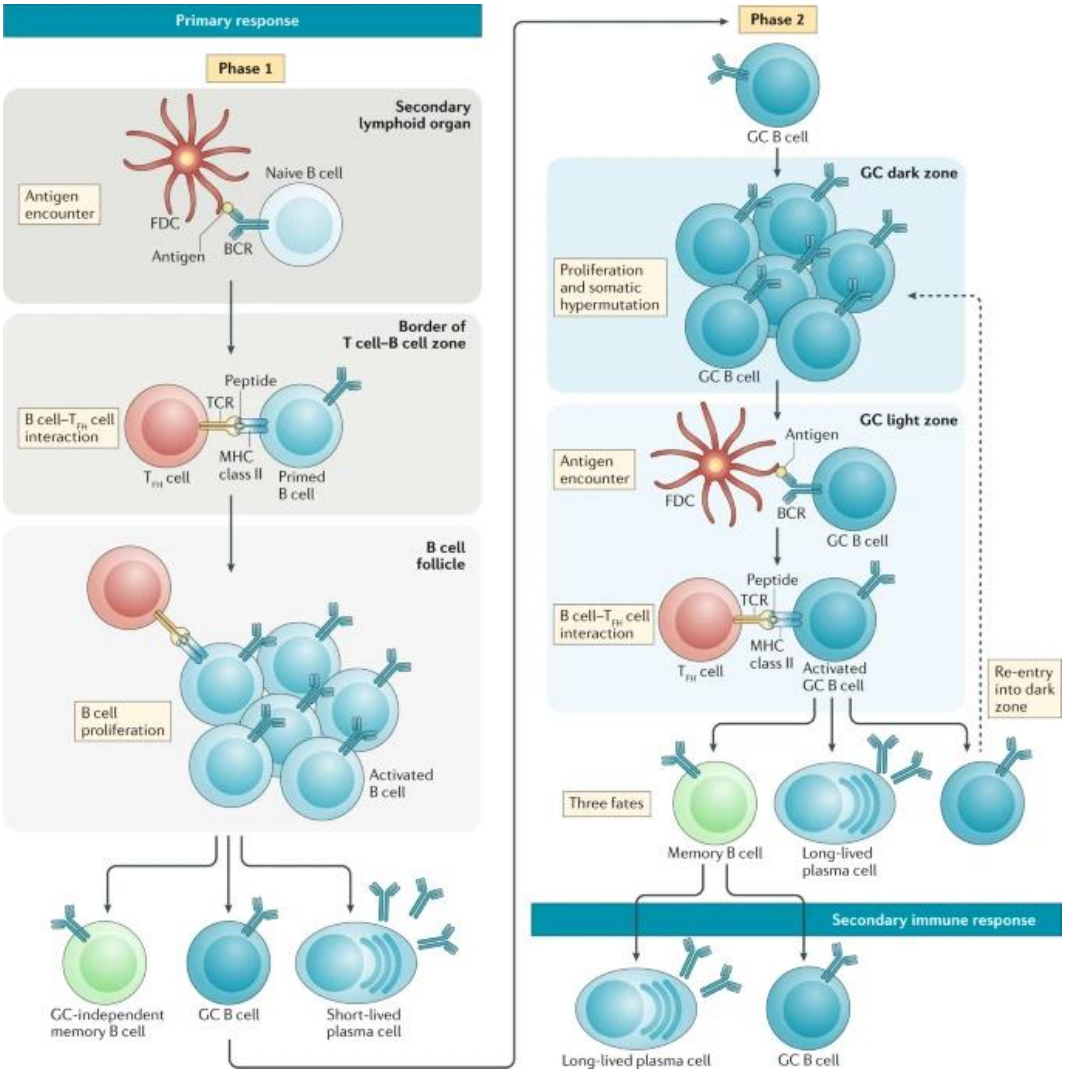


Figure 6: B-cell activation and maturation in secondary lymphoid organs. In phase 1 of the primary response (left), naive B cells exit the circulation, enter B cell follicles in the secondary lymphoid organ and survey the environment for antigen. Antigens encountered on follicular dendritic cells (FDCs) activate B cells through the B cell receptor (BCR), and the antigens are processed and presented to T cells at the T cell–B cell border, driving naive B cells to proliferate and differentiate into three main cell types: germinal centre (GC)-independent memory B cells, GC B cells or short-lived plasma cells. In phase 2 of the primary response (right), newly differentiated GC B cells form GCs and undergo proliferation and somatic hypermutation in the dark zone before exiting to the light zone, where the GC B cells encounter antigen on FDCs, present the antigen to T follicular helper cells (T_{FH} cells) and undergo three main fates: namely, differentiation into memory B cells, differentiation into long-lived plasma cells or re-entry into the GC dark zone. In the secondary response (bottom right), memory B cells respond to antigen and differentiate into long-lived plasma cells or GC B cells that undergo GC reactions. TCR, T cell receptor. Image source: Akkaya, Kwak and Pierce, 2019.

A subset of memory B-cells, known as tissue-resident memory cells, reside in non-lymphoid tissues, such as the lungs, and are maintained without recirculating (Allie and Randall, 2020). Here, they can be reactivated by antigen and proliferate *in situ* to provide protection for the tissue in which they reside. In murine models, lung-resident memory B-cells have been shown to be a common feature of pathogen-experienced lungs and to play an important role in pulmonary antibacterial immunity (Barker *et al.*, 2021). Further research is needed to fully understand this B-cell subset.

The B-cell compartment can be divided into B1 (non-follicular) and B2 (follicular) subsets (Palm and Kleinau, 2021). B1-cells are believed to originate in the bone marrow and reside in peritoneal and pleural cavities; these cells respond early in infection and are involved in the immune defense against mucosal pathogens, such as *S. pneumoniae*. B1-cells can be subdivided into $CD5^+$ cells (B1a), responsible for mainly poly-specific IgM production, and $CD5^-$ cells (B1b), which produce antigen specific IgM, IgA and IgG (Verbinnen *et al.*, 2012). B1b-cells are postulated to constitute the major source of T-cell independent memory B-cells and long-lived plasma cells that confer pneumococcal polysaccharide immunity (Taillardet *et al.*, 2009; Defrance, Taillardet and Genestier, 2011). B1b-cells have been shown to become activated and undergo class switching in response to pneumococcal polysaccharide (Haas *et al.*, 2005; Verbinnen *et al.*, 2012). The B1-cell population is self-renewing and functionality declines with increasing age (Rodriguez-Zhurbenko *et al.*, 2019).

Extrafollicular stimulation in tissues and subsequent activation of B-cell antigen receptors by T-cell independent antigens such as the pneumococcal capsule can result in B-cell proliferation and differentiation into plasma cells and memory B-cells, which confer some anti-bacterial immunity (Taillardet *et al.*, 2009; Defrance, Taillardet and Genestier, 2011). Memory B-cells generated in a T-cell independent manner differ phenotypically and functionally from those generated in a T-cell dependent response and these memory B-cells show very low levels of somatic hypermutation and isotype switching (Obukhanych and Nussenzweig, 2006; Defrance, Taillardet and Genestier, 2011). T-cell independent memory B-cell response is characterized by increased quantity rather than quality of antigen specific clones (Obukhanych and Nussenzweig, 2006). Furthermore, during secondary response, memory B-cell responses to T-cell independent type II antigens, such as the pneumococcal polysaccharide, are regulated by stringent IgG-mediated suppression, specific for the immunising antigen (Obukhanych and Nussenzweig, 2006). T-cell independent B-cell activation requires strong B-cell receptor cross-linking, activation of TLRs, pro-inflammatory cytokine signalling and critical signals by mononuclear cells via the release of B-lymphocyte stimulator protein and a proliferation-inducing ligand (APRIL), which bind with

high affinity to specific receptors on B-cells, inducing CD40-independent class switching in the presence of appropriate cytokines (Litinskiy *et al.*, 2002; Meltzer and Goldblatt, 2006; Defrance, Taillardet and Genestier, 2011).

PPV23 induces a T-cell independent response. It has been shown that immunisation with PPV23 depletes peripheral memory B-cell frequency in adults and attenuates memory B-cell response to subsequent vaccination with PCV7 (Clutterbuck *et al.*, 2012). This decline in memory B-cell frequency extended beyond a 6-month period post PPV23 vaccination. B1b-cell populations were depleted post PPV23 vaccination. In theory, vaccination with PPV23, results in the terminal differentiation of pre-existing memory B-cells into antibody-secreting cells, subsequent depletion of the memory B-cell pool, which is not then renewed in a T-cell independent B-cell response, and thus attenuated responses on re-exposure to the same antigen. Importantly, PPV23 does confer long-term antibody protection in adult humans (Akkaya, Kwak and Pierce, 2019).

The conjugation of polysaccharide to protein carriers, as is the case with PCV, converts the pneumococcal polysaccharide immune response to a T-cell dependent one, enabling immunogenicity in children under 2 years of age (Adderson, 2001; Pollard, Perrett and Beverley, 2009). It has been shown that 2 doses of PCV7 are required in infants (12 months of age) to generate an equivalent memory B-cell response and class switching as seen in adults immunised with one dose of PCV7 (Clutterbuck *et al.*, 2008). Booster responses to PCVs in infancy are thought to be dependent on homologous carrier protein priming (Trück *et al.*, 2016). Moreover, IgM has been shown to dominate the infant antibody responses, and class switching to IgG was serotype dependent (Clutterbuck *et al.*, 2008). In both adults and toddlers, with no preexisting polysaccharide-specific memory B-cells or serum IgG, frequencies of plasma cells and memory B-cells increased by day 7 after vaccination. Although booster immunisation with PCV induces transient polysaccharide-specific memory B-cell at the nasopharyngeal mucosa, these cells re-circulate through the site and are not resident (Clarke *et al.*, 2012). The development of a mucosally administered vaccine might induce resident memory B-cells that are able to confer protection against nasopharyngeal colonisation with the bacteria.

Importantly, at the time of pneumococcal exposure in a human model of infection, naturally acquired capsular polysaccharide-specific memory B-cells, but not levels of circulating or mucosal anti-capsular IgG were associated with protection against pneumococcal acquisition (Pennington *et al.*, 2016). However, increased numbers of circulating capsule-specific IgG secreting plasma cells post-challenge did not correlate with increased levels of mucosal or systemic capsule-specific IgG, suggesting that circulating

antibodies produced by plasma cells may migrate to the nasal mucosa and be responsible for sequestration of bacterial colonisation in the nasopharynx (Nurkka *et al.*, 2001; Roche *et al.*, 2015). Furthermore, it is hypothesised that colonisation is necessary for the development of a sustained plasma cell response in circulation and the continued immune stimulation and IgG production elicited by carriage is what leads to the detection of elevated serum capsule-specific IgG as pneumococci are cleared from the nasopharynx (Pennington *et al.*, 2016). Sampling of the nasal mucosa before and after experimental pneumococcal colonisation in a subsequent study showed that upon colonisation, B-cells were depleted from the nasal mucosa and were associated with an expansion of total and pneumococcal polysaccharide-specific circulating plasmablasts, likely due to recirculation of activated nasal B-cells, suggesting that these nasal B-cells are linked to production of capsule-specific serum IgG (Jochems *et al.*, 2019). Colonisation with pneumococcus has been shown to enhance memory B-cell response to subsequent vaccination with PCV in mice (Rabquer *et al.*, 2007).

Significantly lower percentages of memory B-cells to 3 pneumococcal protein antigens (PhtD, PhtE, and Ply) and reduced antigen-specific IgG is associated with increased propensity to otitis media in children (Sharma, Casey and Pichichero, 2012). In addition, immunisation of mice with a γ-irradiated *S. pneumoniae* whole-cell vaccine conferred serotype-independent protection in lethal challenge models of pneumococcal pneumonia and sepsis but this protection was lost in the absence of B-cells (Babb *et al.*, 2016). In another mouse model, depletion of B-cells resulted in significantly reduced protein-mediated humoral immunity towards *S. pneumoniae* colonisation, but not a complete loss of protection against lung infection and septicemia following challenge with pneumococcus after reconstitution of B-cells (Ercoli *et al.*, 2021). These data suggest some cellular-mediated protective immunity against *S. pneumoniae* exists in the absence of B-cell mediated immune response.

1.6.3 T-cell-mediated immunity to *S. pneumoniae*

Antigens are presented to naïve T-cells by antigen presenting cells, such as dendritic cells or macrophages, which activates T-cells and initiates their development into effector T-cells or memory T-cells. The differentiation of naïve $CD4^+$ T-cells into one of several lineages of helper T-cells (Th1, Th2, Th17, Tregs), as defined by the cytokines they produce and their function, is dependent upon the particular cytokine milieu (Zhu, Yamane and Paul, 2010). Presentation of antigens via MHC class II receptors activate $CD4^+$ helper T-cells. Type 1 helper T-cells release cytokines in the local environment to activate macrophages and direct cytotoxic $CD8^+$ cells to pathogens. Antigen presentation by MHC class I receptors activates cytotoxic $CD8^+$ T-cells, which induce apoptosis of cells containing the antigen presented to them.

T-cells with a memory phenotype rapidly proliferate into helper and cytotoxic T-cells in an anamnestic manner upon contact with a previously encountered antigen. Generation of T-cell memory occurs between 0-20 years of age upon antigen exposure and memory T-cells are the most abundant lymphocyte population in the human body (Farber, Yudanin and Restifo, 2014).

1.6.3.1 CD4+ T-cell mediated immunity

In murine models, it has been shown that protection against nasopharyngeal pneumococcal colonisation and accelerated clearance of the bacteria from the nasopharynx can be induced in the absence of antibody or $CD8^+$ T-cells and independent of the capsular type, but requires the presence of $CD4^+$ at the time of exposure (McCool and Weiser, 2004; Malley *et al.*, 2005; Van Rossum, Lysenko and Weiser, 2005; Lu *et al.*, 2008). However, other studies have shown that humoral and cellular responses are needed for long-term protection against pneumococcal colonisation and infection in mice (Richards *et al.*, 2010; Cohen *et al.*, 2011; Wilson *et al.*, 2015, 2017).

1.6.3.1.1 T-helper 1 (Th1) mediated immunity

The signature cytokine produced by Th1 cells is IFN-γ. They also produce IL-2, TNFα and lymphotoxin (Zhu, Yamane and Paul, 2010). In mice, predominant IgG isotypes produced in response to pneumococcal protein antigen PspA (Van Rossum, Lysenko and Weiser, 2005) or oligosaccharide (OS)-protein conjugates have been those typically produced by a Th1-mediated B-cell response (Lefeber *et al.*, 2003), and production of Th1-associated IgG2a and IgG2b correlated with increased phagocytic capacity (Lefeber *et al.*, 2003). A study investigating T-cell response in 22 patients hospitalised with pneumococcal infections found that IFN-γ-producing T-cells disappear from the circulation in the acute phase of infection but re-appear with treatment and reduced pathology, suggesting that these cells may play a role in the human immune response to pneumococcal infection (Kemp *et al.*, 2002). As previously stated, it has been shown that, *in vitro*, live pneumococci trigger a Th1-biased response via monocyte production of IL-12p40 (Olliver *et al.*, 2011). In addition, peripheral blood mononuclear cells (PBMCs) taken from volunteers in a setting of high pneumococcal prevalence released IFN-γ and IL-17 in response to pneumococcal antigens, demonstrating that exposure to pneumococcus results in T-cell mediated immune memory; the level of memory response did not correlate with current nasopharyngeal carriage of the bacteria (Mureithi *et al.*, 2009). Production of mucosal antibodies (predominantly IgG1, along with lower levels of IgG3 and IgG4 and little or no IgG2) in the nasopharynx of children against pneumococcal proteins PspC and Ply has been shown to be T-cell regulated (Zhang, Bernatoniene, Bagrade, Paton, *et al.*, 2006), however it is unclear to what extent this regulation is mediated by different T-cell subset. Both IFN-γ, a Th1-secreted cytokine,

and IL-10, typically a Th2 cytokine which has an anti-inflammatory effect, have been strongly correlated with the production of anti-protein antibodies in adenoidal mononuclear cells (Zhang, Bernatoniene, Bagrade, Paton, et al., 2006).

1.6.3.1.1 T-helper 2 (Th2) mediated immunity

In contrast to Th1 cells, Th2 cells do not produce IFN-γ or lymphotoxin. They predominantly produce IL-4, IL-5, and IL-13 as well as TNFα (Zhu, Yamane and Paul, 2010). B-cell class switching to IgG and IgE antibody production is driven by Th2-secreted cytokines (Mosmann and Coffman, 1989; Leiva *et al.*, 2001). It is generally accepted that T-cell responses in the first few years of life are Th2-skewed (Adkins, 2003; Adkins, Leclerc and Marshall-Clarke, 2004; Zaghouani, Hoeman and Adkins, 2009; Pettengill, van Haren and Levy, 2014). However, observations surrounding the tendency towards a Th2 bias have mostly been made in neonatal mice. Evidence for a Th2 bias in humans is less well established and the type of stimulus may impact the type of T-cell response generated (Semmes *et al.*, 2021). Dysregulation of the Th2 response can lead to allergies and a predisposition to respiratory conditions such as asthma. Asthma has been associated with a higher risk of susceptibility to invasive pneumococcal disease, particularly in children (Li *et al.*, 2020). Furthermore, in mice, Th2 cytokines such as IL-4 are associated with a risk of bacterial pneumonia (Zhao *et al.*, 2011).

1.6.3.1.3 T-helper 17 (Th17) mediated immunity

Induction of IL-17 production by T-cells is at least in part mediated by IL-23 secretion (Happel *et al.*, 2003; Kolls and Lindén, 2004; Rudner *et al.*, 2007; Kudva *et al.*, 2011). Th17 cells are characterised by their IL-17 secretion. In murine models, the secretion of IL-17 by $CD4^+$ cells plays an important role in the clearance of pneumococcal carriage from the nasopharynx and infection in the lungs via recruitment of monocytes/macrophages and neutrophils to the site of infection (Lu *et al.*, 2008; Zhang, Clarke and Weiser, 2009; Marqués *et al.*, 2012). In the murine lung, pneumococcal infection induced robust Th17 responses at the mucosal site and transfer of $CD4^+$ T-cells from immune mice recovered from a prior infection conferred protection against heterologous pneumonia, which was abrogated in the absence of IL-17A (Wang *et al.*, 2017). Thus, memory Th17 response might provide protection against pneumonia in a serotype-independent manner. Microaspiration of oral commensals in mice, which are rapidly cleared from the lower respiratory tract, induces a prolonged Th17 response that secondarily decreases susceptibility to *S. pneumoniae* (Wu *et al.*, 2021). Whilst antibody-mediated response is also needed for protection against lung infection in mice, protection induced by prior colonisation was lost with the depletion of $CD4^+$ cells or IL-17 (Wilson *et al.*, 2015). Nevertheless, whilst Th17 response is important for

protection against lung infection, antibody-mediated response is necessary for protection against invasive pneumococcal disease in mice (Cohen *et al.*, 2011); suggesting that site of infection determines the basis of protective response. Notably, the increase in secretion of type I IFNs caused by influenza A infection inhibits Th17-mediated response and thus is one mechanism by which influenza may increase susceptibility to secondary bacterial pneumonia (Kudva *et al.*, 2011).

Pneumolysin promotes $CD4^+$ T-cell response associated with protection against nasopharyngeal carriage (Zhang *et al.*, 2007; Mureithi *et al.*, 2009). In the absence of pneumolysin expression, IL-17A production in human tonsillar mononuclear cells is annulled (Lu *et al.*, 2008). Furthermore, in both the absence and presence of antibodies and complement, IL-17A increased pneumococcal killing by human neutrophils. Importantly, induction of IL-17A expression may be dependent on prior exposure to *S. pneumoniae* as expression was induced by pneumococcal antigens in tonsillar cells from children and peripheral blood cells from adults but not in umbilical cord blood (Lu *et al.*, 2008). This is consistent with previous findings that IL-17A is a key cytokine produced by $CD4^+$ memory T-cells in response to pneumococcal antigens in a population with high pneumococcal prevalence (Mureithi *et al.*, 2009).

Experimental human pneumococcal carriage in a cohort of healthy adults increased the proportion of IL-17A-secreting CD4+ memory T-cells in the lung, and was associated with enhanced killing capacity of opsonised pneumococci by alveolar macrophages (Wright *et al.*, 2013). In addition, Th17 response was shown to be the dominant naturally acquired T-cell response to pneumococcal protein antigens in young and elderly adults; this response was dependent upon the presence of antigen presenting cells (Schmid *et al.*, 2011). The role of Th17 mediated immunity against pneumococcal colonisation in children has also been demonstrated. High levels of pneumococcal nasopharyngeal colonisation is associated with reduced IL-17A secretion in Fijian children (Hoe *et al.*, 2015). Comparison of Th17 responses to *S. pneumoniae* in children and adults in Sweden (a high-income setting) and Bangladesh (a low-income setting with higher prevalence of *S. pneumoniae*) found that children in Sweden produced lower levels of IL-17A in response to pneumococcal whole cell antigen (WCA) than did Swedish adults, however this difference was not observed in the Bangladeshi cohort (Lundgren *et al.*, 2012). In samples from Bangladeshi participants, Th17 responses from both adults and children were significantly higher than those in Sweden. Again, these results suggest increased prior exposure to pneumococcus may enhance subsequent Th17 response to the pathogen.

1.6.2.1.4 T-regulatory T-cells

Regulatory T-cells (Tregs) secrete immunomodulatory cytokines IL-10, transforming growth factor-β (TGFβ) and IL-35 (Vignali, Collison and Workman, 2008). TGFβ signalling in Tregs is required for their *in vivo* expansion and immunosuppressive capacity (Huber *et al.*, 2004). A critical role of TGFβ by Tregs in the prevention of dissemination of pneumococci from the lungs to the blood in mice has been reported (Neill *et al.*, 2012). Further to this, Neill and colleagues found that TGFβ1 signalling prolongs pneumococcal carriage and promotes clearance of the bacteria from the nasopharynx, substantiating the role of Tregs and TGFβ1 signaling in immune tolerance and providing a mechanism by which pneumococcus colonises the human nasopharynx without inducing damaging host inflammation (Neill *et al.*, 2014). In human participants exposed to experimental pneumococcal challenge, TGFβ1 and IL-10 levels were elevated in nasal washes of colonised individuals as compared to their non-colonised counterparts (Neill *et al.*, 2014). In a study investigating the expression of Treg-related markers in adenoidal mononuclear cells (MNC) and PBMCs from children, significantly higher levels of Tregs with an effector/memory phenotype were found in adenoidal cells taken from children colonised with pneumococcus as compared to non-colonised children (Zhang *et al.*, 2011). Induction of adenoidal Treg proliferation by WCA produced IL-10 but not IL-17 and in Treg-depleted mononuclear cells, $CD4^+$ T-cell proliferation was higher in carriage positive participants than carriage negatives. The increased presence of effector/memory Tregs with a potent inhibitory effect in adenoidal tissue of colonised children might further point to the role of Tregs in promoting prolonged duration of carriage. Importantly, the balance between Th17 and Treg mediated immune response is of critical importance in the control of colonisation in the nasopharynx. Frequencies of Tregs and Th17 cells are inversely correlated in nasopharynx-associated lymphoid tissue (NALT) and the frequency of Tregs declines with age whilst Th17 frequency and Th17:Treg ratio increases with age (Mubarak *et al.*, 2016). A Th17-skewed response also correlates with protection against nasopharyngeal colonisation in children.

1.6.3.2 CD8+ T-cell mediated immunity

$CD8^+$ T-cell mediated immunity is typically associated with intracellular pathogens. $CD8^+$ T-cells have been shown to play a pivotal role in protection against pneumococcal pneumonia, caused by serotype 3, in mice (Weber, Tian and Pirofski, 2011). The underlying mechanism by which $CD8^+$ T-cells play a role in immune response to pneumococcus could be through capsular polysaccharide induction of $CD8^+CD28^-$ T-cells, which exhibit immunosuppressive properties on $CD4^+$ T-cells, dampening inflammatory response (Mertens *et al.*, 2009). $CD8^+$ T-cell activation appears to be dependent upon enhanced T-cell receptor (TCR) cross-linking (Mertens *et al.*, 2009). Tissue-resident memory $CD8^+$ cells in the nose

correlate with protection against experimental colonisation in a human challenge model (Jochems *et al.*, 2019). These cells are highly functional; among biopsied nasal cells, 94.8% produced TNFα and/or IFN-γ following *in vitro* stimulation with phorbol myristate acetate (PMA) and ionomycin, compared with 36% of blood $CD8^+$ T cells. Nevertheless, the role of $CD8^+$ T-cells in the adaptive immune response against *S. pneumoniae* needs further characterisation.

1.6.3.3 Unconventional T-cell mediated immunity

TCR-γδ T-cells are a minor T-cell lineage present in peripheral tissues, which act as sentinels at mucosal sites and secrete IL-17. TCR-γδ T-cell deficient mice have showed dampened IL-17 response to *S. pneumoniae* and a severely decreased capacity to control lung infection with the bacteria (Jing *et al.*, 2010). Attenuated neutrophil recruitment to the infected lungs is observed in mice lacking TCR-γδ T-cells, which mediate neutrophil recruitment trough production of MIP-2 and TNFα (Nakasone *et al.*, 2007). In a murine model of *S. pneumoniae*-mediated lung inflammation, a >30-fold increase in absolute numbers of γδ T-cells in the lungs was observed at the peak response following *S. pneumoniae* challenge and γδ T-cells were critical modulators of inflammatory response in the lungs by restoring mononuclear phagocyte numbers to homeostatic levels following bacterial clearance (Kirby *et al.*, 2007). *S. pneumoniae* lung infection in mice promotes a significant increase in γδ T-cells, which secrete high levels of IFN-γ and IL-17A (Wanke-Jellinek *et al.*, 2016). γδ T-cell response in the murine lung is inhibited by type I IFN produced in the presence of primary influenza infection and this inhibition can lead to fatal secondary *S. pneumoniae* infection (Li, Moltedo and Moran, 2012). In comparison to wild-type mice, mice deficient in type I interferon receptor demonstrated enhanced neutrophil recruitment and expression of IL-17 in response to secondary bacterial infection in the lungs together with increased bacterial clearance. Lung γδ T-cells were responsible for almost all IL-17 production, which is supported by the findings of McNeela *et al.*, 2010 . Overall, these data point to the importance of the role of this T-cell subset in the immune response to *S. pneumoniae* infection. However, the role of γδ T-cells in the immune response to *S. pneumoniae* in humans needs further elucidation, as do the mechanisms by which pneumococcus induces their activation.

Baseline circulating blood mucosal associated invariant T (MAIT) cell functionality associated with resistance to pneumococcal colonisation in a human challenge model, suggesting a role for this cell type in immune response to pneumococcus (Jochems *et al.*, 2019).

Tissue-resident memory T-cells (T_{RM}) are constrained within tissues and do not recirculate, unlike their effector memory and central memory counterparts. It is thought that distinct patterns of

transcription factors may induce the development of resident memory T-cells in tissues (Mueller and Mackay, 2015). Both $CD4^+$ and $CD8^+$ tissue resident memory T-cells have been described. T_{RM} cells have been identified in the airway epithelium and lungs, as well as the skin, bone marrow, spleen, intestine and lymph nodes (Farber, Yudanin and Restifo, 2014; Turner and Farber, 2014; Mueller and Mackay, 2015; Kumar *et al.*, 2017). Despite originally being defined by expression of CD69 and CD103 (Sathaliyawala *et al.*, 2013; Farber, Yudanin and Restifo, 2014; Turner and Farber, 2014; Mueller and Mackay, 2015; Kumar *et al.*, 2017), definitive markers of T_{RM} cells (in both humans and mice) require further validation, which will help to clarify relative proportions of resident and circulating memory T-cells in human tissues (Mueller and Mackay, 2015). $CD8^+$ and $CD4^+$ T_{RM} cells have been shown to confer protection against infection with respiratory viruses in mice (Teijaro *et al.*, 2011; Anderson *et al.*, 2012). Intranasal administration of live or killed pneumococcus in mice generated *S. pneumoniae*-responsive IL-17A-producing $CD4^+$ mucosal T_{RM} cells, which mediated long-lived, neutrophil-dependent protection against subsequent pneumococcal nasal challenge (O'Hara *et al.*, 2019). Most notably, parenteral administration of killed pneumococcus also generated IL-$17A^+$ $CD4^+$ T_{RM} cells at the nasal mucosa, in contrast to the paradigm that generation of mucosal T_{RM} requires mucosal vaccination (Belyakov and Ahlers, 2009; Holmgren and Svennerholm, 2012; Aguilo *et al.*, 2016). As previously stated, tissue-resident memory $CD8^+$ cells in the nose correlate with protection against experimental colonisation in a human challenge model (Jochems *et al.*, 2019). Novel vaccines should be designed to induce both this tissue-resident and circulating T-cell response for optimal protection against infections. The generation of tissue-resident memory cells is one of the principal advantages of mucosally administered vaccines.

1.7 Pneumococcal vaccines

1.7.1 Current pneumococcal vaccines

There are currently three pneumococcal vaccines licensed for use, which elicit humoral responses against various capsular polysaccharide serotypes.

Protective against 23 (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F) of the >100 pneumococcal serotypes, the pneumococcal polysaccharide vaccine (PPV) (Pneumovax® 23; Merck), is routinely given to adults aged 65 and over as well as high risk groups but is poorly immunogenic in children under 2 years of age, until which age maturation of capsule-specific antibodies is not observed (Pollard, Perrett and Beverley, 2009). PPV induces T-cell independent B-cell and plasma cell responses, leading to an increase in expression of anti-capsular IgG. There are concerns surrounding PPV however as multiple studies have documented PPV23-induced hypo-responsiveness,

following various vaccination strategies in both adult and child populations (Papadatou and Spoulou, 2016). The use of the vaccine has been linked to depletion of memory B cell pools, which may be one mechanism by which hyporesponsiveness is induced (Poolman and Borrow, 2011; Clutterbuck *et al.*, 2012). This also makes the vaccine unsuitable for use in HIV-infected individuals (Garmpi *et al.*, 2019). Furthermore, although the vaccine has been shown to reduce incidence of invasive pneumococcal disease, the efficacy of PPV in high risk groups and against non-bacteremic pneumonia, is less obvious (Blasi *et al.*, 2012). A study conducted in Japan found that PPV23 effectiveness against all pneumococcal pneumonia was 27.4% and 33.5% effectiveness against pneumococcal pneumonia caused by PPV23 serotypes was reported (Suzuki *et al.*, 2017). Although subgroup analysis did not find any significant differences, protection was greater in people under 75 years of age. No effectiveness against all-cause pneumonia or mortality was demonstrated. The efficacy of the vaccine against invasive disease is also debated, particularly in the elderly and chronically ill persons (Feldman and Anderson, 2014).

Two formulations of the pneumococcal conjugate vaccine (PCV) are currently licensed for use in infants: PCV10 (introduced in 2009; GlaxoSmithKline) and PCV13 (introduced in 2010; Pfizer), which target 10 and 13 pneumococcal serotypes respectively. Due to the conjugation of purified polysaccharides to carrier proteins (non-typeable *Haemophilus influenzae* protein D (NTHi protein D), diphtheria toxoid, and tetanus toxoid in the case of PCV10 and a nontoxic variant of diphtheria toxin known as CRM197 in the case of PCV13), the PCVs elicit a T-cell response and result in enhanced memory-B cell formation, affinity maturation, class switching, and levels of IgG (Clutterbuck *et al.*, 2012). PCV10 and PCV13 protect against serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F with the addition of serotypes 3, 6A and 19A to the PCV13 formulation. Prior to the introduction of PCV10, the 7-valent PCV7 was licensed for use in 2000 covering serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. The widespread use of PCV has led to a clear decrease in the incidence of vaccine-type pneumococcal carriage in the population and thus a decrease in the incidence of invasive pneumococcal disease in vaccinated children as well as un-vaccinated children and adults through herd immunity (Whitney *et al.*, 2003; Waight *et al.*, 2015). In the Danish population, the decline in the incidence of invasive pneumococcal disease following the introduction of PCV13 was accompanied by an almost 30% decline in pneumococcal-related mortality in non-vaccinated persons (Harboe *et al.*, 2014). Moreover, the introduction of PCV has been associated with a decrease in the incidence of clinically defined and radiologically-confirmed pneumonia (Silaba *et al.*, 2019). However, whilst the introduction of PCV correlates with a reduction in cases of pneumonia in low-income settings, the effect of the vaccine in high-income settings is less clear (Schuck-Paim *et al.*, 2019). In addition, although PCVs have showed efficacy of 76 to 97% against invasive pneumococcal disease in most studies,

efficacy against radiographically confirmed pneumonia or primary endpoint pneumonia is much lower, ranging from 17 to 37% (Madhi, Whitney and Nohynek, 2008).

Under current NHS guidelines, in the UK, infants born after 1st January 2020 are offered 2 doses of PCV13 at 12 weeks and at 1 year of age. Previously, PCV was given to babies at 8 and 16 weeks, with a booster at 12-13 months of age. Over 65s and high-risk groups are offered one dose of PPV23.

The effectiveness of pneumococcal vaccines, particularly PCVs, in HIV-positive persons needs further robust evaluation. Whilst there is a general paucity of data, current evidence suggests decreased immunogenicity in HIV-positive individuals compared to HIV-negative. Vaccination strategies in this group need further optimisation and there is a great need for vaccines with improved robustness and sustained immunogenicity in HIV-infected groups (Crum-Cianflone *et al.*, 2010; Garmpi *et al.*, 2019; Dhingra and Singh, 2020).

As of March 2020, PCV has been introduced into the National Immunization Program (NIP) of 146 countries, including 138 universal, 5 subnational and 3 risk programs (Hopkins Bloomberg, 2020). A further 15 countries have announced plans to introduce PCV into their NIP, whilst 33 countries have yet to decide regarding the introduction. Established in 2000, Gavi, a global vaccine alliance, enables the equitable and sustainable use of vaccines by aiding lower-income countries to procure newest vaccines at the same time as high-income countries. By the end of 2019, >80% of eligible Gavi countries (60 low-income countries) had introduced PCV into their routine immunisation programs. However, a 2018 WHO/UNICEF estimate suggested 54% of infants in Gavi countries (43.5 million) are not receiving PCV because they live in countries that have not introduced PCV into their NIP or they are not being reached by the immunisation services in their country.

1.7.2 Drawbacks of current pneumococcal vaccines

There are various drawbacks of current vaccines, not least the limited evidence of efficacy against non-invasive pneumonia. Firstly, current vaccines are complex and therefore costly to produce, which is a major global concern and can limit access to vaccines in low- and middle-income countries. Serotypes included in vaccine formulations are also based on those most prevalent in high income countries and are poorly matched to those in low income countries where the burden of disease is highest (Pichichero, Khan and Xu, 2016). Additionally, herd protection conferred by PCVs in the elderly seems to have reached its ceiling in many countries, which is of particular concern given the aging global population and the

uncertainty surrounding the most effective immunisation strategies against pneumococcal disease in the elderly (Feldman and Anderson, 2020).

Furthermore, the evidence for increased prevalence of non-vaccine serotype replacement in the community is cause for concern and points to gradual loss of vaccine effectiveness over time (Weinberger, Malley and Lipsitch, 2011; Olaya-Abril *et al.*, 2013). Evidence suggests that rates of invasive pneumococcal disease due to non-PCV13 serotypes were increasing in the UK in 2014, particularly in children under five years of age (Waight *et al.*, 2015). The incidence of non-vaccine type invasive pneumococcal disease in children and adults following the introduction of PCV7 also increased in North America, Europe, South America at varying degrees (Feikin *et al.*, 2013). Whilst the introduction of PCVs with broader coverage may limit the incidence of non-vaccine type disease, it is reasonable to hypothesise that serotype replacement will continue to pose a threat to overall PCV effectiveness. A recent study found that in high-income settings (Løchen, Croucher and Anderson, 2020), the introduction of multi-valent PCVs and subsequent serotype replacement has meant that there is no-longer dominant strains causing invasive pneumococcal disease as was the case before the introduction of PCV (serotype 14) and PCV10/13 (serotype 19A). Thus, increasing vaccine valency may have reduced effectiveness and prove difficult given the diversity of predominant or emerging serotypes across different countries. Non-vaccine serotypes 8, 9N, 15A and 23B are increasing in some European countries, North America, and Australia, although their incidence trends vary and there are various other non-vaccine types affecting each country and different age-groups.

Residual vaccine type prevalence following varying vaccination strategies differs across African countries, however the reduction in carriage rates do not match those seen in Europe and North America (Swarthout *et al.*, 2020). In Malawi, high residual vaccine-type carriage has been observed between 3-7 years post-PCV introduction and vaccine-type half-life is similar between vaccinated and unvaccinated children (Swarthout *et al.*, 2020). In addition, with the increasing prevalence of unencapsulated serotypes, with disease causing capability (Keller, Robinson and McDaniel, 2016), the need for capsule independent vaccines is further validated. Further evidence suggests that the herd protection conferred by childhood vaccination with PCV in Germany has reached its limit, particularly in the over 60 age group, and the incidence of invasive pneumococcal disease is on the rise (van der Linden, Imöhl and Perniciaro, 2019). Vaccine type pneumococcal disease also continues to have a high burden in the UK adult population, despite routine vaccination of children with PCV and ongoing herd protection (Chalmers *et al.*, 2016). Prevalence of serotype 3, known to cause invasive disease, is also increasing in countries around the world

despite its inclusion in the PCV13 formulation (Feldman and Anderson, 2020). The efficacy of the vaccine against serotype 3 is lesser than for other serotypes (Linley *et al.*, 2019). Rather alarmingly, a study in Switzerland found that incidence of hospitalisations of adults with non-pneumococcal and pneumococcal pneumonia increased significantly from the pre-vaccine era to the PCV7 era and the PCV13 era (Albrich *et al.*, 2019). A study of paediatric invasive pneumococcal disease in Portugal between 2012-2015 found that PCV13 serotypes continued to account for the majority of cases (57.8%), with no obvious decline in the incidence of paediatric invasive pneumococcal disease caused by the serotypes included in the PCV13 formulation (Silva-Costa *et al.*, 2019).

There is thus a distinct need for improved novel vaccines against pneumonia and pneumococcal disease, which are serotype-independent.

1.7.3 Serotype-independent vaccine strategies

Protein vaccines, whole cell pneumococcal vaccines, and recombinant bacteria expressing pneumococcal antigens are promising strategies for novel vaccines with serotype-independent protection (Miyaji *et al.*, 2013; Pichichero, 2017). Protein antigen-based vaccines are considered safer than whole cell or viral vector vaccines due to the absence of genetic materials and thus reduced chance of causing disease (Wang *et al.*, 2015). However, this subunit vaccine approach can also dampen immunogenicity and thus immunostimulatory agents (adjuvants) are generally necessary to ensure protein-based antigen vaccines generate a sufficient immune response. A summary of pneumococcal vaccine candidates in clinical trials is given in Appendix A (Pichichero, 2017; Lagousi *et al.*, 2019).

Given the success of PCVs in reducing the burden of invasive pneumococcal disease worldwide, it is unlikely they will ever be fully replaced. However, it is likely that they will be used in parallel with multi-valent protein vaccines, which address the increasing need for capsular serotype-independent vaccines (Scott *et al.*, 2021).

There is substantial evidence that mucosal immunisation with pneumococcus stimulates both a local and systemic immune response and thus mucosal vaccines may provide improved protection against pneumococcal pneumonia compared to current parenterally administered vaccines as well as eliminating the need for needles (Jambo *et al.*, 2010). A vast array of mucosa prophylactic vaccines using protein-based antigens are being explored for various diseases, although no intranasal or oral protein-based vaccines have as yet been approved (Wang *et al.*, 2015).

Mucosal response to pneumococcus is a key step in preventing nasopharyngeal carriage of the bacteria and could thereby reduce transmission of the bacteria as well as incidence of otitis media and pneumonia. Whilst polysaccharide-directed immune response is unlikely to affect pre-existing commensal carriage due to phase variation of the capsule, protein induced immune response may be able to hamper initiation of colonisation. In mice, intranasal immunisation with a cholera toxin B subunit-PsaA fusion protein protected against pneumococcal colonisation and did not significantly alter the oral or nasopharyngeal microbiota of the mice (Pimenta *et al.*, 2006). However, correlation between anti-protein humoral response and elimination of nasopharyngeal carriage of the bacteria in humans is yet to be clearly demonstrated (Xu *et al.*, 2017).

1.7.3.1 Protein-based vaccines

Pneumococcal protein antigens that are universally expressed across pneumococcal serotypes may induce humoral and cellular mediated immune response with broad protection and provide a cheaper alternative to current vaccines with less complex manufacturing (Lagousi *et al.*, 2019).

There is evidence to suggest that designing vaccines to include immunogenic regions of full-length proteins, such as antigenic protein fragments or peptides in their natural conformation, alone or in combination with full-length proteins could overcome potential drawbacks of using the full-length protein (Lagousi *et al.*, 2019). Firstly, the manufacturing costs incurred by the need for detoxification of full-length proteins are reduced. Moreover, overexpression or misfolding of the 3-dimensional structure of full-length recombinant proteins, which could hamper immunogenicity, is avoided (Lagousi *et al.*, 2019). Further, potential toxicity induced, for example by pneumolysin, is lessened as antigenic fragments associated with autoimmunity or toxicity can be excluded (Welsh and Fujinami, 2007). It has also been shown that peptides are more readily endocytosed and presented by dendritic cells to $CD4^+$ and $CD8^+$ T-cells, leading to enhanced T-cell activation (Zhang *et al.*, 2009; Rosalia *et al.*, 2013). It is likely, that combinations of antigens will need to be included in protein-based vaccine formulations to overcome differential levels of antigen expression across serotypes as well as to ensure sufficient immunogenicity (Gámez *et al.*, 2018). The combination of full-length proteins with protein fragments has demonstrated protective efficacy against multiple diseases in murine models (Jambo *et al.*, 2010).

One of the most universally used and comprehensive approaches to identifying novel protein-based vaccine candidates is reverse vaccinology (Meinke, Henics and Nagy, 2004; Giefing *et al.*, 2008; Pichichero, Khan and Xu, 2016). Human sera are analysed before and after natural or experimental challenge with the bacteria. Highly reactive antigens that generate a robust antibody response can be

characterized, cloned, and purified to be taken forward in vaccine formulations used to immunise animal models of disease. Vaccine candidates should be (i) conserved across serotypes, (ii) surface exposed/accessible, (iii) immunogenic, (iv) expressed during colonisation or infection and (v) protective in models of disease.

1.7.3.1.1 Humoral response to pneumococcal proteins

Naturally acquired protection against invasive pneumococcal disease has been shown to be dependent on antibodies against protein antigens, rather than the capsule (Wilson *et al.*, 2017). Moreover, induced protection against reacquisition of pneumococcus in Bangladeshi infants with a heterologous strain is postulated to be due to anti-protein response because of its serotype-independent nature (Granat *et al.*, 2009). It has been demonstrated that colonisation with pneumococcus induces both mucosal and serum IgG to pneumococcal proteins.

A study in infants found that anti-protein IgG levels in serum were increased following colonisation, and humoral response to some proteins was associated with decreased doctor visits for respiratory tract infections (Lebon *et al.*, 2011). Increased IgG against proteins BVH-3, NanA, PmpA, PsaA, SIrA, SP0189 and SP1003 were increased in young children who experienced fewer respiratory tract infections in the third year of life, suggesting that antibodies against these proteins may provide some protection against respiratory tract infections or are markers of other protective agents. However, only anti-PspC IgG was associated with protection against pneumococcal colonisation. High levels of anti-Ply antibodies in mothers has been correlated with reduced odds of infant colonisation between 7-12 weeks of age; suggesting that transplacental transfer of Ply antibodies confers some protection against colonisation in infants (Holmlund *et al.*, 2006). In a study conducted in 112 children aged 2-12 years undergoing adenoidectomy, adenoidal mononuclear cell supernatants were analysed for antigen-specific antibodies following culture with pneumococcal culture supernatants or recombinant proteins (Zhang, Bernatoniene, Bagrade, Pollard, *et al.*, 2006). Whilst pneumococcal carriage rate declined with age, levels of anti-PspC, Ply and PspA antibodies in serum increased. Importantly, carriage negative children had higher levels of circulating and salivary anti-PspC and anti-Ply IgG than colonised children, suggesting that antibodies to these proteins may protect against colonisation in children aged 2 years and older. Furthermore, antigen-specific IgG production by adenoidal mononuclear cells in response to antigen stimulation was greater in colonised children, again pointing to an immune priming effect of colonisation. Antibody production against pneumococcal proteins in infants in the Philippines has been shown to begin at around 4-5 months of age and is associated with the presence of pneumococcal carriage (Holmlund *et*

al., 2009); importantly, the initiation of the development of antibodies in children in countries with lower risk of exposure to pneumococcus occurs at around 1 year of age (Syrjänen *et al.*, 2001; Zhang, Bernatoniene, Bagrade, Pollard, *et al.*, 2006). Colonisation with pneumococcus induces anti-protein IgG to PspA, PsaA, Ply, PhtB, PhtE, PpmA, NanA, PhtD, PhtD C, PspC, and LytC in children <1 – 2 years of age (Obaro *et al.*, 2000; Rapola *et al.*, 2000; Bogaert *et al.*, 2006; Holmlund *et al.*, 2006, 2007, 2009; Simell *et al.*, 2006). PspC in particular appears to be highly immunogenic in children (Holmlund *et al.*, 2009).

Previously, intranasal exposure to pneumococcus serotype 6B using an EHPC model induced an increase in levels of serum IgG to six pneumococcal protein antigens 14 days post exposure in volunteers who established carriage (n = 29) and fourteen antigens in volunteers who did not establish carriage (Ferreira *et al.*, 2013). The level of induction in serum IgG levels was greater in the carriage positive cohort. Increased anti-protein IgG for 13 antigens persisted in the carriage positive cohort but not the carriage negative cohort 5 weeks post inoculation. There was no significant difference in levels of anti-protein IgG prior to experimental challenge (baseline) between individuals who developed carriage and those who did not. Ten carriage positive individuals were re-challenged with the homologous pneumococcal strain up to 11 months post clearance of the initial carriage event and all were protected against re-colonisation. In 8 out of 10 volunteers, protection against carriage reacquisition was associated with significantly altered levels of IgG to both proteins and the polysaccharide capsule. However, it is unclear to what extent humoral mediated immunity plays a role as compared to cellular-mediated immunity, particularly $CD4^+$ T helper type 17 cells. Notably, 2 out of the 10 volunteers did not have significantly increased levels of IgG.

It is important to recognise that the expression of any given pneumococcal protein may vary considerably during colonisation compared to when the pathogen is invasive. Thus, an adequate and functional mucosal immune response to protein antigens requires expression of the target antigen by the bacteria in order for the response to be protective (Pichichero, Khan and Xu, 2016).

PspA is one of the most important protein antigens when it comes to inducing a protective immune response and is thus a well-established vaccine candidate. The protein has been divided into six groups or “clades” based on the amino acid sequence of a protective “B” region found within the alpha-helical region, just upstream of the proline-rich region (Nabors *et al.*, 2000). The B region spans approximately 192-270 amino acids and elicits cross-protective immunity. The six clades vary by >20% of their amino acid sequence. The protein clades are then further grouped into 3 families. Family one includes clades 1 and 2, family 2 comprises clades 3-5 and family 3 equates to clade 6, however family 3 is far less common than families 1 and 2. As the protein is highly variable across clades, immune system

cross-reactivity against variations of the protein is essential to confer broad protection. It has been hypothesised that natural exposure to circulating serotypes of pneumococcus primes the human immune system to generate anti-PspA antibodies with broad cross-reactivity (Nabors *et al.*, 2000). PspA clades 1-5 are more closely related than is clade 6 to any of the other clades. Clades 4 and 5 are more highly related than any other clade pairs, followed by clades 1 and 2 (Nabors *et al.*, 2000). Antibodies against PspA clades 4 (PspA4) and 5 (PspA5) have been shown to recognize pneumococci expressing PspA from other clades (Moreno *et al.*, 2010). In a murine model, PspA4 and PspA5 were able to induce protection against lethal challenge with one pneumococcal strain expressing PspA from family 1 and one strain expressing family 2 PspA, indicating that these protein antigens have the potential to induce broad protection against different pneumococcal serotypes, an essential feature for a serotype-independent vaccine. Moreover, PspA4 and PspA5 induced antibodies that were able to bind and mediate C3 deposition on both family 1 and family 2 bearing pneumococcal strains whereas anti-PspA2 and anti-PspA1 antibodies were only effective against family 1 bearing strains. In contrast, antibodies against clades 2 and 3 show little cross-reactivity with PspA4 or PspA5; anti-PspA3 antibodies in particular appear to have no cross-reactivity against PspA of any other clade whilst anti-PspA2 antibodies show minimal cross-reactivity with clade 1 but none towards clades 3-6 (Nabors *et al.*, 2000; Darrieux *et al.*, 2008). Antibodies raised towards a PspA hybrid including PspA clade 1 N-terminal fused to the B region of clade 4 have elicited strong recognition of isolates containing clades 1, 4 and 5, and weaker reactions with clades 2 and 3 (Darrieux *et al.*, 2008). Thus, PspA hybrid proteins including B and whole N-terminal regions from different clades are likely needed to confer broad cross-reactivity and protection, which is of relevance when designing vaccines to include PspA (Darrieux *et al.*, 2007, 2008; Akbari *et al.*, 2019).

In the aforementioned EHPC study (Ferreira *et al.*, 2013), carriage induced a significant increase in the level of serum IgG to the following protein antigens 14 days post initial exposure: PspC, PspA-UAB055, PcpA, PhtD, PiuA and RrgBT4. In the absence of carriage, pneumococcal challenge elicited increased levels of IgG antibodies against PspC, LytC, PspA-UAB055 (clade 2), PcsB, PhtD, Ply (pneumolysin), PsaA, RrgBT4, SP2194, SP0057, SP0096, Spr1, Spr2021 and Stkp. Increased mucosal IgG to PspA was induced by carriage from 7 days post exposure, reaching significantly higher levels than baseline at day 28, 35 and 42. There was no induction in mucosal IgG against PspC in the carriage positive cohort. Furthermore, there was no significant change in levels of antibodies against PspA or PspC in the absence of carriage. Homologous re-challenge did not alter IgG levels to either protein. Interestingly, the clade 1 6B pneumococcus used to inoculate participants expresses 61% amino acid sequence homology with PspA-UAB055 (clade 2) and 40% with PspA-UAB099 (clade 3) but only IgG towards the clade 2 PspA was

significantly increased in response to experimental exposure. These results further point to the low reactivity of clade 3 PspA but high reactivity of clades 1 and 2. Increased mucosal anti-PspA IgA was observed 28 days after challenge for carriage-positive and carriage-negative volunteers, with a progressive increase in the carriage positive cohort until the increase was significant as compared to baseline at day 42. For the carriage negative cohort, levels of IgA were significantly higher 28 days post-inoculation but declined beyond this to levels that were not significantly higher than at baseline. Notably, passive-transfer of post-carriage sera conferred 70% protection against invasive pneumococcal disease in a lethal murine model of pneumococcal bacteraemia following challenge with a heterologous strain.

Again, using the EHPC model in which volunteers were intranasally exposed to serotype 6B pneumococcus, a study in an elderly cohort found that there were no differences in the baseline levels of any antibody between colonised and non-colonised participants, however, antibody titres against several pneumococcal proteins were increased following pneumococcal colonisation, including PspC, PspA-UAB055, RgrA-Tigr4, PiuA and PcpA. No increase in anti-capsular IgG was observed following colonisation (Adler *et al.*, 2021). This data may suggest that for elderly cohorts, pneumococcal immune response could be protein mediated. Moreover, serum levels and binding capacity of IgM against PspA and an unencapsulated pneumococcal strain isolated from hospital patients with and without lower respiratory tract infections were unchanged with increasing age, further substantiating that protein-based pneumococcal vaccines may be the superior method of providing protective immunity in the elderly (German *et al.*, 2018).

In similarity to PspA, PspC has been classified into 11 groups based on sequence comparison (Iannelli, Oggioni and Pozzi, 2002). PspC antibodies also show cross-reactivity against PspC variants of a different group (Moreno *et al.*, 2012). In animal models, anti-serum produced in response to recombinant PspC cross-reacted with both PspC and PspA from 15 pneumococcal isolates and the cross-reactive antibodies afforded protection against challenge with a strain expressing only PspA and not PspC. The cross-reactive antibodies were targeted to the proline-rich domain present in both proteins (Brooks-Walter, Briles and Hollingshead, 1999). However, it has been demonstrated that variation within the PspC antigen promotes immune evasion and could confer a fitness benefit during infection. In an antibody-dependent opsonophagocytic assay, recombinant anti-PspC antibodies were specific for their cognate PspC variant; only pneumococci expressing a recombinant PspC variant homologous to recombinant antibody specificity was killed efficiently whereas killing efficacy was not evident for pneumococci expressing a mismatched PspC variant (Georgieva *et al.*, 2018). A study conducted in the Gambia found that higher

levels of anti-PspC antibodies within the first 8 weeks of age (conferred via passive transfer of maternal antibodies to the child *in utero*), but not anti-PspA or Ply antibodies, were associated with protection against colonisation in infants (Mendy *et al.*, 2017).

In addition to PspA and PspC, Ply (pneumolysin), PhtD and PcpA are leading vaccine candidates (Feldman and Anderson, 2014; Kaur *et al.*, 2014). It has been demonstrated that human antibodies against pneumococcal proteins PhtD, PcpA, and Ply, which contain highly conserved regions, reduce adherence of pneumococcus to human lung epithelial cells and nasopharyngeal colonisation in mice (Kaur *et al.*, 2014). In a phase I clinical trial of a pneumococcal histidine triad protein D (PhtD) vaccine candidate in adults, administration of a second injection significantly increased the levels of anti-PhtD antibodies (Seiberling *et al.*, 2012). A vaccine comprising pneumococcal non-typeable *Haemophilus influenzae* protein D conjugate vaccine combined with pneumolysin and PhtD was tested in a phase II clinical trial in infants in The Gambia (Odutola *et al.*, 2017). Antibodies against both proteins were potently induced, however little protection was conferred against non-vaccine type pneumococcal carriage. Incremental efficacy in preventing acute otitis media over PCV13 in Native American infants was also not demonstrated by the vaccine (Hammitt *et al.*, 2019).

A comprehensive ANTIGENome analysis was conducted using serum antibodies of exposed, but not infected, individuals and convalescing patients (Giefing *et al.*, 2008). Serum samples for antigen screening were collected from 97 patients convalescing from invasive pneumococcal diseases, with an average age of 54 years, and from 40 healthy adults between 20 and 45 years of age. Eighteen protein vaccine candidates were preselected for animal studies, and 4 of them showed significant protection against lethal sepsis. In particular, protein required for cell wall separation of group B streptococcus (PcsB) and serine/threonine protein kinase (StkP) were found to be extremely highly conserved across clinical isolates (>99.5% identity). The proteins were also shown to be immunogenic during pneumococcal infections in children aged 2 months-18 years, inducing opsonophagocytic/bactericidal antibodies. 88 sera samples obtained from children not suffering from any infectious disease at the time of sampling were analyzed for anti-protein IgG levels; StkP-specific antibody levels showed a continuous increase from 2-4 years onwards, with peak median value in the 8–12-year old age group, whereas PcsB-specific IgG was low in infants and very young children (<12–24 months old) but median IgG levels increased after 2 years of life and remained at sustained levels up to 18 years of age. The proteins were subsequently found to confer cross-protection against four pneumococcal serotypes in lethal pneumonia and sepsis mouse models. Given the immunogenicity of both proteins in children and older adults, and because the proteins are

expressed during invasive disease, as well as during colonisation and exposure, it is possible that a vaccine comprising these proteins could address the needs of both target populations. A multi-antigen vaccine (MAV) comprising PspA and Ply has been shown to induce a robust antibody response against multiple pneumococcal serotypes, including non-PCV serotypes (Chan *et al.*, 2019). The vaccine conferred protection against pneumococcal pneumonia in rodents and passive transfer of serum from MAV-immunised rabbits to naïve mice conferred protection against sepsis caused by homologous and heterologous *S. pneumoniae*. These results illustrate the potential serotype-independent protection that could be conferred by protein-antigen based vaccines.

In HIV-infected individuals, the mucosal response to pneumococcal protein antigens does not appear to be defective, as anti-PspA and anti-Ply IgG have been found to be present in BAL fluid of HIV-positive adults (Collins *et al.*, 2013). This is of paramount importance if novel vaccines are to be effective in one of the most at-risk groups for pneumococcal disease (Siemieniuk, Gregson and Gill, 2011; Harboe *et al.*, 2014; Munier *et al.*, 2014). However, it is essential that functional protective response is evaluated to determine the true protective effect against pneumococcal pneumonia.

A pangenome-wide proteome microarray found that of over 2,000 pneumococcal proteins screened, 208 strongly bound antibodies in 35 adult human sera samples from Massachusetts, USA (Croucher *et al.*, 2017). The vast majority of proteins could be classified as either variants of PspA, PspC, ZmpA or ZmpB loci or more conserved proteins involved in adhesion, enzymatic degradation, solute binding, or cell wall synthesis. A panproteome-wide analysis of the changes in IgG binding following administration of a unencapsulated pneumococcal whole-cell vaccine identified elevated IgG binding to 72 functionally-distinct proteins post-vaccination (Campo *et al.*, 2018). However, induction of IgG response was generally small, potentially due to participants in the trial being healthy adults with likely pre-existing immunity to pneumococcus as well as the study being under-powered to detect immunogenicity of the vaccine.

1.7.3.1.2 Cellular-mediated response to pneumococcal proteins

It has been shown that human pneumococcal carriage enhances the proportion of lung and circulating IL-17A-secreting CD4+ memory T-cells, which may enhance innate cellular immunity to pneumococcal challenge (Wright *et al.*, 2013). Investigations in murine models also found that IL-17A and CD4+ T-cells were integral to the recruitment of innate immune cells responsible for pneumococcal clearance (Zhang, Clarke and Weiser, 2009). Furthermore, acquired immunity to pneumococcal colonisation in mice is also associated with the Th17 subset of CD4+ T-cells (Lu *et al.*, 2008) and T-cell mediated protection against colonisation can be induced in a protein antigen-specific fashion (Trzciński *et al.*, 2008). The ratio of Th17

CD4+ cells and regulatory T-cells in the human nasopharynx has been shown to increase with age and decrease with colonisation frequency, suggesting further a role of Th17 cells in pneumococcal clearance (Mubarak *et al.*, 2016). Moreover, an increased presence of Tregs in the adenoids of children is associated with increased pneumococcal colonisation of the nasopharynx, which may be attributed to the dampening effect of Tregs on Th17 CD4+ cells (Zhang *et al.*, 2011).

Pneumolysin has been shown to elicit a prominent $CD4^+$ T-cell proliferative response in human nasopharynx-associated lymphoid tissue (NALT) and peripheral blood from children (Gray *et al.*, 2014). A significant memory Th17 response to pneumolysin in NALT and a moderate Th17 response in peripheral blood was also observed, which was more prominent in carriage negative children compared to carriage positive children, suggesting a protective effect of this response against pneumococcal carriage. No difference was shown in pneumolysin-induced Th1 response between the carriage negative and positive children. Moreover, pneumolysin activated human monocytes and murine macrophages that was in part dependent on TLR- 4.

IL-17A and IL-22 responses against pneumococcal WCA were analysed in adenoidal samples from 33 children (Lu *et al.*, 2018). Significant, age-dependent IL-17A and IL-22 responses to WCA were observed; responses positively correlated with age. In 13 adenoidal samples, IL-17A and IL-22 production was elicited in response to WCA and 56 recombinant proteins. WCA exhibited the highest responses and IL-17A responses were generally in the range of 50 to 100 pg/ml. IL-22 responses were generally higher than IL-17A responses, mostly ranging from 200 to 600 pg/ml. In a murine model, 16 protein antigens elicited Th17-dependent protection against pneumococcal colonisation (Lu *et al.*, 2018). Comparison of IL-17A and IL-22 response to pneumococcal protein antigens in mouse and human cell screens found that antigens that more consistently induced cytokine (IL-17A and IL-22) responses across both cell screens were more likely to be protective against colonisation in murine models. Importantly, while protection against colonisation by any single antigen did not approach that of pneumococcal whole-cell vaccine in mouse models, a combination of three antigens significantly improved on the protective efficacy of any individual protein antigen. These findings substantiate that a candidate protein-based vaccine would likely need to comprise several antigens to maximize protection and coverage.

In addition, Th17 responses in human primary cell cultures from blood and adenoidal tissue (nasal associated lymphoid tissue) of children were investigated (Oliver *et al.*, 2019). PspC, PsaA and PspA generated a significantly increased IL-17A and IL-22 response from peripheral blood mononuclear cells (PBMCs) than did WCA. However, PhtD elicited no IL-17A or IL-22 response, which could explain the lack

of effectiveness of a PhtD vaccine in protecting against pneumococcal colonisation (Odutola *et al.*, 2017) and acute otitis media (Hammitt *et al.*, 2019) in phase II clinical trials. Neither WCA nor any of the 5 protein antigens elicited an IL-17A or IL-22 response in adenoidal mononuclear cells.

Using a proteomic screening approach, a library of candidate human $CD4^+$ Th17 cell antigens was identified (Li *et al.*, 2012). A murine model of pneumococcal colonisation showed that Th17-based immunity almost equally reduced colonisation by both an antigen-positive pneumococcal strain and a co-colonising antigen-negative strain. Additionally, unlike antibody target antigens in the pneumococcus, bioinformatic analysis found that the DNA sequences of Th17 cell antigens demonstrated no detectable signs of being under selective pressure. This evidence points to the importance of Th17-mediated immunity in limiting carriage of antigen-positive pneumococci and that this form of T-cell immunity does not favour diversifying selection in the target antigens. These results suggest evolution of escape from Th17-based vaccines may be slower than from antibody-based vaccines (Li *et al.*, 2012).

The GEN-004 vaccine consisting of 3 pneumococcal protein antigens, SP0148, SP1912 and SP2108, was tested using the EHPC model (Skoberne *et al.*, 2016). The protein antigens were selected following screening of an expression library containing >96% of predicted pneumococcal protein antigens. Antigens which were recognized by Th17 cells from mice immune to pneumococcal colonisation were selected (Moffitt *et al.*, 2011). Whilst the vaccine did consistently reduce carriage acquisition by 18% to 36% versus placebo, the differences were not statistically significant because the study was powered to detect 50% protection. No reduction in colonisation density or duration up to 14 days post-inoculation was observed. Unfortunately, a lack of analysis of Th17-mediated response to the vaccine means the effect of the vaccine on this cannot be deduced (Skoberne *et al.*, 2016; Jochems *et al.*, 2017).

1.7.3.1.3 Protein-based pneumococcal vaccines to date

As outlines in Appendix A, several protein-based vaccines have been taken forward into clinical trials. Table 2 includes a summary of pneumococcal proteins with vaccine potential (Feldman and Anderson, 2014). GlaxoSmithKlein also have a number of PCV/protein vaccines in development (Appendix A). Whilst there are various protein vaccines in the pipeline, the true effectiveness of these vaccines is yet to be determined. It is likely that any protein-based vaccine will need to include multiple highly immunogenic proteins (Ogunniyi *et al.*, 2007). Whilst a given protein may induce immunity against infection of a given site, for example the lungs, heart or brain, other proteins may be involved in preventing adherence to host tissues and colonisation. Vaccines comprising multiple proteins with different functions may therefore be necessary to provide broad protection against *S. pneumoniae* infection (Scott *et al.*, 2021).

Moreover, as multiple colonising events occur in childhood, resulting in a protein-mediated adaptive immune response (Wilson *et al.*, 2015; Ramos-Sevillano, Ercoli and Brown, 2019), it is likely that immune response against several proteins across various serotypes is developed over time rather than being serotype specific. Thus, multiple proteins would be needed to confer serotype-independent protection. In addition, as some leading protein vaccine candidates, such as PspA and PspC, show high levels of variation (Croucher *et al.*, 2017), it seems unlikely that any one antigen would be conserved enough to provide broad protection and so a multi-valent approach is essential.

A trivalent vaccine of pneumolysoid (a pneumolysin derivative) and epitopes of PspC and PspA has been tested in multiple mouse models and consistently demonstrated significantly broad and strong protection against multiple diseases, such as sepsis, pneumonia, and meningitis, caused by different serotypes (Chen *et al.*, 2015). A candidate trivalent vaccine comprising recombinant PcpA, PhtD, and pneumolysin has been shown to be safe and immunogenic in adults, toddlers, and infants in a phase I randomized control study (Brooks *et al.*, 2015). The study proceeded to a phase II trial, which investigated the vaccine reactogenicity, safety, and immunogenicity when co-administered with the existing PHiD-CV vaccine. However, as previously mentioned, little protection was conferred against nasopharyngeal pneumococcal carriage in Gambian infants (Odutola *et al.*, 2017) and incremental efficacy in preventing acute otitis media over PCV13 in Native American infants was not demonstrated (Hammitt *et al.*, 2019). PnuBioVax is a promising multi-protein vaccine undergoing developed by ImmunoBiology Limited. The vaccine contains protein antigens PspA2, pneumolysin (Ply), PsaA, PiaA, PiuA, RrgB and RrgA (TIGR4 protein sequences). It has been shown to be safe and immunogenic in a phase I trial in which a ≥2-fold increase in antibody titres against Ply, PsaA, PiaA, PspA and pilus proteins RrgB and RrgA was observed for most participants (n = 36) (Entwisle *et al.*, 2017). Additionally, a robust antibody response against PspA, Ply, PsaA and PiuA was elicited in rabbits in response to the vaccine and immunisation promoted killing of the vaccine pneumococcal strain, TIGR4, as well as heterologous strains (serotypes 6B, 19F and 15B) in an opsonophagocytic killing assay (Hill *et al.*, 2018).

Table 2: Pneumococcal protein adhesins and invasins with vaccine potential (Feldman and Anderson, 2014).

Protein	Ligand
Pneumococcal surface protein A (PspA)	E-cadherin
Pneumococcal surface protein C (PspC)	Polymeric Ig receptor FH domain of complement factor H Vitronectin
Choline-binding protein E (CbpE)	Plasminogen
α-Enolase	Plasminogen
Fibronectin-binding protein A (PfbA)	Fibronectin and plasminogen
Pneumococcal adherence and virulence factors A and B (Pav A/B)	Fibronectin and plasminogen
Polyhistidine triad (Pht) proteins	Unknown
Pneumococcal serine-rich repeat protein (Psrp)	The intermediary filament protein, KR10
Pneumococcal Choline-Binding Protein A (PcpA)	Uncertain
Pneumococcal pili, PI-1, and PI-2	Collagen, fibronectin and laminin
Type IV pilus	DNA
Neuraminidases A and B (Nan A/B)	Expose cryptic adhesion sites on target cells
Pneumococcus-specific glycosyl hydrolase 25 (GHIP)	Exposes cryptic adhesion sites on target cells
Hyaluronate lyase (SpnHL)	Hydrolyses extracellular matrix
NADH oxidase	Contactin 4, chondroitin 4, sulphotransferase, laminin
Pneumococcal protein endopeptidase O (PepO)	Plasminogen and fibronectin
Zinc metalloproteinase B (ZmpB)	Collagen IV
Cell wall-associated serine protease (PtrA)	Collagen IV
Autolysin A (LytA)	Promotes autolysis and release of Ply
Pneumolysin (Ply)	Facilitates invasion via pore-forming activity

1.7.3.1.4 Protein-based vaccine delivery systems

The need for adequate delivery system formulations for pulmonary vaccines has been demonstrated by a study conducted by Gordon *et al.*, (2008) in which the effect of intramuscular versus inhaled PPV23 on pulmonary mucosal immunoglobulin levels was compared. Whilst intramuscular administration of the vaccine resulted in a significant increase in polysaccharide-specific IgG against serotypes 1, 9V and 14 and type 1 IgA in both serum and bronchoalveolar lavage (BAL) 1-month post immunisation (n = 17), inhaled vaccine produced no response (n = 16). Moreover, the study illustrated that inhaled vaccines would be better focused on targeting pneumococcal surface proteins rather than capsular polysaccharide. Studies have shown that delivery of antigenic material directly to the lung has the capability to induce enhanced immune response and is a promising method for transport delivery (Alpar *et al.*, 2005).

Mucosal vaccination enables direct interaction between the vaccine and the mucosal immune system along the route of the respiratory tract; particulates depositing along the respiratory tract can be taken up and processed locally. Deposition of particles along the respiratory tract is size-dependent (Table 3) and determined by various mechanisms including inertial impaction, Brownian diffusion, gravitational sedimentation and electrostatic effects (Yang, Peters and Williams, 2008). Larger particles (>8µm) have greater impact on the upper respiratory tract, depositing from nasal passages to larger bronchioles in a size-dependent manner, whilst fine particles (<1–3µm) penetrate lung tissue and deposit in the alveoli (Thomas, 2013). Importantly, particles should be large enough in size to avoid clearance by lymphatic drainage (>100nm) but small enough to avoid clearance by alveolar macrophages (<500nm) (Fifis *et al.*, 2004; Scherließ and Janke, 2021).

Table 3: Correlation between the areas of lung deposition, the mechanism of deposition and particle size (Yang, Peters and Williams, 2008).

Site	Size (µm)	Mechanism	Comment
Large airways	5–9 (slow inhalation), 3–6 (fast inhalation)	Impaction	Most deposition in segmental airways
Smaller airways	1–5	Gravitational sedimentation	Improved with slow and deep breathe
Respiratory bronchioles	1–3	Gravitational sedimentation	Improved with slow and deep breathe
Alveoli	≤0.5	Brownian diffusion	Most exhaled

To be successfully delivered directly to mucosal sites in the nose or lungs, protein-based vaccines need to be packaged into specially designed delivery systems. These delivery systems must enhance antigen affinity for the mucosal epithelium and subvert mucociliary clearance through mucoadhesion or mucopenetration (Zaman, Chandrudu and Toth, 2013). Enhancing retention time at the mucosal site through mucoadhesion is postulated to inherently increase chance of interaction with local immune cells. Polymers such as Carbopol and sodium agglutinate exhibit hydrophobic properties and form hydrogen bonds with mucus, enhancing absorption (Zaman, Chandrudu and Toth, 2013). Other mucoadhesive polymers, which have been of interest include the biodegradable and biocompatible synthetic polymer polylactide-co-glycolide (PLGA) and natural polymer chitosan. Chitosan interacts with mucus via ionic bonds and also habors immunostimulatory properties through activation of the STING-cGAS pathway to stimulate type-1 interferon dependent dendritic cell maturation and enhancement of antigen-specific Th1 responses (Carroll *et al.*, 2016). In addition, delivery systems should facilitate translocation of antigens across the epithelium and endocytosis by antigen-presenting cells for presentation of antigens to the adaptive arm of the immune system (Paranjpe and Müller-Goymann, 2014; Gonçalves *et al.*, 2019).

Antigens are actively transported through tight junctions in M-cells located in the epithelium to reach antigen presenting cells for processing and presentation (Ogasawara *et al.*, 2011). It is also necessary to consider how to avoid potential clearance of molecules by alveolar macrophages in the lung (Paranjpe and Müller-Goymann, 2014). Molecular adjuvants are generally necessary in order to generate a great enough immune response to antigens as immunogenicity of antigens alone can be insufficient (Wang *et al.*, 2015) (Figure 7). Importantly however, further consideration must be given to any possibility of transport or accumulation of adjuvants present in nasally administered vaccines to olfactory bulbs of the brain via olfactory neurons (Alpar *et al.*, 2005).

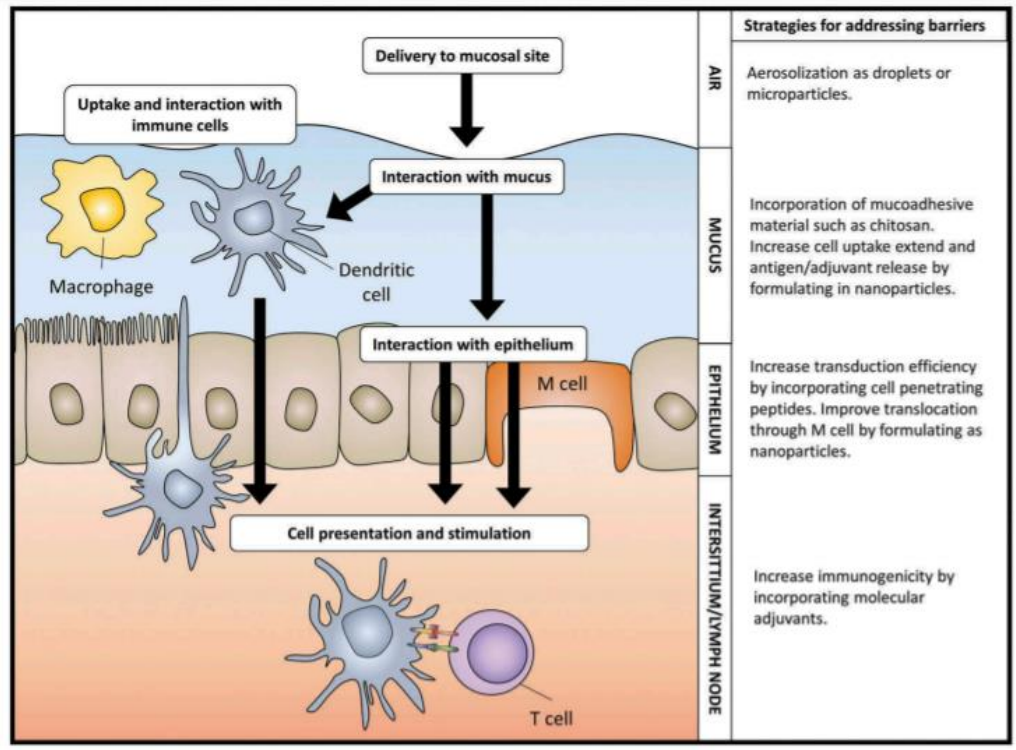


Figure 7: Overview of the barriers associated with vaccine delivery at the mucosal surface after administration through the nasal or pulmonary route. Common strategies for improving the vaccine efficacy through overcoming identified barriers have been listed. Image source: Gonçalves et al., 2019.

The most promising protein-based antigen delivery systems are bacterium-like particles, nanogels and nanoparticles (Gonçalves *et al.*, 2019).

1.7.3.1.4.1 Bacterium-like particles

Lactic acid bacteria have been explored as live vectors, which could deliver antigens to mucosal sites for the development of novel intranasal pneumococcal vaccines (Oliveira *et al.*, 2006; Hanniffy *et al.*, 2007).

Nasal inoculation of mice with *L. plantarum* NCDO1193 and *L. helveticus* ATCC15009 expressing the pneumococcal PsaA antigen induced both systemic anti-PsaA IgG and mucosa anti-PsaA IgA (Oliveira *et al.*, 2006). However, both *L. casei* and *L. lactis* expressing PsaA did not induce detectable levels of IgA in nasal or bronchial mucosa nor elevated systemic IgG, although *L. lactis* PsaA did induce higher levels of anti-PsaA IgG as compared to its control strain carrying an empty pT1NX vector. These results indicate that some *lactobacillus* strains are intrinsically more suited to mucosal vaccine delivery. Fifteen days post final inoculation, mice were challenged with *S. pneumoniae* strain 0603. Mice inoculated with *L. helveticus* PsaA showed significantly reduced pneumococcal colonisation as compared to mice inoculated with saline as well as mice inoculated with *L. helveticus*-pT1NX. Mice inoculated with *L. plantarum* PsaA showed decreased colonisation compared to the saline group but not compared to the respective control group carrying the empty vector. Administration of *L. casei* pT1NX led to an inhibition of colonisation when compared with the saline group but, in similarity to what was observed for *L. plantarum,* reduction in colonisation was not significantly different from the results obtained for the *L. casei* pT1NX group; potentially suggesting an immunising effect of the *lactobacillus* itself. Subsequent work showed that nasal immunisation of mice with *L. casei* expressing the N-terminal region of clade 1 PspA induced anti-PspA1 IgG in sera, with significantly higher titres than those observed for the groups inoculated with *L. casei*-pT1NX or saline (Campos *et al.*, 2008). However, no detectable increase in anti-PspA IgA was observed. Moreover, 5-day survival rate for mice immunised with *L. casei*-PspA1 was significantly higher than for mice inoculated with saline following lethal intraperitoneal challenge with A66.1 pneumococcus (33% survival vs 0% survival). Notably, it has been demonstrated that an intranasal lactococcal vaccine (*L. lactis*) expressing PspA affords improved protection against challenge with pneumococcus as compared to vaccination with purified antigen administered intranasally or injection with the adjuvant alum (Hanniffy *et al.*, 2007). Protection was associated with a shift toward a Th1 immune response.

Bacterium-like particles are based on acid-treated non-recombinant *Lactococcus lactis* bacteria, such as *L. lactis,* which have “Generally Regarded As Safe” status from the Food and Drug Administration (Braeckel-Budimir, Haijema and Leenhouts, 2013). Pretreatment of the bacteria in hot acid destroys cellular components, including DNA and cell-wall components, with the exception of peptidoglycan. This results in a non-living bacterium-like particle (BLP) that maintains the size and shape of the bacteria prior to the acid treatment with a diameter of approximately 1–2µm and a peptidoglycan outer surface. Bacterium-like particles can be used both as immunostimulatory molecules and delivery systems for antigen-based vaccines.

In BALB/c mice, intranasal immunisation with a PspA-BLP pneumococcal vaccine, comprised of PspA2 from pneumococcal family 1 and PspA4 from pneumococcal family 2, induced both high levels of serum IgG and mucosal secretory IgA antibodies with broad coverage of binding to pneumococcal strains expressing PspA clades 1-5 (Lu *et al.*, 2019). Antibody levels were significantly higher in the group immunised with BLP-PspA as compared to the BLP control group. Immunisation with the vaccine conferred protection against fatal intranasal challenge with both PspA family 1 and family 2 pneumococcal strains regardless of serotype. Following vaccination with BLP, BLP-PspA or PPV23, mice were challenged with either pneumococcal strain ATCC10813 (PspA family 1, clade 2) or ATCC6303 (PspA family 2, clade 5). Mortality was monitored for 14 days after challenge. For both the ATCC10813 and ATCC6303 challenged cohorts, the BLP group showed no protection against lethal challenge with pneumococcus, whereas mice in the BLP-PspA and PPV23 groups showed a high level of protection. Mice immunised with BLP-PspA reached 100% protection, higher than that observed in the PPV23 vaccinated group. Thus, it was demonstrated that BLPs are promising delivery systems for pneumococcal antigen-based vaccines administered intranasally, inducing systemic and mucosal humoral response against pneumococcus.

1.7.3.1.4.2 Nanogels

A nanometer-sized hydrogel (nanogel) consisting of a cationic cholesteryl group-bearing pullulan (cCHP) combined with PspA was developed and tested in murine pneumococcal airway infection models (Kong *et al.*, 2013). cCHP is a universal protein-based antigen-delivery vehicle for adjuvant-free nasal vaccination (Fukuyama *et al.*, 2015). Immunisation with the vaccine induced high levels of circulating anti-PspA IgG as well as nasal and bronchial IgA response. Systemic and nasal mucosal Th17 responses were also elicited. Survival rates after lethal challenge with *S. pneumoniae* Xen10 over a 2-week period for mice immunised with cCHP-PspA, PspA-CT (cholera toxin), PspA alone, or PBS were evaluated. Serotype Xen10 expressed homologous PspA as the nanogel vaccine (clades 1 and 2). The survival rate for both the cCHP-PspA and PspA-CT groups was 100%, compared to 0% in the group immunised with PspA alone and 20% in the group immunised with PBS (8 days post-challenge). Reduced colonisation and invasion in the upper and lower respiratory tracts by Xen10 pneumococcus was observed following vaccination. Mice nasally inoculated with cCHP-PspA were also protected from lethal challenge with serotype 3 *S. pneumoniae* strain 3JYP3670, which expresses clade 4 PspA, demonstrating a level of cross protection against pneumococcal strains expressing PspA from differing families. Importantly, no evidence of PspA delivery by nanogel to either the olfactory bulbs or the central nervous system after intranasal administration was observed, further attesting to the safety and effectiveness of the nanogel-based PspA nasal vaccine system as a mucosal vaccine against pneumococcal respiratory infection.

Comparatively, another cationic cholesteryl group-bearing pullulan nanogel (cCHP nanogel) containing PspA was established and its safety and efficacy against pneumococcal infection in macaques was examined (Fukuyama *et al.*, 2015). Nasal vaccination with cCHP-PspA induced PspA specific IgG in serum with protective activity as well as mucosal secretory IgA response. In similarity to responses observed in the murine models inoculated with a cCHP-PspA vaccine, nasal cellular immune response was mediated through secretion of Th2 and Th17 cytokines by $CD4^+$ T-cells. Furthermore, no deposition of fluorescently labelled PspA in the olfactory bulbs or brain was seen following vaccination.

1.7.3.1.4.1 Nanoparticles

Nanoparticles offer a novel means of vaccine delivery due to their easy preparation, biodegradability, non-toxic properties, biocompatibility, and the ability to adapt surface properties (Pati, Shevtsov and Sonawane, 2018). Antigens can either be adsorbed onto the surface of a nanoparticle or be encapsulated within it (Figure 8).

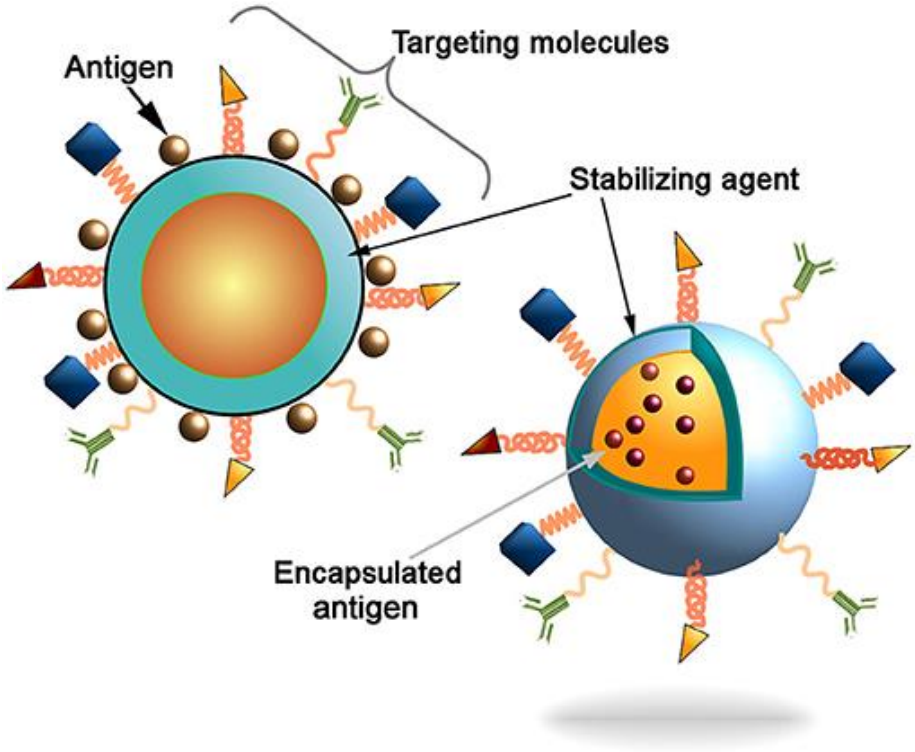


Figure 8: Schematic representation of the nanocarriers. Antigen can be conjugated to the nanoparticles surface or incapsulated into core of the particles. Decoration of the nanoparticles surface with targeting molecules (e.g., antibodies, Fab-fragments, peptides, etc) could further increase the delivery of particles into the antigen presenting cells (APCs) to induce innate and adaptive immune responses. Image source: Pati, Shevtsov and Sonawane, 2018.

Various nanoparticle formulations have been explored, including polymeric nanoparticles and liposomes (Paranjpe and Müller-Goymann, 2014; Wang *et al.*, 2015; Pati, Shevtsov and Sonawane, 2018; Jin *et al.*, 2019). Polymers which have already been extensively explored for therapeutic pulmonary drug delivery, include the synthetic polymers poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), poly(ε-caprolactone) (PCL) and natural polymers alginate, chitosan and gelatin base (Menon *et al.*, 2014; Paranjpe and Müller-Goymann, 2014).

A PGA-co-PDL (poly(glycerol adipate-co-ω-pentadecalactone)) nanoparticle with surface adsorbed PspA4 encapsulated in L-leucine microparticles has been synthesized in order to develop a pneumococcal protein vaccine which can be inhaled as a dry powder to be delivered directly to the lungs (Kunda, Alfagih, Miyaji, *et al.*, 2015). The incorporation of L-leucine microparticles enables modification of particle size to ensure successful pulmonary delivery (Kunda, Alfagih, Miyaji, *et al.*, 2015). Immunisation of mice with the nanoparticle-nanocomposite microparticle formulation (NP/NCMP PspA4) induced both systemic and lung anti-PspA4 IgG (Rodrigues *et al.*, 2018). Antibodies exhibited efficient binding to pneumococcal strains expressing family 2 PspA clades, though not family 1 clades. Whilst subcutaneous injection with purified protein and mucosal immunisation with the NP/NCMP PspA4 vaccine induced comparable levels of protection against lethal intranasal challenge with serotype 3 (expressing clade 5, family 2 PspA), NP/NCMP PspA4 vaccination induced earlier control of infection. No reduction in bacterial burden in the lungs following challenge with serotype 19F, expressing clade 1 PspA (family 1) was observed following either immunisation with the vaccine or subcutaneous administration of purified protein. Importantly, mucosal immunisation with the NP/NCMP PspA4 vaccine induced much higher anti-PspA4 IgG titres in the lungs than did mucosal immunisation with the purified protein, which induced a negligible IgG response. Moreover, no increase in IgG titres was observed following immunisation with the empty NP/NCMP formulation (no PspA4 adsorbed). There was no significant increase in IgA levels in any of the groups, although a slightly stronger induction was observed in the NP/NCMP PspA4 group. These results suggest that mucosal immunisation with the nanoparticle formulation may be a promising method of mucosal vaccine delivery, inducing both local and systemic antibody response.

Another nanoparticle formulation which has been explored for the development of a mucosal pneumococcal vaccine is a complex of polysorbitol transporter adjuvant with PspA (Kye *et al.*, 2019). The polysorbitol transporter (PST) adjuvant forms nano-sized complexes with the PspA. Whilst intranasal immunisation with PspA alone was unable to induce any protection against pneumonia infection in murine models, the PspA/PST complex induced robust anti-PspA antibody responses both systemically

and in the lung, which was significantly higher than that induced by PspA alone. Vaccination with PspA/PST induced a comparable IgG response to that induced by a PspA-cholera toxin formulation. PspA-specific serum IgA was also detected following immunisation with PspA/PST. Moreover, all mice immunised with PspA/PST vaccine survived lethal challenge with pneumococcus 12 weeks post-immunisation. In particular, long-term T- and B-cell immune responses were observed, with a preferential shift towards Th2 or T follicular helper (Tfh) cell response mediated through antigen-presenting cells via activating a peroxisome proliferator-activated receptor gamma (PPAR-γ) pathway. These results further attest to the potential of these nanoparticle-based vaccines and demonstrate the necessity of a successful adjuvant for mucosal administration of protein subunit vaccines.

Finally, the natural polymer chitosan has garnered much enthusiasm as a promising nanoparticle-based vaccine delivery system due to its biodegradable, biocompatible and mucoadhesive properties as well as being easily modifiable and adaptable in size and shape (Singh *et al.*, 2018; Jin *et al.*, 2019). The mucoadhesive properties of chitosan are believed to be mediated through adherence to mucins via electrostatic interactions between the positively charged polymer and the negatively charged cell sialic acid of the mucins (Singh *et al.*, 2018). As previously discussed, chitosan has also been shown to act as a successful adjuvant (Carroll *et al.*, 2016). Chitosan-PsaA nanoparticles were developed and used to intranasally immunise mice (Xu *et al.*, 2015). Higher anti-PsaA IgG titres were observed in the sera of mice immunised with chitosan-PsaA as compared to intranasal immunisation with naked PsaA. Furthermore, increased IgA levels were detected in the nasal wash, bronchoalveolar lavage fluid and middle ear lavage of mice immunised with the chitosan-PsaA vaccine as compared to mice vaccinated with the naked PsaA, thereby suggesting an enhancing of mucosal immune response to protein by the chitosan. 100% of 10 mice vaccinated with chitosan-PsaA survived lethal invasive challenge with serotypes 3 and 14 pneumococcus compared to 60% in the naked PsaA group infected with pneumococcus serotype 3 and only 40% in the naked PsaA group infected with pneumococcus serotype 14. Significantly increased protection against pneumococcal acute otitis media was also conferred by the chitosan-PsaA vaccine as compared to naked PsaA or chitosan alone, whereas no difference in level of protection was observed between the naked PsaA and chitosan groups. Thus, immunisation with a chitosan-protein based vaccine may provide protection against pneumococcal infection.

Further investigation is needed to establish whether nanoparticle-based vaccine formulations have similar immunising effects in humans as seen in murine models and it is also necessary to establish safety of these vaccines regarding translocation to and accumulation within the central nervous system.

There is varying evidence surrounding the likelihood of extrapulmonary translocation of nanoparticles to the circulation and extrapulmonary organs (Yang, Peters and Williams, 2008). It is postulated that the well-established harmful effects of air pollution on human cardiovascular health could be in part due to the phenomena of translocation of inhaled particulates from the lungs into the circulation. The translocation of inhaled nanoparticulates to extrapulmonary organs, as well as the olfactory bulb in rats suggests that further investigations need to be carried out in order to determine whether the same is observed in humans (Oberdörster *et al.*, 2008; Zhu *et al.*, 2009; X. He *et al.*, 2010). The occurrence of such is likely dependent on nanoparticle properties (Yang, Peters and Williams, 2008). It is generally accepted that nanoparticles with a diameter of less that 35nm can cross the blood-brain barrier (Dawson, Salvati and Lynch, 2009).

1.8 Experimental Human Pneumococcal Carriage model

It is important to note that in many clinical trials, prevention of nasopharyngeal carriage is used as a measurable endpoint for testing novel vaccines as placebo-controlled trials are not ethically justifiable (Ferreira, Jambo and Gordon, 2011). The immunological correlates of protection against colonisation in humans are not yet well understood, limiting the development of effective novel vaccines (Jochems *et al.*, 2017), however human challenge models can help to elucidate mechanisms of protection against carriage.

An experimental human pneumococcal carriage (EHPC) model was developed as previously described (Gritzfeld *et al.*, 2013). Volunteers are inoculated in each nostril with pneumococcus and are classified as carriage positive if they become colonised with the inoculation strain at any time-point during the study after challenge. If volunteers do not establish carriage throughout the study, they are deemed carriage negative. Colonisation is determined by classical microbiology from nasal wash samples taken at multiple time-points. Blood samples are taken from volunteers prior to inoculation (screen) and post-inoculation. The genetic stability of the inoculation serotype 6B ST138 over 35 days has been evaluated and minimal variation was observed, attesting to the stability of the EHPC model over this time-frame (Gladstone *et al.*, 2015).

The EHPC model provides a highly controllable platform to test immune response to challenge with pneumococcus in various cohorts of participants (Rylance *et al.*, 2019; Adler *et al.*, 2021), to conduct co-colonisation studies, such as with influenza virus (Jochems *et al.*, 2018), and to enable the testing of existing and novel vaccines, informing vaccination strategies and development (Collins *et al.*, 2015). The model has now been established in Malawi, where it can be used to determine immunological correlates

of protection against pneumococcal colonisation as well as vaccine efficacy in this population (Morton *et al.*, 2021).

1.9 Thesis objectives

The identification of serotype-independent cellular and humoral correlates of protection against pneumococcal infection is needed to accelerate development of serotype-independent vaccines.

The first aim of this work was to develop a multiplex assay allowing rapid detection of protein-specific antibody responses to a library of 75 highly conserved purified pneumococcal protein antigens (Chapter 2). First, it was necessary to determine correlates of natural protection against pneumococcal colonisation of the nasal mucosa (Chapter 3). Then, establish which pneumococcal protein antigens are immunogenic, generating a significant humoral immune response (Chapter 3).

Proteins which correlated with protection from carriage, or which generated a significant increase in systemic protein-specific IgG post colonisation with pneumococcus were selected as potential vaccine candidates. Cellular response to these proteins *in vitro* was then investigated to determine whether the proteins induced adaptive immune response (Chapter 4). In this way, we aim to inform novel protein-based vaccine development with serotype-independent coverage.

Finally, this work assessed the potential efficacy of novel nanoparticle-based vaccines in stimulating adaptive immunity to pneumococcal protein antigens *in vitro* (Chapter 5).

Chapter 2

Developing and validating a multiplex assay using Luminex xMAP technology

2.1 Abstract

The identification of serotype-independent correlates of protection against pneumococcal infection will accelerate development of vaccines with broad protection. An Experimental Human Pneumococcal Challenge (EHPC) model biobank of samples and a large library of purified pneumococcal proteins were used to develop a multiplex assay to measure systemic protein-specific IgG responses against pneumococcus (Spn). A multiplex Luminex multi-analyte profiling (xMAP) assay was developed, optimised and validated. Antibodies in clinical samples bind to analytes conjugated to microspheres, enabling detection of protein-specific antibody binding. Serum samples from EHPC volunteers intranasally inoculated with serotype 6B (Spn6B) were used for assay validation. Net median fluorescence intensity (MFI) values averaged across duplicates were used to report anti-protein IgG levels. The assay successfully detected serum antibody binding to the full library of pneumococcal proteins. Multiplex (22-plex) and singleplex assays showed excellent correlation. Average median intra-assay coefficient of variation (CV) for all proteins was 1.56%. Median inter-assay CV of average net MFI values for proteins was 22.45%. Volunteers susceptible to experimental colonisation with Spn6B elicited significantly increased antibody levels to three immunogenic antigens 29 days post-challenge compared to pre-challenge. This observation was not detected in volunteers who were protected against colonisation. Minimal difference was identified between susceptible and protected cohorts at baseline. The findings of this work using the novel assay are in accordance with results obtained in previous studies by ELISA or MSD technology. A multiplex assay has thus been developed allowing rapid determination of protein-specific antibody responses to a large library of purified pneumococcal vaccine candidates before and after experimental challenge with *S. pneumoniae*.

2.2 Introduction

Enzyme-linked immunosorbent assays (ELISAs) have routinely been used to measure serum levels of anti-pneumococcal IgG. However, ELISAs are limited in that a separate test is required to measure antibodies against different capsular serotypes or different proteins. Multiplex assays enable rapid detection of antibody response to multiple analytes simultaneously using the minimum amount of

sample, which is often limited, greatly reducing time and expense as compared to singleplex assays. In 2011, Goldblatt *et al.* compared a new multiplex electrochemiluminescence-based detection assay with the existing WHO consensus ELISA for the quantification of pneumococcal capsular polysaccharide IgG (Goldblatt *et al.*, 2011). The electrochemiluminescence (ECL)-based detection assay was based on the Meso Scale Discovery (MSD) technology that includes integrated screen-printed carbon ink electrodes on the bottoms of plate wells. Polysaccharides bind directly to the carbon surface onto specific spots, with up to 10 spots per well. A Sulfo-tag label emits light upon electrochemical stimulation when an electrical current is passed through the plate (Marchese *et al.*, 2009). Because polysaccharides bind directly to the carbon surface on the bottom of wells, the ECL detection assay does not require conjugation of pneumococcal capsular polysaccharides to a solid phase. Thus, regarding the detection of anti-capsular polysaccharide IgG, MSD technology may be less affected by the potential impact on the antigenicity capacity of polysaccharides, i.e. the ability to specifically bind with anti-polysaccharide IgG, than bead-based technologies such as Luminex (Marchese *et al.*, 2009). Good agreement between ELISA and MSD techniques and two testing laboratories for the detection of IgG against seven pneumococcal serotypes was observed (Marchese *et al.*, 2009). MSD technology has previously been used to detect antibody levels against 27 pneumococcal proteins and showed an increase in circulating IgG against a number of proteins post-colonisation with Spn6B in both young and elderly adult cohorts (Ferreira *et al.*, 2013; Adler *et al.*, 2020).

A study comparing Luminex and MSD technologies for human cytokine profiling found that whilst MSD had better sensitivity, Luminex was more precise (Chowdhury, Williams and Johnson, 2009). MSD technology has a lower limit of quantification for much fewer analytes in human plasma than does Luminex technology. Luminex assays also require less labor time than MSD assays (Günther *et al.*, 2020). In a comparison of three multiplex cytokine assays to detect cytokine concentrations in small volume samples taken from a pediatric cohort with epilepsy, Luminex xMAP technology had much higher recovery of values extrapolated outside of range for all analytes than did MSD; <15% of values extrapolated outside of range compared to 40%, respectively (Numis *et al.*, 2021). The study found that Luminex may optimise yield in plasma for proteins of particular interest in epilepsy research as well as improving precision compared to standard sensitivity and MSD assays. Overall, Luminex technology is considered more robust in multiplexing than MSD technology as many more analytes can be run per specimen, although MSD is perhaps more sensitive (*eagle-i search*, 2019). Importantly, Luminex assays are also customizable.

Luminex multi-analyte profiling (xMAP) technology (Luminex Corporation, Austin, Texas, USA) enables the detection and quantification of multiple analytes simultaneously. The high-throughput immunoassay technology produces comparable results to ELISA but with greater efficiency, speed, and dynamic range. In addition, multiplexing is less expensive per target result. In the context of this project, the Luminex technology enables the measurement of levels of antibody binding to multiple proteins in a single well. Here, the development of a 22-plex assay is described. Microsphere populations are impregnated with an internal dye, which fluoresces at a wavelength specific to that population, or region. A light source inside the Luminex® 100/200™ analyser excites the internal dye, enabling the identification of each microsphere region. A chosen analyte is conjugated to a specific microsphere region. Antibodies present in samples bind the analytes conjugated to the microspheres. Fluorescently labelled detection antibodies then bind the primary antibody. The fluorescently labelled antibody is also excited by the light source inside the analyser, thus binding events of the primary antibody to the specific analyte can be quantified (Figure 9).

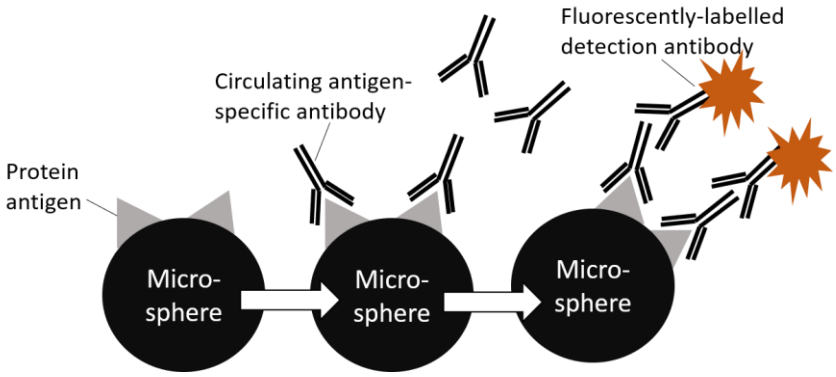


Figure 9: Application of Luminex xMAP technology to detect antigen-specific antibodies in circulation. Microsphere populations are impregnated with an internal dye, which fluoresces at a wavelength specific to that population, or region. A light source inside the Luminex 100/200™ analyser excites the internal dye, enabling the identification of each microsphere region. Antigen-specific antibodies present in samples bind the analytes conjugated to the microspheres. Fluorescently labelled detection antibodies then bind the primary antibody. The fluorescently labelled antibody is also excited by the light source inside the analyser, thus binding events of the primary antibody to the specific analyte can be quantified.

Previously, multiplex immunoassays utilising Luminex technology have been developed to assess humoral response to pneumococcal polysaccharides and were shown to be a viable method of quantifying circulating IgG (Pickering *et al.*, 2002; Lal *et al.*, 2005; Pavliakova *et al.*, 2018). Notably, these assays focus on anti-capsular IgG rather than anti-protein response. Shoma and colleagues previously developed a Luminex xMAP assay for the detection of serum antibodies against 17 pneumococcal proteins and showed that anti-protein antibody levels exhibited extensive inter-individual variability in 36 young children

suffering from invasive pneumococcal disease (Shoma *et al.*, 2010). The assay was subsequently used to detect changes in IgG levels against the 17 proteins across the first 36 months of life in 57 infants (Lebon *et al.*, 2011). Similarly, a 2015 study developed a Luminex xMAP multiplex assay to quantify levels of serum IgG against 64 pneumococcal proteins in 54 children (aged 6 months – 12 years) with pneumococcal pneumonia compared to 21 children with non-pneumococcal infectious disease and 31 healthy children (Jiménez-Munguía *et al.*, 2015). The study identified significantly reduced IgG response to one protein (RrgB) in children with pneumococcal pneumonia under 4 years of age compared to control groups.

In the current work, a multiplex Luminex assay has been developed and validated using a library of 75 purified pneumococcal protein antigens. The aim of this work was to develop a rapid means of measuring anti-protein IgG in a biobank of samples taken from EHPC volunteers inoculated with pneumococcus to identify serotype-independent correlates of protection and accelerate development of novel vaccine candidates with broad coverage.

2.3 Materials and Methods

2.3.1 Protein antigens

A library of 70 pneumococcal protein antigens, was developed by the Malley laboratory at the Division of Infectious Diseases, Boston Children's Hospital, Harvard Medical School, MA, USA (Lu *et al.*, 2018). The library was developed to identify novel protein-based vaccine candidates. Protein antigens were selected by bioinformatic analysis *in silico.* Beginning with the TIGR4 strain, a serotype 4 isolate and a commonly used model strain, 42 pneumococcus sequences were chosen from the integrated microbial genomes website. 335 proteins were identified with a secretion signal peptide and 15 proteins with possible cell wall anchor motifs were identified. The following parameters, which were chosen *a priori*, were used to narrow the library down to 70 proteins:

1. Conserved across all 42 sequences defined as >90% identity at the amino acid level.
2. Exclusion of proteins with >40% homology with proteins in the human genome.
3. To concentrate on proteins most likely to be accessible to antibodies in the presence of a polysaccharide capsule, proteins containing an extracellular domain smaller than 100 amino acids were also excluded.
4. Antigens which had previously been studied were excluded to ensure identification of novel candidates. Antigens excluded on this basis included PsaA, SP2018 and SP0148, StkP and PcsB, and Pht family proteins.

Protein functions are given in Table 4. Extracellular domains without transmembrane regions or signal peptides were cloned. TIGR4 genomic DNA was used as a template to amplify extracellular domains of the selected proteins using PCR. Using the CloneEZ PCR cloning kit, the domains were then integrated into pET21b expression vectors. *E. coli* transformants containing the cloned proteins were used for protein expression. Proteins were purified from supernatants of lysed cell pellets over a Ni-NTA column and were eluted in imidazole buffer. Elutions containing each protein were combined and purified over a gel filtration column in 20 mM Tris-HCl, 150 mM NaCl (pH 8.0). Due to difficulty in purifying the full length of proteins SP0648 and SP1154 (>250kDa), each amino acid sequence was divided into three parts and each fragment was purified separately. Predictions of the secondary structures of the proteins by BCL::Jufo (Simultaneous Prediction of Protein Secondary Structure and Trans-Membrane Spans. Available online: http://www.meilerlab.org/index.php/bclcommons/show/b_apps_id/2) were used to ensure proteins were truncated in un-conserved sequence regions. Furthermore, two possible extracellular domains of protein SP0662 were cloned and designated SP0662-1 and SP0662-2. Thus, the final library comprised 81 proteins and peptides, of which 70 purified proteins were included in the panel developed during this project. Plasmid inserts were confirmed by sequencing (Genewiz Inc.).

The alpha-helical N-terminal domain of PspA1, PspA4, PspC6, PspC9, which are well-established vaccine candidates (Miyaji *et al.*, 2013), were provided by the Miyaji laboratory (Laboratório de Bacteriologia, Instituto Butantan, São Paulo, Brazil) to include in the assay. PspA1, PspC6 and PspC9 were cloned using DNA from strain BHN418 (serotype 6B pneumococcus), the sequence of which [GI:557376079] is available from https://www.ncbi.nlm.nih.gov/nuccore/557376079. It is important to note that these proteins were therefore homologous to the Spn6B strain used to challenge EHPC volunteers in this work. PspA4 was cloned from strain 255/00 (serotype 14, GenBank accession number EF649969). Proteins were cloned into expression vectors and produced in *E. coli* as described previously (Darrieux *et al.*, 2008). All cloning procedures were performed in *E. coli* DH5α grown in Luria–Bertani medium supplemented with ampicillin (100 μg/ml^{-1}). DNA fragments encoding the N-terminal regions of the proteins were amplified by PCR from the genomic DNA. The gene products were cloned into a pGEMT-Easy vector (Promega) and the correct sequences were confirmed by DNA sequencing. The pGEMTEasy/pspA constructs were digested with restriction endonucleases and the resulting fragments were subcloned into linearized pAE-66His vector, which was used to transform BL21(DE3) *E. coli* competent cells. The recombinant proteins were purified from the soluble fraction by affinity chromatography using Ni^{2+}-charged resin in an Äkta prime apparatus. Elution was carried out with

imidazole. The purified fractions were analysed by SDS-PAGE, dialysed against 10mM Tris-HCl (pH 8), 20mM NaCl, 0.1 % glycine.

The final protein library used to develop the assay described in this project is shown in Table 4.

Table 4: Library of 75 highly conserved pneumococcal proteins used to develop a multiplex assay utilising Luminex xMAP technology.

Protein	Function	Protein	Function
SP0043	Competence factor transport protein ComB	SP1386	Spermidine/putrescine ABC transporter
SP0079	Potassium uptake protein, Trk family protein	SP1404	Hypothetical protein
SP0084	Sensor histidine kinase	SP1479	Peptidoglycan N-acetylglucosamine deacetylase A
SP0092	ABC[1] transporter, substrate-binding protein	SP1500	Amino acid ABC[1] transporter substrate-binding protein
SP0098	Hypothetical protein	SP1545	Hypothetical protein
SP0127	Hypothetical protein	SP1560	YbbR-like lipoprotein, putative
SP0149	Lipoprotein	SP1652	Efflux ABC transporter, permease protein
SP0191	Lipoprotein, putative	SP1683	Carbohydrate ABC transporter substrate-binding protein
SP0198	Hypothetical protein	SP1826	ABC transporter, substrate-binding protein
SP0249	PTS[2] system, IIB component	SP1872	Iron-compound ABC transporter, iron-compound-binding protein
SP0321	PTS system, IIA component	SP1897	Sugar ABC transporter, sugar-binding protein
SP0346	Capsular polysaccharide biosynthesis protein Cps4A	SP1942	Transcriptional regulator, putative
SP0402	Signal peptidase I	SP2083	Sensor histidine kinase PnpS
SP0435	Translation elongation factor P	SP2151	Carbamate kinase
SP0564	Hypothetical protein	SP2192	Sensor histidine kinase
SP0582	Hypothetical protein	SP2197	ABC transporter, substrate-binding protein, putative
SP0601	ABC transporter, transmembrane protein Vexp3	SP2207	Competence protein ComF, putative
SP0604	Sensor histidine kinase VncS	SP2218	Rod shape-determining protein MreC
SP0617	Matrixin family protein	SP2145	Antigen, cell wall surface anchor protein,
SP0620	Amino acid ABC transporter, amino acid-binding protein, putative	SP1534	Manganese-dependent inorganic pyrophosphatase, putative
SP0629	D-Ala-D-Ala carboxypeptidase, metallo peptidase	SP2070	Glucose-6-phosphate isomerase

[1] ATP-binding cassette
[2] Phosphotransferase system

SP0648-1	Beta-galactosidase	SP1002	Adhesion lipoprotein
SP0648-2	Beta-galactosidase	SP0148	ABC transporter, substrate-binding protein
SP0648-3	Beta-galactosidase	SP0314	Hyaluronate lyase precursor
SP0659	Thioredoxin family protein	SP0336	Penicillin-binding protein
SP0662-2	Sensor histidine kinase	SP0369	Penicillin-binding protein 1A
SP0678	Rhodanese-like domain	SP0930	Choline binding protein E
SP0724	Hypothetical protein	SP1492	Cell wall surface anchor family protein
SP0742	Hypothetical protein	SP1833	Cell wall surface anchor family protein, metal binding
SP0757	Cell division protein FtsX	SP2108	Maltose/maltodextrin ABC transporter, maltose/maltodextrin-binding protein
SP0785	HlyD family secretion protein	SP0662-1	Sensor histidine kinase, putative
SP0787	Antimicrobial peptide ABC transporter permease	SP2099	Penicillin-binding protein 1B
SP0878	SpoE family protein	PspC6	Pneumococcal surface protein C (clade 6); binds complement factor H to evade C3 opsonization
SP0899	Hypothetical protein	PspC9	Pneumococcal surface protein C (clade 9); binds complement factor H to evade C3 opsonization
SP1032	Iron-compound ABC transporter	PspA1	Pneumococcal surface protein A (clade 1); cell wall-associated protein involved in inhibiting complement-mediated opsonization and preventing lactoferrin-mediated clearance
SP1069	Hypothetical protein	PspA4	Pneumococcal surface protein A (clade 4); cell wall-associated protein involved in inhibiting complement-mediated opsonization and preventing lactoferrin-mediated clearance
SP1154-2	IgA1-specific metallopeptidase	PdB	Pneumolysin mutant
SP1376	Shikimate 5-dehydrogenase		

2.3.2 Luminex assay development and optimisation

2.3.2.1 Protein coupling to Luminex MagPlex microspheres

Protein antigens were covalently coupled to carboxylated magnetic Luminex MagPlex microspheres, or beads, using the xMAP Antibody Coupling Kit as per the manufacturer's instructions. A two-step carbodiimide reaction between the carboxyl group on the surface of the microsphere and the amine groups of the proteins enable coupling of the proteins to the microspheres. Chemical reactions involved in the coupling process are depicted in Figure 10.

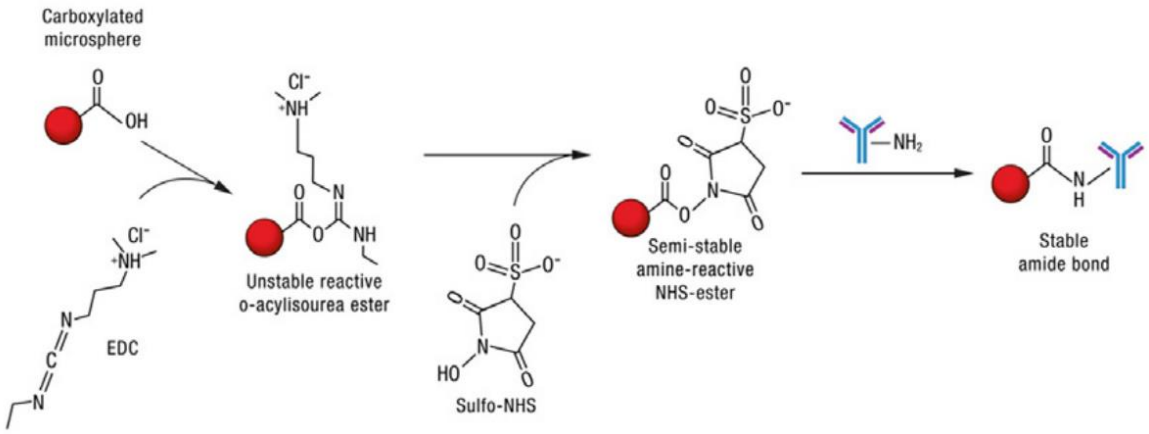


Figure 10: Protein coupling to carboxylated MagPlex microsphere reaction. A carbodiimide derivative, EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride), first reacts with the carboxyl groups on the microsphere surface prior to coupling the protein antigen to form an active O-acylisourea intermediate. Sulfo-NHS (N-hydroxysulfosuccinimide) is used to form a more stable amine-reactive ester, which reacts with the primary amine groups of protein antigens to form an amide linkage (covalent bond). It should be noted that in the reaction protocol, in order to maximize the efficiency of the reaction, Sulfo-NHS is added prior to EDC as the EDC microsphere conjugate is of limited stability. Image taken from Luminex xMAP Antibody Coupling Kit User Manual (2016).

Stock microspheres were re-suspended by vortexing and sonication for 10 seconds. $1x10^6$ of stock microspheres were added to each reaction tube and washed three times with activation buffer. Microspheres were then re-suspended in 480µl of activation buffer, reaction tubes were again vortexed and sonicated. 10µl of Sulfo-NHS was then added to each tube followed by 10µl EDC. Reaction tubes were vortexed for a minimum of 10 seconds, protected from light and rotated on a rotator for 20 minutes (rotation speed 15-30 rpm). Microspheres were washed a further three times with activation buffer. The appropriate volumes of activation buffer and protein were then added to each tube to achieve the desired protein antigen concentration. Reaction tubes were vortexed for 20 seconds, protected from light and rotated at 15-30 rpm for 2 hours. Microspheres were washed three times with wash buffer, re-suspended in 300µl wash buffer, protected from light, and stored at 4°C until further use. Microspheres were stored for at least 12 hours post-coupling before coupling verification was carried out as per manufacturer's recommendation.

2.3.2.2 Coupling verification

For coupling verification, the pooled sera of 10 EHPC volunteers were used. Serum samples were collected 29 days post-inoculation with Spn6B. All volunteers were colonised with the bacteria following inoculation. Volunteers were selected on the basis that they showed high antibody responses against whole cell pneumococcal ELISA and thus it was assumed that the pooled sera of these volunteers would

elicit responses against pneumococcal antigens included in the assay. Thereby, successful coupling of antigens to microspheres could be confirmed. Successful protein antigen coupling to microspheres was verified using this stock pooled sera throughout the optimisation process. Coupling was verified as described in 2.3.2.3.

2.3.2.3 Assay optimisation

Stock protein-coupled microspheres were diluted in PBS-1%BSA (bovine serum albumin) (assay buffer) to achieve 50 beads/µl. 2,500 (50µl) beads of each protein-coupled microsphere population were then added to each well of a 96-well plate. The number of beads added to each well and conditions used were chosen based upon the manufacturer's protocol and protocols used in similar studies (Martins, Augustine and Hill, 2006; Shoma *et al.*, 2010; Verkaik *et al.*, 2008, 2009; Jiménez-Munguía *et al.*, 2015). 50µl of pooled sera diluted in assay buffer was added to each well, with the exception of blank wells to which assay buffer was instead added to distinguish background median fluorescence intensity (MFI) values. The plate was covered to protect microspheres from light and incubated overnight at 4°C on a plate shaker (300rpm). Following incubation, the plate was clipped into place on the Luminex Magnetic Plate Separator and rapidly and forcefully inverted over a biohazard receptacle to expel the liquid from the wells. Microspheres were washed three times with 200µl assay buffer. Biotinylated human IgG Fc antibody (Invitrogen™ Novex™, catalogue number: A18821, lot: 62, 129, 090718) was diluted to 2µg/ml in assay buffer and 50µl was then added to each well. The plate was then incubated in the dark for 30 minutes at room temperature on a plate shaker. Following incubation, liquid was expelled from wells and microspheres were washed three times as previously. Strepdavidin R-phycoerythrin conjugate (SA-PE) (Invitrogen, catalogue number: S866, lot: 1973501) was diluted idem as for the biotinylated secondary antibody and 50µl of SA-PE solution was added to each well. The plate was incubated as previously for 10 minutes. Liquid was expelled from wells and microspheres were then washed three times and re-suspended in 50µl assay buffer. The plate was incubated for five minutes before analysing the contents of each well on the Luminex 100/200™ analyser. All samples were run in duplicate. All data reported are in units of average net MFI (average of replicates) with background MFI subtracted (average net MFI - blank wells).

2.3.3 Luminex assay validation

2.3.3.1 Measuring humoral response in healthy adults inoculated with serotype 6B pneumococcus

Serum samples from healthy non-smoking volunteers between the ages of 18 and 50 who were intranasally inoculated with 80,000 CFU in 0.1ml solution of *S. pneumoniae* serotype 6B strain BHN418

per naris (full sequence available GenBank: ASHP00000000) were analysed. Serum samples were taken prior to inoculation (baseline) and 29 days post-inoculation. Volunteers were inoculated as part of a previous study (EudraCT 2014-004634-26) (split into 2 parts), which examined the effect of Live Attenuated Influenza Vaccine (LAIV) on colonisation with serotype 6B pneumococcus (Jochems *et al.*, 2018; Rylance *et al.*, 2019). The samples used in this work were from the control cohorts of these studies. Participants in the study control groups were given nasal placebo of normal saline paired with intramuscular influenza vaccination (Fluarix Tetra; TIV; GlaxoSmithKline). In comparison, test groups received nasal LAIV paired with intramuscular placebo of normal saline (AstraZeneca). Participants either received inoculation with Spn6B 3 days after receiving an influenza vaccine (study part 1) or 3 days prior to receiving an influenza vaccine (study part 2). The order in which participants received TIV and inoculation with *S. pneumoniae* showed no effect on carriage rate or colonisation density. All participants gave written informed consent, with approval from the North West NHS Research Ethics Committee (14/NW/1460). Colonisation was determined by classical microbiology and the presence of Spn6B in nasal wash samples taken at any timepoint between inoculation and 29 days post-inoculation. Complementary molecular methods (*LytA* qPCR) to detect the presence of bacteria were also used to confirm carriage results. No volunteers had previously received any pneumococcal vaccine.

Serum samples from participants in the control arms receiving intramuscular Fluarix Tetra (17 carriage positive and 20 carriage negative) were analysed and antibody levels against nine proteins from the library (panel 4) were quantified. This panel was chosen for validation of the assay as it included the proteins PspA and PspC, which are well established protein vaccine candidates and are known to induce a significant change in anti-protein IgG following pneumococcal challenge (McCool *et al.*, 2002, 2003; Ferreira *et al.*, 2013; Adler *et al.*, 2021).

For measurement of serum IgG response in adults inoculated with Spn6B, a 'master-mix' of each protein panel was made containing 250 beads/µl of each protein-coupled bead population (stored in wash buffer). These stocks were protected from light and stored at 4°C. From this stock solution, the required volume of stock microspheres was removed and diluted 1:5 in 1%BSA-PBS (assay buffer) to achieve 50 beads/µl of each protein-coupled bead population. 25µl of bead solution was added to each well on a 96-well plate. To this, 25µl of serum diluted in assay buffer was added. The same stock of day 29 pooled sera as previously described (diluted 1:50 in assay buffer) was used as an internal positive control across all plates and assay buffer was used as a negative control to detect background median fluorescence intensity (MFI) values. The assay was then carried out in the same way as described in 2.3.2.3. Notably,

the internal positive control both confirmed immunodetection and indicated inter-assay variation of the panel of proteins against which serum IgG was being analysed. In the case of high variation between plates of which results would be combined in one dataset, experiments would be repeated to ensure comparability of results, however this was not necessary as inter-assay variance for all proteins remained low (<20% CV). Baseline and post-challenge samples for the same volunteer were always analysed on the same plate.

2.3.3.1.1 Data cleaning and statistical analysis

If any paired samples had less than 50 events of a given analyte in each well, the analyte was removed from analysis. The bead count of 50 microspheres is based on recommendations by the Luminex Corporation that a recovery of less than 50 events renders a result unreliable as well as previous work showing that lower bead counts elicit higher CV values (Bjornstal *et al.*, 2011). The coefficient of variation (CV) of replicates was calculated for all analytes. Coefficient of variation is calculated as $(Standard\ deviation/Mean) * 100$. Whilst standard deviations generally increase or decrease proportionally as the mean of values increases or decreases, CV is a standardisation of the standard deviation, allowing comparison of variability estimates irrespective of the magnitude of analyte concentration (Reed, Lynn and Meade, 2002). If any analyte/region had greater than 25% CV of replicates and both replicates had greater than 50 events per well, the analyte was removed from analysis. If only one well had greater than 50 microspheres for a given analyte and the value for that well appeared to be comparable to the trend of results, the value of the analyte taken from the well with greater than 50 microspheres was taken as opposed to the average of replicates. All statistical analysis was carried out in RStudio (version 1.0.153). Statistical significance was determined via a Mann-Whitney or Wilcoxon test for unpaired and paired groups, respectively, followed by Benjamini Hochberg correction for multiple comparisons.

2.3.3.2 Validation of Luminex assay results by ELISA

Serum samples from five carriage positive EHPC volunteers were tested using an ELISA. As previously, the pooled sera were used as a positive control. 100µl of carbonate/bicarbonate buffer (pH 9.6) containing 2µg/ml of protein was used to coat a 96-well plate. The plate was incubated overnight at 4°C. The plate was then incubated at 37°C for 30 minutes. After washing three times with PBS-Tween, 200µl of 5% skimmed milk diluted in PBS was added to each well and the plate was again incubated at 37°C for 30 minutes. The plate was then washed 3 times with PBS-Tween. Serial dilutions of serum samples were performed in PBS-1%BSA. Serum samples were diluted from 1:50 to 1:40,000 concentration.

Samples were then incubated overnight at room temperature. The plate was again washed 3 times. 100µl/well of PBS-1%BSA containing 1:5000 anti-human IgG conjugated with horse-radish peroxidase was added and the plate was incubated for 1 hour at 37°C. A final three washes were performed. TMB substrate (3,3',5,5'-Tetramethylbenzidine) was added at a volume of 100µl/well left to develop for 10 minutes at room temperature. The reaction was stopped using 50µl/well of sulphuric acid (2 Normal) and the plate was analysed using a FLUOstar® Omega microplate reader at a wavelength of 450nm.

2.4 Results

2.4.1 Assay optimisation

2.4.1.1 Protein-coupled microspheres are recognised by immune sera

The coupling reaction (2.3.2.1) was first carried out using *Staphylococcus aureus* enterotoxin B (SEB) protein. *S. aureus* is a ubiquitous gram-positive bacterium, which is estimated to persist in 20% of the general population and colonises a further 60% intermittently (Kluytmans, Van Belkum and Verbrugh, 1997). Staphylococcal enterotoxins are classified as superantigens and are frequently used as positive controls. It was therefore assumed that there would be a high level of circulating SEB-specific antibodies present in the pooled sera of 10 EHPC volunteers (see pooled sera described in 2.3.2.2). Thus, it would be reasonable to assume that if no antibody binding was identified when the pooled sera of these volunteers were added to microspheres, coupling was un-successful. Successful coupling of SEB to microspheres was thereby confirmed as described in 2.3.2.2 (Figure 11). As is shown in Figure 11, a serum dilution of 1:50 generated the highest level of detection of circulating anti-SEB IgG. At this serum concentration, little difference in detection of IgG was observed between the three protein coupling concentrations of SEB tested (2µg, 5µg and 10µg per million microspheres). In addition, a titration of secondary biotinylated antibody and streptavidin-phycoerythrin (SA-PE) showed that there was minimal difference in average net MFI when either 2µg/ml or 4µg/ml secondary biotinylated antibody/SA-PE was used at a protein concentrations of 2µg, 5µg or 10µg per million microspheres. Therefore a concentration of 2µg/ml of secondary biotinylated antibody/SA-PE was deemed optimal – optimal here being defined as minimal amount of reagent required to obtain consistent results (Figure 11).

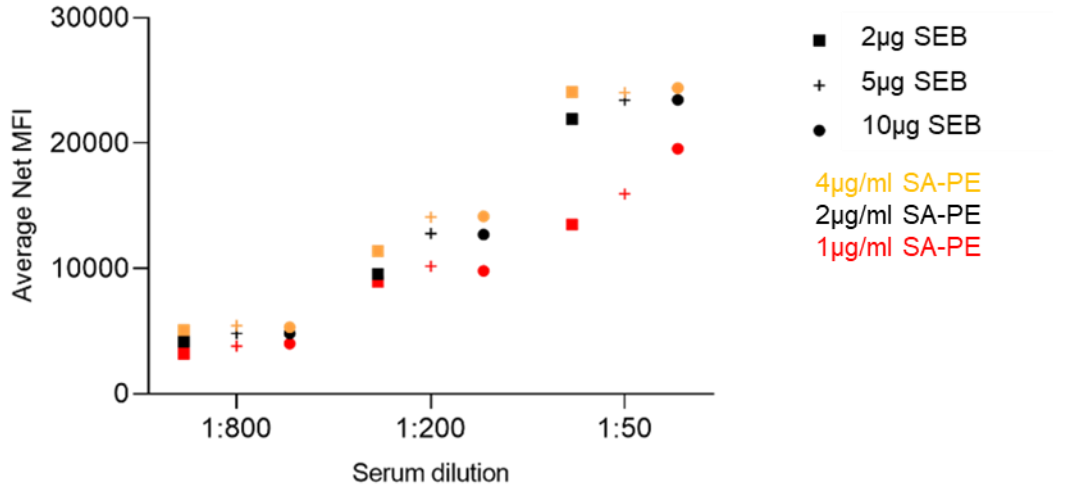


Figure 11: Optimisation of serum concentration, coupling concentration of *Staphylococcus aureus* enterotoxin B (SEB) and concentration of secondary biotinylated antibody and SA-PE to detect circulating protein-specific IgG, measured as average net MFI, using a Luminex assay. Pooled sera were diluted from 1:50 to 1:800 concentration in PBS-1%BSA (bovine serum albumin) and incubated with SEB-coupled MagPlex microspheres for 2-hours at room temperature. Three concentrations of secondary biotinylated antibody and streptavidin-phycoerythrin (SA-PE) were tested: 1µg/ml (red), 2µg/ml (black) and 4µg/ml (orange). Three concentrations of SEB were coupled to microspheres: 2µg, 5µg and 10µg per million microspheres. 1:50 serum dilution and 2µg/ml secondary antibody/SA-PE was optimal to detect circulating anti-PdB IgG. MFI = median fluorescence intensity.

Hence, coupling of pneumococcal proteins to microspheres was carried out. Pneumolysin Toxoid B (PdB), a pneumolysin mutant with a tryptophan-to-phenylalanine substitution at position 433 and 0.1% haemolytic activity of wild-type pneumolysin (Paton *et al.*, 1991), was first conjugated to microspheres. The PdB was produced as described previously (Ferreira *et al.*, 2006). As shown in Figure 12, a titration was performed to determine optimal protein concentration per million microspheres. There was little difference in the average net MFI between protein concentrations. Similarly, pooled sera were titrated using a series of two-fold dilutions ranging from 1:50 to 1:1600 to find the optimal serum dilution. A serum dilution of 1:50 was deemed optimal, with the caveat that this would potentially be amended once individual serum samples, rather than pooled sera, were to be analysed. Over-night incubation at 4°C was found to be superior to 2-hour incubation at room temperature for detection of antigen-specific IgG binding.

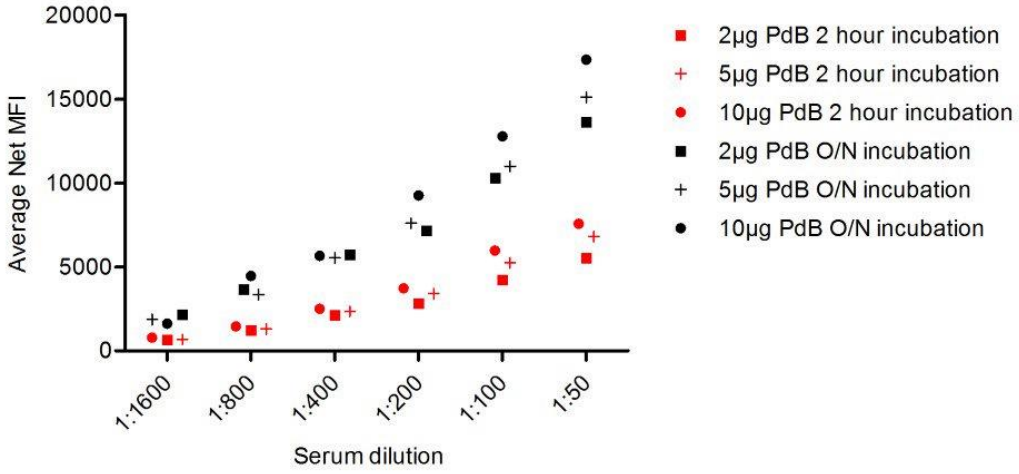


Figure 12: Optimisation of incubation period, serum concentration and coupling concentration of pneumolysin mutant (PdB) to MagPlex microspheres to detect circulating protein-specific IgG, measured as average net MFI, using a Luminex assay. Pooled sera were diluted from 1:50 to 1:1600 concentration in PBS-1%BSA (bovine serum albumin) and incubated with PdB-coupled MagPlex microspheres for either 2-hours at room temperature or over-night (O/N) at 4°C. 3 concentrations of PdB were coupled to microspheres: 2µg, 5µg and 10µg per million microspheres. 1:50 serum dilution and O/N incubation was optimal to detect circulating anti-PdB IgG and a coupling concentration of either 5µg or 10µg gave highest fluorescence values. MFI = median fluorescence intensity.

2.4.1.2 Protein coupling to different microsphere regions elicits comparable results

It was then necessary to confirm that similar MFI readings were obtained for the same protein (PdB) coupled to a different microsphere region (Figure 13). Comparable results were obtained for both regions (<25% CV between values obtained for each region at all serum dilutions).

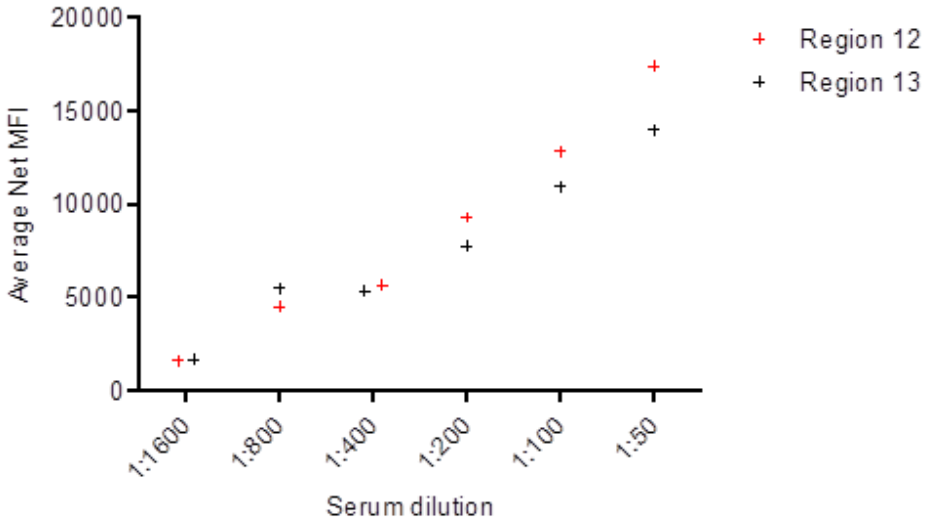


Figure 13: Verification that MagPlex microsphere regions 12 and 13 give comparable MFI readings to detect circulating protein-specific IgG using a Luminex assay at a range of serum dilutions. Pooled sera were diluted from 1:50 to 1:1600 concentration in PBS-1%BSA (bovine serum albumin) and incubated with PdB-coupled MagPlex microspheres (regions 12 and 13) over-night at 4°C. Comparable net MFI readings were obtained for both microsphere regions at all serum dilutions. MFI = median fluorescence intensity.

2.4.1.3 Determining optimal protein coupling concentration and serum dilution for detection of anti-protein IgG binding

Subsequently, pneumococcal surface protein A (PspA) (clade 1) was coupled to microspheres at concentrations of 1µg, 2µg, 5µg and 10µg per million microspheres and previous experiments were repeated to evaluate whether results were reproducible with a second protein known to be highly immunogenic. Results verified that overnight incubation at 4°C was superior to a 2-hour incubation at room temperature for obtaining the highest signal (Figure 14). Similarly, 1µg, 2µg and 5µg concentration of protein showed little variation (Figure 14). Notably, the saturation point for PspA was reached at a serum concentration of around 1:400 (Figure 14). However, at 10µg coupling concentration of PspA, peak MFI value was achieved at 1:400 serum dilution and declined with increasing serum concentration beyond this (Figure 15). As is discussed later, it is conceivable that this is a result of the prozone effect. Alternatively, microspheres coupled with 10µg PspA per million microspheres may have aggregated on the bottom of wells, impairing antibody binding, although this was mitigated by constant agitation of the plate on a plate-shaker.

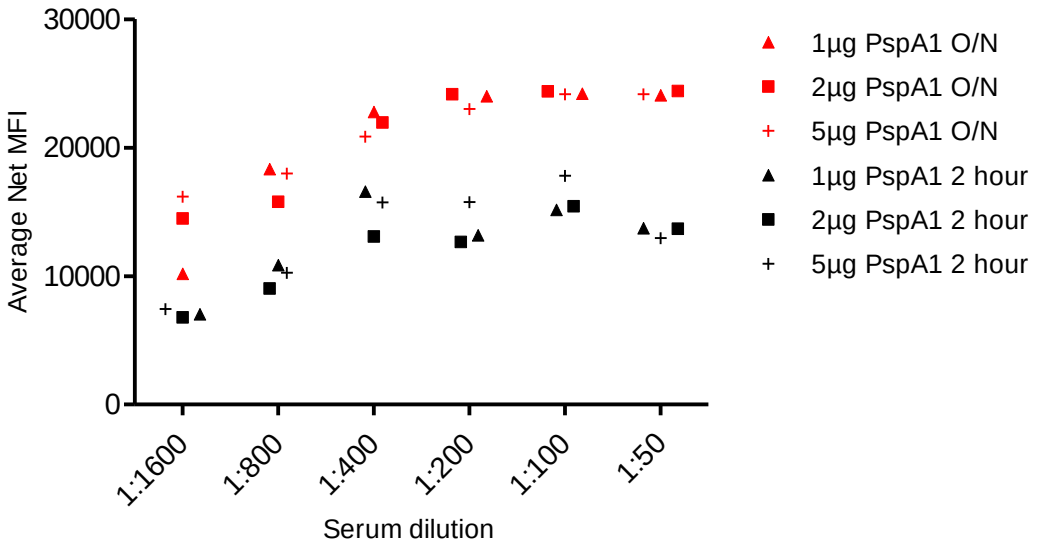


Figure 14: Optimisation of incubation period, serum concentration and coupling concentration of PspA (clade 1) to detect circulating protein-specific IgG, measured as average net MFI, using a Luminex assay. Pooled sera were diluted from 1:50 to 1:1600 concentration in PBS-1%BSA (bovine serum albumin) and incubated with PspA1-coupled MagPlex microspheres for either 2-hours at room temperature or over-night (O/N) at 4°C. 3 concentrations of PspA1 were coupled to microspheres: 1µg, 2µg and 5µg per million microspheres. Peak signal was reached at 1:400 serum dilution; no increase in MFI was obtained with more concentrated serum. O/N incubation was optimal to detect circulating IgG. Minimal difference in MFI values was observed with differing PspA1 concentrations. MFI = median fluorescence intensity.

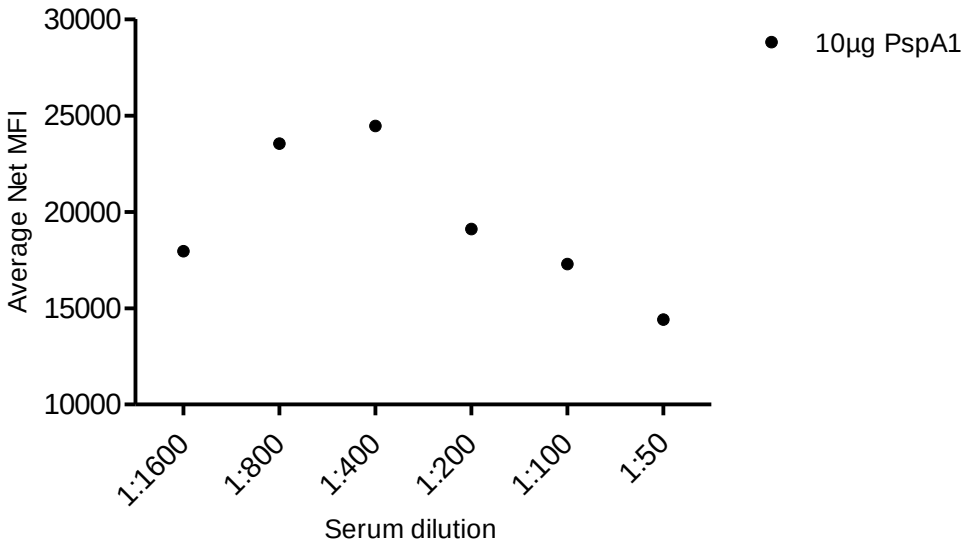


Figure 15: Overnight incubation of 10µg of PspA (clade 1) per million MagPlex microspheres with varying serum concentrations showed a decline in average net MFI value with increasing serum concentration above 1:800. Pooled sera were diluted from 1:50 to 1:1600 concentration in PBS-1%BSA (bovine serum albumin) and incubated with PspA1-coupled MagPlex microspheres over-night at 4°C. At 10µg per million microspheres coupling concentration of PspA, peak signal was achieved at 1:800 serum dilution and declined with increasing serum concentration beyond 1:400. MFI = median fluorescence intensity.

A further four pneumococcal protein antigens were coupled to microspheres at concentrations 2µg, 5µg and 10µg per million microspheres (Figure 16). Proteins SP0149 and SP1154-2 were coupled to microsphere region 12 and SP0321 and SP1534 were coupled to microsphere region 13.

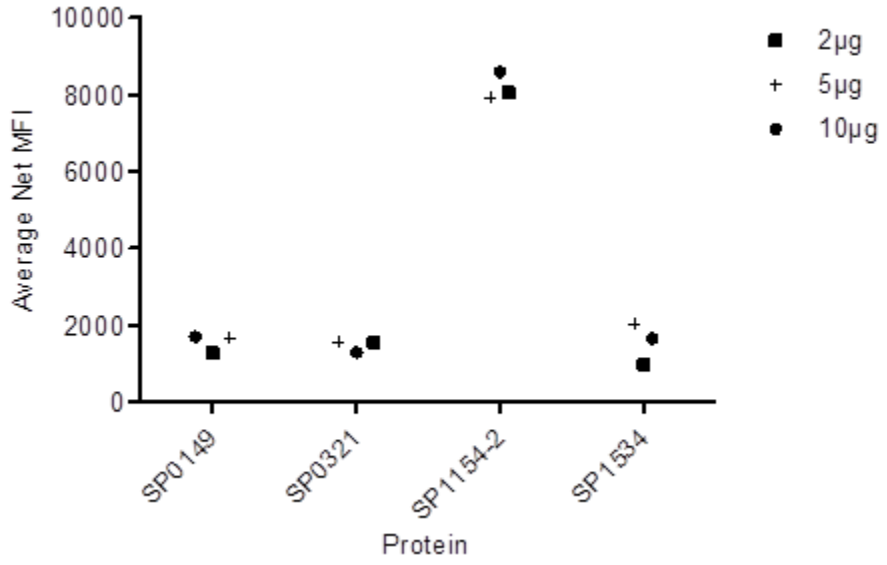


Figure 16: Verification that 5µg per million microspheres is an appropriate protein concentration for coupling to MagPlex microspheres to detect circulating anti-protein IgG, measured as average net MFI, for proteins SP0149, SP0321, SP1154-2, SP1534 at 1:50 serum dilution using a Luminex assay. Pooled sera were diluted 1:50 in PBS-1%BSA (bovine serum albumin) and incubated with protein-coupled MagPlex microspheres over-night at 4°C. Proteins were coupled at 2µg, 5µg and 10µg per million microspheres. Minimal difference in MFI values obtained for each protein concentration was observed. MFI = median fluorescence intensity.

Again, minimal difference in average net MFI values was observed for the different protein concentrations. It was decided that subsequent proteins would be coupled at 5µg per million microspheres to streamline the coupling process as this concentration seemed suitable given results with all proteins tested.

2.4.1.4 Multiplexing has no effect on antibody binding as compared to singleplex assay

The next stage in the optimisation process was to determine that no difference in MFI values was seen for antigens when the assay was run in singleplex and multiplex. Twenty-two antigens were chosen at random (Table 5) to determine the correlation between assays. Overall, average net MFI values for the singleplex and 22-plex assays showed a strong correlation (Pearson r = 0.99, p <0.0001) (Figure 17).

Table 5: Luminex panel of 22 randomly selected proteins for assay optimisation.

Protein	Region
PspA1	12
SP0321	13
SP0198	14
SP0601	15
SP0092	18
SP0191	19
SP0564	20
SP0346	21
SP1545	22
SP2792	25
SP2197	26
SP2218	27
SP1942	28
SP1032	29
SP0617	30
SP0620	33
SP0678	34
SP1560	35
SP2099	36
SP0930	37
SP1479	38
SP1492	39

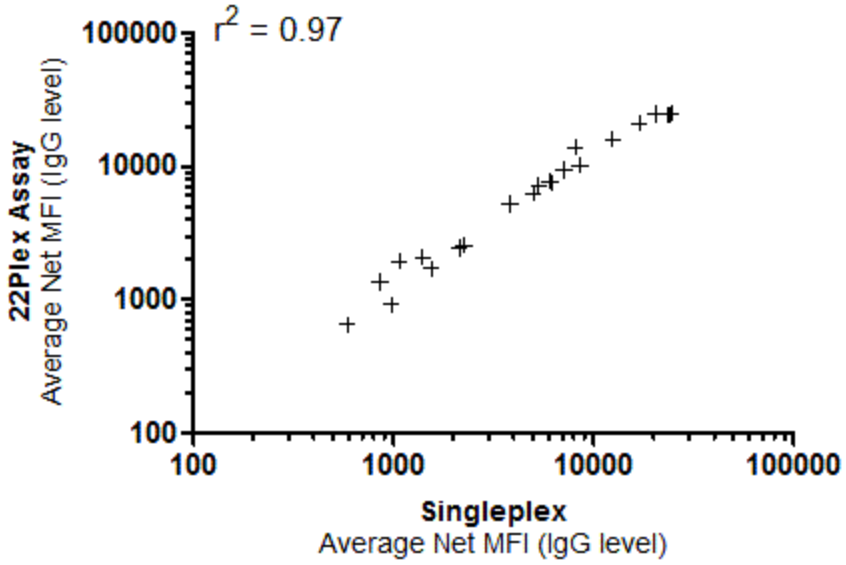


Figure 17: Correlation between median fluorescence intensity (MFI) readings for singleplex and multiplex assay. Twenty-two protein-coupled MagPlex microspheres of different regions were incubated with pooled sera as singleplex or 22-plex. Good correlation was seen between singleplex and multiplex assays. Pearson r = 0.99 (95% confidence interval = 0.97 to 0.99), $p < 0.0001$.

2.4.1.5 Summary of assay optimised conditions

The following conclusions were therefore drawn from the optimisation steps:

1. Use 2µg/ml of biotinylated antibody and SA-PE for detection of antibody (IgG) binding to antigens
2. 1:50 serum dilution is optimal for antigen-specific IgG binding detection
3. Overnight incubation at 4°C elicits optimal antigen-specific IgG binding detection
4. Different microsphere regions are comparable
5. 5µg protein per million microspheres is a suitable protein coupling concentration
6. Single and multiplex (22-plex) assays correlate well

Thus, each of the proteins in the library was coupled to a microsphere region. Regions were randomly assigned to proteins. Given the availability of 22 possible regions, the proteins were divided into four panels (Table 6). Note that the pneumolysin mutant (PdB) used in the optimisation process was included and added to the full protein library to bring the total number of proteins included to 75.

Table 6: Protein panels developed for Luminex multiplex assay.

Panel 1		Panel 2		Panel 3		Panel 4	
Protein	**Region**	**Protein**	**Region**	**Protein**	**Region**	**Protein**	**Region**
SP0149	12	**PdB**	12	**SP1154-2**	12		
SP0321	13	**SP1534**	13	**SP0878**	13		13
SP0198	14	**SP1069**	14	**SP0249**	14	**SP0314**	14
SP0601	15	**SP0084**	15	**SP0582**	15	**SP1826**	15
SP0092	18	**SP0659**	18	**SP0648-3**	18		18
SP0191	19	**SP1652**	19	**SP1376**	19	**SP1872**	19
SP0564	20	**SP2108**	20	**SP0724**	20		20
SP0346	21	**SP0079**	21	**SP2151**	21	**SP1404**	21
SP1545	22	**SP0402**	22	**SP0336**	22		
SP2192	25	**SP0629**	25	**SP1500**	25	**SP0648-2**	25
SP2197	26	**SP0757**	26	**SP2083**	26	**PspC6α (BHN418)**	26
SP2218	27	**SP0785**	27	**SP1833**	27	**PspC9α (BHN418)**	27
SP1942	28	**SP0787**	28	**SP0043**	28	**PspA1α (BHN418)**	28
SP1032	29	**SP0899**	29	**SP0098**	29	**PspA4α (255/00)**	29
SP0617	30	**SP1386**	30	**SP0127**	30		30
SP0620	33	**SP2145**	33	**SP0435**	33		33
SP0678	34	**SP1897**	34	**SP0604**	34		34
SP1560	35	**SP0662-1**	35	**SP0648-1**	35		35
SP2099	36	**SP0662-2**	36	**SP0148**	36		36
SP0930	37	**SP1683**	37	**SP2207**	37		37
SP1479	38	**SP0369**	38	**SP1002**	38		38
SP1492	39	**SP2070**	39	**SP0742**	39		39

2.4.2 Assay Validation

2.4.2.1 Validating optimal serum concentration for detection of anti-protein IgG binding for all proteins

Serum samples obtained 5 days prior to inoculation with Spn6B (baseline) were pooled to create a stock of baseline sera. Baseline samples were taken from the same volunteers as those used to create the day 29 pooled sera. Baseline pooled sera and day 29 pooled sera were used to validate the multiplex assay. To determine if 1:50 was the optimal serum dilution to use going forward, each stock of pooled sera was titrated from 1:10 to 1:1600 dilution. Each of the four panels were tested in full 22-plex with baseline and day 29 sera. Following testing of titrations, the serum dilution chosen with which to proceed

was 1:50. A 1:50 serum dilution enabled detection of IgG antibodies to all proteins in the panel, both in baseline and day 29 samples, and was also below saturation point for most of the proteins (Appendix B). It is notable that proteins PspA and PspC, which are highly immunogenic, saturate at a much lower serum dilution as compared to the other proteins in the panel.

2.4.2.2 Determining assay reproducibility as determined by intra- and inter-assay variability

Intra- and inter-assay variability (CV) were calculated from the average net MFI values obtained from pooled day 29 sera samples for each protein run in three separate assays (all samples were run in duplicate). Initially, inter-assay CV was >50% for most of the proteins (data not shown). It was assumed that pooling small amounts of beads for each experiment was leading to large error, causing this high level of variation. Thus, a large stock of a 'master-mix' of each panel was made containing 50 beads/µl of each protein-coupled bead population and the same solution was used across three plates. Inter-assay variability experiments were subsequently repeated, and variation was reduced. Across the three plates, the median intra-assay CV (CV of replicates) for the entire protein library averaged 1.56%. The average intra-assay CV across three assays for each of the proteins is shown in Figure 18. The median inter-assay CV of average net MFI values for each protein across three assays was higher at 22.45%. 23 of the 75 proteins (30.67%) had an inter-assay CV >25%, 4 of which showed ≥30% variation (Figure 19). The inter-assay variation observed is comparable to similar studies conducted previously with *Staphylococcus aureus* (Verkaik *et al.*, 2008) and pneumococcal antigens (Lal *et al.*, 2005; Jiménez-Munguía *et al.*, 2015). Any proteins with an inter-assay CV >25% may be considered less reproducible.

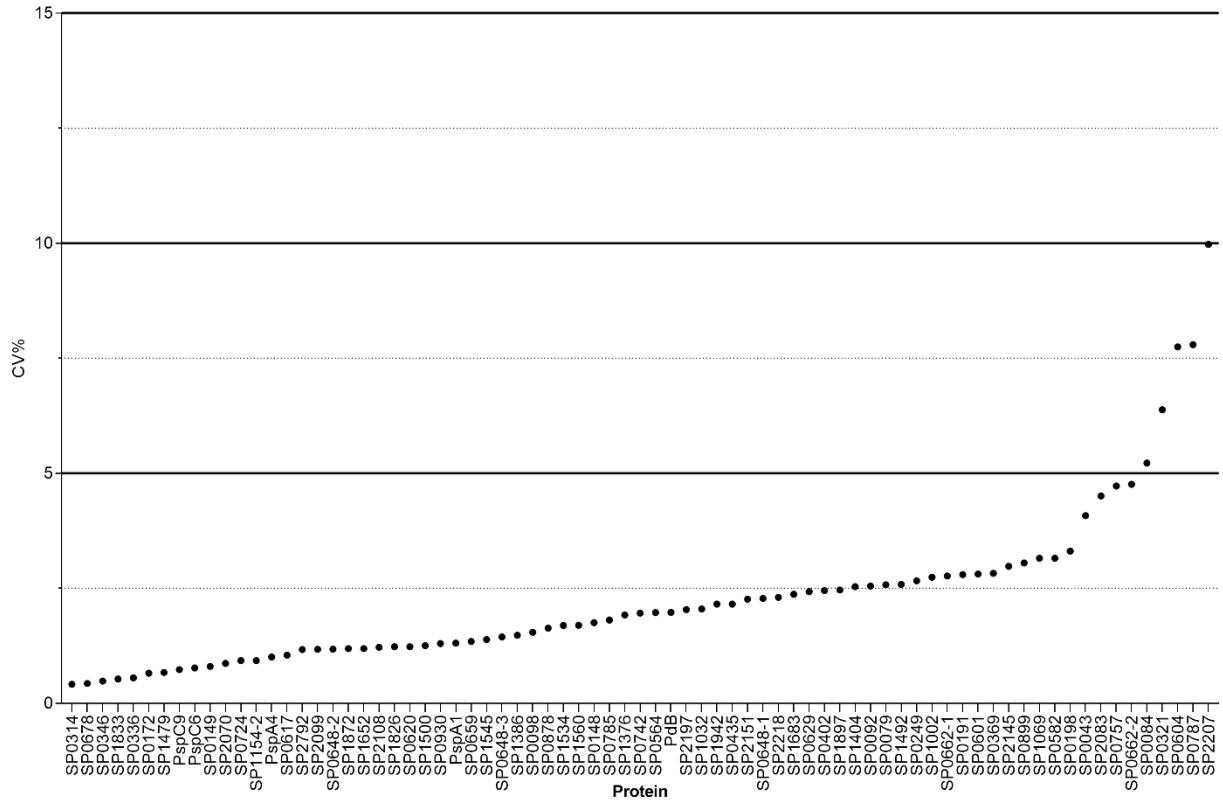


Figure 18: Percentage intra-assay coefficient of variation (CV) of average net median fluorescence intensity (MFI) values, indicating levels of protein-specific IgG in pooled sera, for a library of 75 pneumococcal proteins across three multiplex Luminex assays. 75 pneumococcal proteins were coupled to MagPlex microspheres and incubated with pooled sera. Across the three assays, the median intra-assay CV averaged 1.56%.

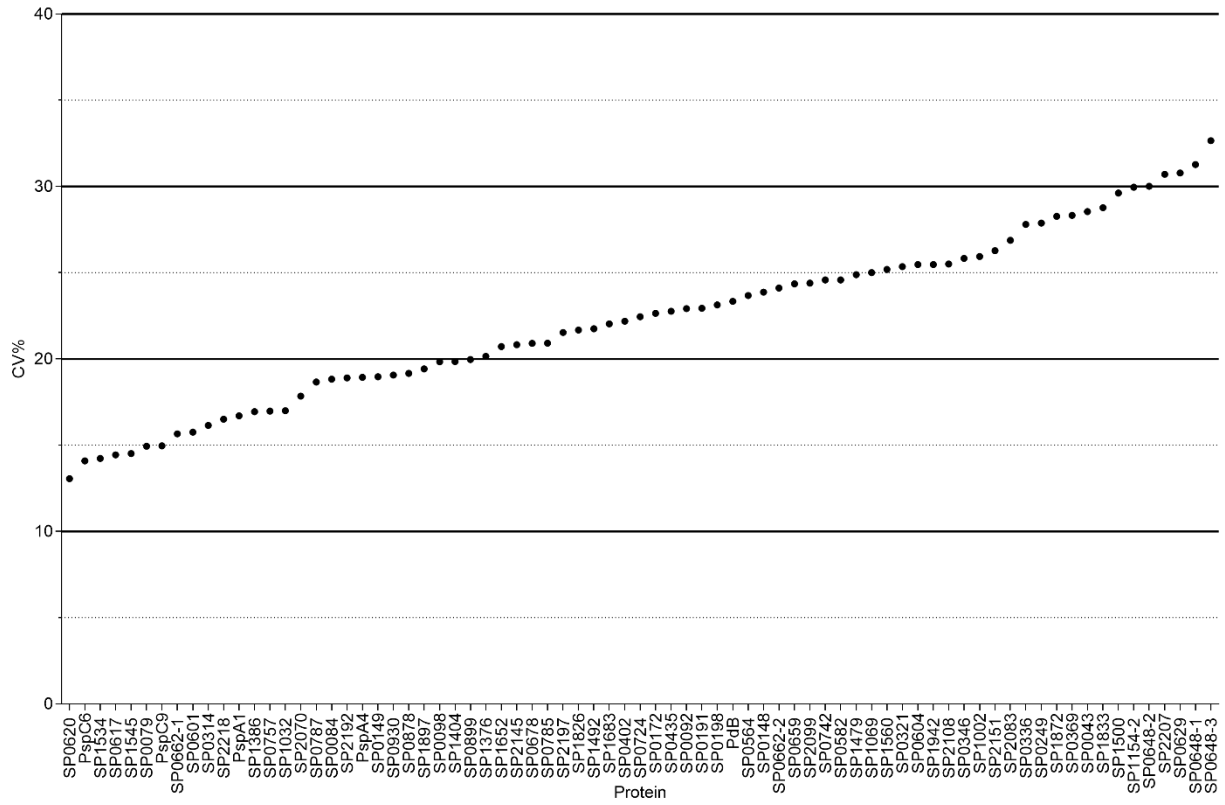


Figure 19: Percentage inter-assay coefficient of variation (CV) of average net median fluorescence intensity (MFI) values, indicating levels of protein-specific IgG in pooled sera, for a library of 75 pneumococcal proteins across three multiplex Luminex assays. 75 pneumococcal proteins were coupled to MagPlex microspheres and incubated with pooled sera. Across the three assays, the median inter-assay CV was 22.45%. 23 of the 75 proteins (30.67%) had an inter-assay CV ≥25%, 4 of which showed ≥30% variation.

2.4.2.3 No heterophilic antibody binding is observed in the assay

Human sera contain heterophile antibodies that may bind non-specifically to Luminex microspheres, in the absence of coupled antigen, causing interference with the assay (Martins *et al.*, 2004). Carboxylated beads have been shown to elicit less non-specific binding than other bead types, such as Penta-His beads (Waterboer, Sehr and Pawlita, 2006; Verkaik *et al.*, 2008). A previous study defined non-specific background by bead-binding antibodies as MFI >250 (Waterboer, Sehr and Pawlita, 2006). We ran un-coupled region 12 microspheres with pooled sera at a range of dilutions and showed that average net MFI readings for unconjugated microspheres were below 250 at all serum concentrations, suggesting very little heterophilic antibody binding in this assay with our participant sera (Table 7). Notably, Waterboaer and colleagues have stated that non-specific background varies depending on serum origin and sampling conditions (Waterboer, Sehr and Pawlita, 2006). The values of background MFI in negative control wells containing assay buffer and protein-coupled microspheres but absent of any human sera were also consistently low.

Table 7: Average net MFI obtained when pooled immune sera was incubated with un-coupled microspheres.

Serum concentration	Average net MFI
1:100	50.15
1:200	42.15
1:400	33.15
1:800	12.65
1:1600	4.15

2.4.2.4 Protein panels are interchangeable

To confirm that panels are interchangeable, and results are consistent irrespective of which proteins are run in multiplex with each other, a panel of 22 proteins was designed based on protein antigens previously shown to correlate with protection against pneumococcal colonisation in mice (Lu *et al.*, 2018) and humans (unpublished data), (Table 8). The panel was run alongside the original panels using a 1:50 dilution of day 29 pooled sera and this was repeated across three separate assays. As previously, all samples were run in duplicate. Across the three assays, the median CV between the average net MFI values obtained for each protein when run in the new panel as compared to the original panel was consistently low, ranging from 2.82% to 7.39% (Table 9). Thus, results validated that protein-coupled microspheres can be interchanged across panels, provided fluorescing wavelengths of microspheres to which proteins are coupled do not overlap. Median intra-assay variation for the new panel across three assays averaged 2.39% and median inter-assay variation of average net MFI values for each protein in the new panel was also low at 9.40% (Table 10).

Table 8: Luminex panel designed to incorporate proteins previously shown to correlate with protection against pneumococcal colonisation.

Protein	Region
SP1154-2	12
SP1534	13
SP1069	14
SP2099	15
SP0648-3	18
SP1652	19
SP2108	20
SP0079	21
SP1032	22
SP1500	25
SP0757	26
SP0785	27
SP0787	28
SP0899	29
SP1386	30
SP0435	33
SP0742	34
SP0648-1	35
SP0662-2	36
SP1683	37
SP1479	38
SP2070	39

Table 9: Percentage coefficient of variation (CV) of average net median fluorescence intensity (MFI) readings, indicating levels of circulating protein-specific IgG in pooled sera, for 22 pneumococcal proteins coupled to Luminex MagPlex microspheres when run in different panels and across three separate assays.

	Assay 1			Assay 2			Assay 3		
Protein	**Original panel MFI**	**New panel MFI**	**%CV**	**Original panel MFI**	**New panel MFI**	**%CV**	**Original panel MFI**	**New panel MFI**	**%CV**
1154-2	18215.50	17695.25	**2.05**	13475.40	12337.15	**6.24**	19165.08	18449.33	**2.69**
SP1534	1382.82	1305.32	**4.08**	675.05	633.55	**4.48**	739.58	761.33	**2.05**
SP1069	6089.56	5680.56	**4.91**	3465.35	3113.85	**7.56**	4813.95	4785.95	**0.41**
SP2099	9741.88	8122.50	**12.82**	6250.25	5022.80	**15.40**	9267.60	7400.55	**15.84**
SP0648-3	17052.44	15937.69	**4.78**	8989.25	8484.25	**4.09**	13682.35	13432.85	**1.30**
SP1652	4093.00	3757.75	**6.04**	1805.45	1580.95	**9.38**	2364.90	2284.40	**2.45**
SP2108	9748.44	9222.44	**3.92**	6429.95	5804.45	**7.23**	8880.00	8262.25	**5.10**
SP0079	2372.50	2250.50	**3.73**	809.90	698.15	**10.48**	992.10	929.10	**4.64**
SP1032	24641.13	24698.50	**0.16**	18108.30	18882.20	**2.96**	25073.30	25051.35	**0.06**
SP1500	5450.25	4855.75	**8.16**	3519.05	3112.05	**8.68**	5244.15	4699.65	**7.74**
SP0757	864.69	962.44	**7.57**	467.25	476.50	**1.39**	558.36	622.86	**7.72**
SP0785	24627.00	24457.75	**0.49**	18876.25	17623.25	**4.85**	24662.70	24632.20	**0.09**
SP0787	5115.06	5168.06	**0.73**	2754.25	2767.50	**0.34**	3594.70	3039.95	**11.82**
SP0899	4174.63	4034.63	**2.41**	1856.30	1652.80	**8.20**	2584.30	2501.55	**2.30**
SP1386	3152.13	3233.88	**1.81**	1049.22	939.22	**7.82**	1271.75	1311.75	**2.19**
SP0435	4228.50	4530.00	**4.87**	1252.20	1102.20	**9.01**	1665.95	1508.95	**6.99**
SP0742	5478.63	6829.00	**15.52**	2867.78	3195.56	**7.65**	3822.17	4185.85	**6.42**
SP0648-1	24132.00	24090.25	**0.12**	14837.11	14360.61	**2.31**	22688.15	22362.90	**1.02**
SP0662-2	3123.13	3071.88	**1.17**	1357.50	1210.25	**8.11**	1862.10	1489.35	**15.73**
SP1683	9025.63	8830.63	**1.54**	5547.86	5220.36	**4.30**	7639.31	7508.56	**1.22**
SP1479	11725.63	11632.88	**0.56**	6541.10	6229.35	**3.45**	9729.30	9331.30	**2.95**
SP2070	2191.63	2148.63	**1.40**	964.28	857.28	**8.31**	1174.67	1060.42	**7.23**
Median			**3.07**			**7.39**			**2.82**
Average			**4.04**			**6.46**			**4.91**

Table 10: Average intra-and inter-assay percentage coefficient of variation (CV) for a panel of 22 proteins across three separate assays using Luminex technology. Data shows intra-assay variation averaged across three assays and inter-assay variation of average net median fluorescence intensity values obtained for each protein across three assays.

Protein	Average intra-assay CV (%) across three assays	Average inter-assay CV (%) across three assays
SP1154-2	0.81	13.23
SP1534	4.11	2.56
SP1069	2.68	11.33
SP2099	3.05	13.23
SP0648-3	1.15	14.26
SP1652	4.36	9.63
SP2108	1.14	11.89
SP0079	3.80	4.18
SP1032	0.81	3.72
SP1500	2.11	12.05
SP0757	2.78	5.63
SP0785	0.87	5.63
SP0787	8.54	6.53
SP0899	2.17	9.16
SP1386	2.36	3.84
SP0435	3.50	6.69
SP0742	2.90	6.55
SP0648-1	1.19	15.08
SP0662-2	7.56	17.77
SP1683	0.83	10.33
SP1479	1.11	12.62
SP2070	3.81	2.97

2.4.2.5 Impact of reducing microsphere numbers per well

To determine if the assay could function with a lower number of microspheres per well for the purpose of conserving reagents and reducing costs, the average net MFI for 22 proteins in response to the pooled sera was measured and compared using 2,500 and 1,250 of each protein-coupled microsphere population per well. As indicated in Table 11, the CV between the average net MFI values obtained for each protein when multiplexed with 2,500 and 1,250 microspheres of each protein-coupled population per well was consistently <10%, except for proteins SP2099 and SP0662-2, which were slightly higher at 12.74% and 15.88% respectively. This level of variation was deemed acceptable and subsequent experiments used 25µl of 50 beads/µl (1,250 beads total per well).

Consequently, to reduce inter-assay variation and prolong stocks, a single large 'master-mix' of each panel was made containing 250 beads/µl of each protein-coupled bead population (stored in wash buffer). These stocks were protected from light and stored at 4°C. From this master mix, every time an assay was run, the required volume of stock was removed from the master-mix and diluted 1:5 in assay buffer to achieve 50 beads/µl of each protein-coupled bead population and 25µl of this solution was added per well.

Table 11: Percentage coefficient of variation (CV) of average net median fluorescence intensity (MFI) readings, indicating levels of circulating protein-specific IgG in pooled sera, for 22 pneumococcal proteins coupled to Luminex MagPlex microspheres when multiplexed with 2,500 microspheres per well and 1,250 microspheres per well.

Protein	MFI 2,500 beads per well	MFI 1,250 beads per well	%CV
SP1154-2	10357.75	11469	7.20
SP1534	875.5	866.5	0.73
SP1069	3898	4271.75	6.47
SP2099	8480.75	10160.5	12.74
SP0648-3	11800.5	13216.75	8.01
SP1652	1647.5	1617	1.32
SP2108	5243.25	5489.25	3.24
SP0079	757	735.75	2.01
SP1032	14473.75	15407	4.42
SP1500	7032.75	7589.5	5.38
SP0757	710	728	1.77
SP0785	14096.25	15971.5	8.82
SP0787	2845.25	2640.25	5.29
SP0899	2309.25	2363	1.63
SP1386	1570.5	1528.5	1.92
SP0435	1363.75	1360	0.19
SP0742	2257.75	2259.5	0.05
SP0648-1	12171	13453.5	7.08
SP0662-2	1670	1332.75	15.88
SP1683	4558.25	4956	5.91
SP1479	6018.25	6340	3.68
SP2070	1118	1170.5	3.24

2.4.2.6 Humoral response in healthy adults inoculated with serotype 6B pneumococcus

Due to CV of replicates >25% (see 2.3.3.1.1), several analytes (18%) were removed from analysis and the actual sample size for each protein is given in Appendix C. There was no significant difference between levels of anti-protein IgG in carriage positive and carriage negative cohorts at baseline (Figure

20). Nor was there a significant difference between levels at baseline and 29 days post challenge in either cohort (significance was also not shown prior to multiple correction) (Figures 21, 22).

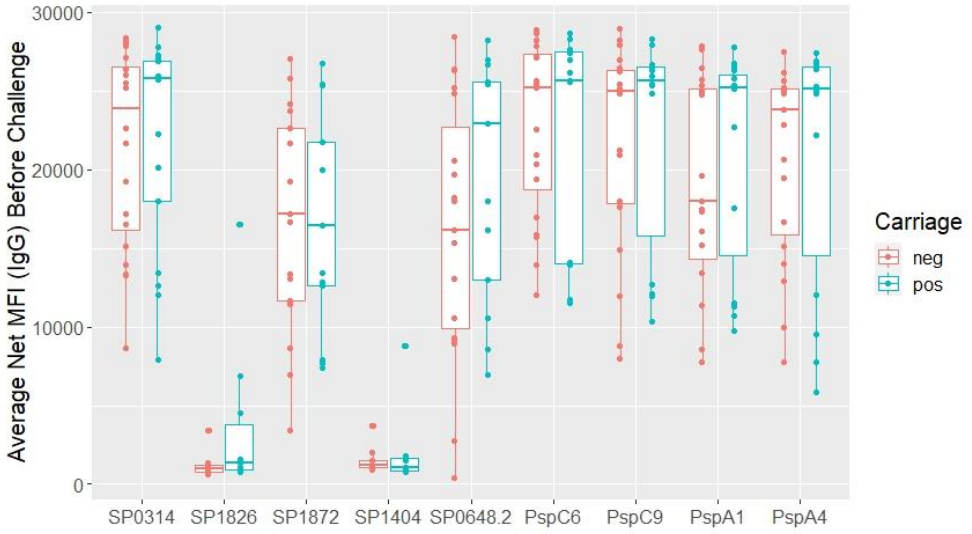


Figure 20: Protein-specific serum IgG response, determined by average net median fluorescence intensity (MFI), at baseline in a cohort of health adults aged 18-50 before inoculation with *S. pneumoniae* serotype 6B (Spn6B). Volunteers were intranasally inoculated with Spn6B in a human challenge model. Serum samples were taken before challenge and 29 days post-challenge. No significant difference in levels of baseline protein-specific IgG was detected between participants protected against experimental colonisation (carriage neg) and those who were susceptible to colonisation (carriage pos). MFI = median fluorescence intensity. Bars indicate interquartile range. Carriage negative n = 20, carriage positive n = 17.

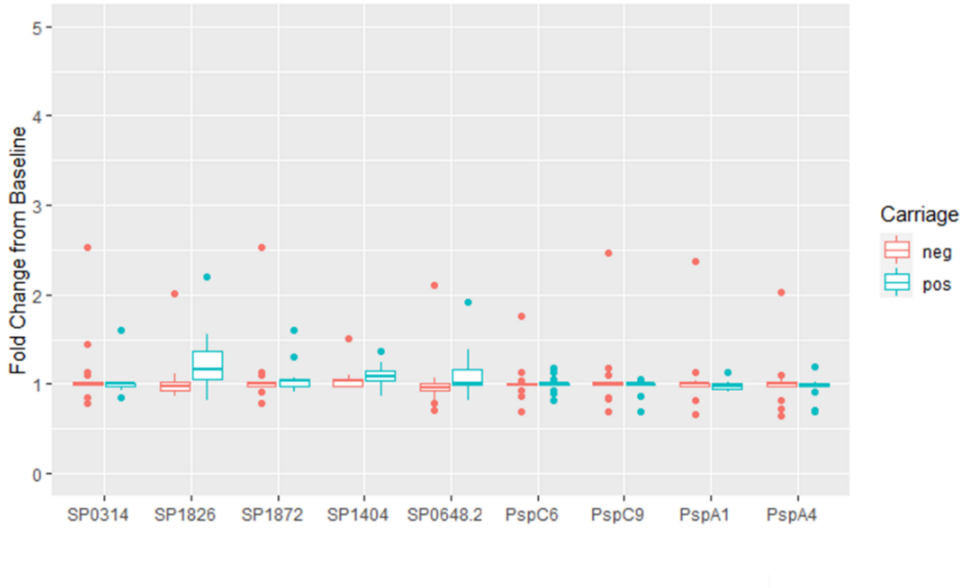


Figure 21: Fold change in protein-specific serum IgG response, measured as average net median fluorescence intensity (MFI), between baseline and 29 days after experimental challenge with *S. pneumoniae* serotype 6B (Spn6B) in a cohort of healthy adults aged 18-50. Volunteers were intranasally inoculated with Spn6B in a human challenge model. Serum samples were taken before challenge and 29 days post-challenge. No significant increase in anti-protein IgG from baseline (Wilcoxon paired test followed by Benjamini Hochberg correction) was observed for any protein in carriage positive or carriage negative participants following inoculation. Bars indicate interquartile range. Outliers were removed from graphs. Carriage negative n = 20, carriage positive n = 17. * = $p < 0.05$, ** = $p < 0.005$.

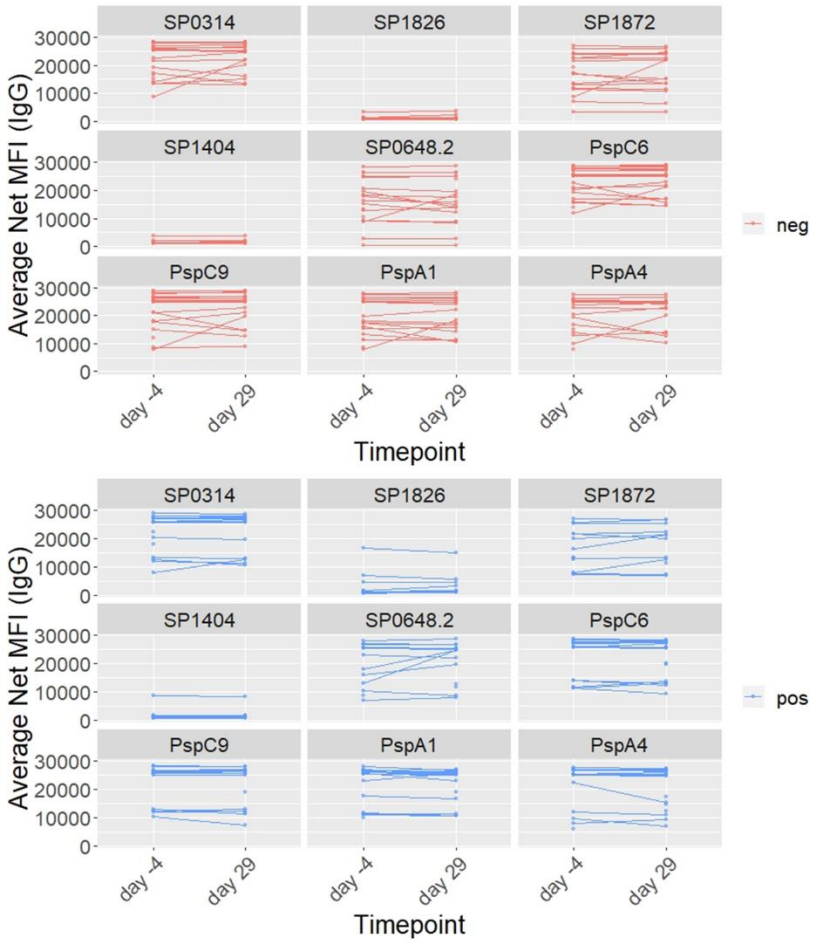


Figure 22: Protein-specific serum IgG response at baseline (day -4) and 29 days after experimental challenge with *S. pneumoniae* serotype 6B (Spn6B) in a cohort of health adults aged 18-50. Volunteers were intranasally inoculated with Spn6B in a human challenge model. Serum samples were taken before challenge and 29 days post-challenge. No significant increase in anti-protein IgG, measured as average net MFI, from baseline (Wilcoxon paired test followed by Benjamini Hochberg) was observed for any protein in either carriage negative or carriage positive participants following inoculation with Spn6B. MFI = median fluorescence intensity. Carriage negative (top) n = 20, carriage positive (bottom) n = 17. * = $p < 0.05$, ** = $p < 0.005$.

Despite clear detection of antibody binding pre- and post-challenge, it was concerning that the expected response to proteins PspA and PspC, based on previous studies, was not seen in the carriage positive cohort, suggesting that the assay was perhaps not sensitive enough to detect these changes. It was hypothesised that serum samples were too concentrated, at least for these highly immunogenic antigens, leading to high saturation of the assay. It is notable that where higher serum concentrations have been used previously to detect pneumococcal protein-specifc serum IgG using a Luminex-based assay, it was the presence of anti-protein IgG being measured in different cohorts as opposed to any change in IgG levels from baseline in response to recent challenge with the bacteria (Shoma *et al.*, 2008; Jiménez-Munguía *et al.*, 2015).

2.4.2.7 ELISA validation

Following these results, an ELISA was used to determine whether there were in fact changes in protein-specific antibody responses post-challenge with Spn6B that were not being detected using the Luminex assay, even at lower serum concentrations. Results indicated that a serum dilution of 1:10,000 to 1:20,000 was most appropriate to detect change in PspA1-specific IgG levels (Figure 23A). The ELISA confirmed that a change in circulating anti-PspA1 IgG post-colonisation was present but was being undetected due to oversaturation in the Luminex assay. Results from the optimisation ELISA suggested that a serum concentration of 1:20,000 enabled the detection of changes in IgG levels between baseline and 29 days post colonisation with Spn6B in these sera samples (Figure 23B). The same serum samples were then run using the Luminex assay for panel 4 proteins at the same range of dilutions to determine if similar results were detected. Comparable results were shown using the Luminex assay (Figure 24), confirming its validity and the fact that oversaturation was masking the detection of change in IgG levels. Although in the Luminex assay, 1:80,000 serum dilution detected a slightly greater median fold change in anti-PspA1 IgG than other serum dilutions, for less immunogenic proteins, the lower serum concentration offered no advantage over 1:20,000 for detecting a change in IgG levels post-challenge. A serum dilution of 1:20,000 also seemed superior to a dilution of 1:40,000 for detecting fold change in IgG (based on median fold change). In the interests of maintaining a serum concentration able to detect IgG for less immunogenic proteins, whilst also elucidating a distinct change in anti-protein IgG for more immunogenic proteins between baseline and 29 days post-colonisation, 1:20,000 serum concentration was deemed optimal. A serum concentration of 1:20,000 was thus used going forward.

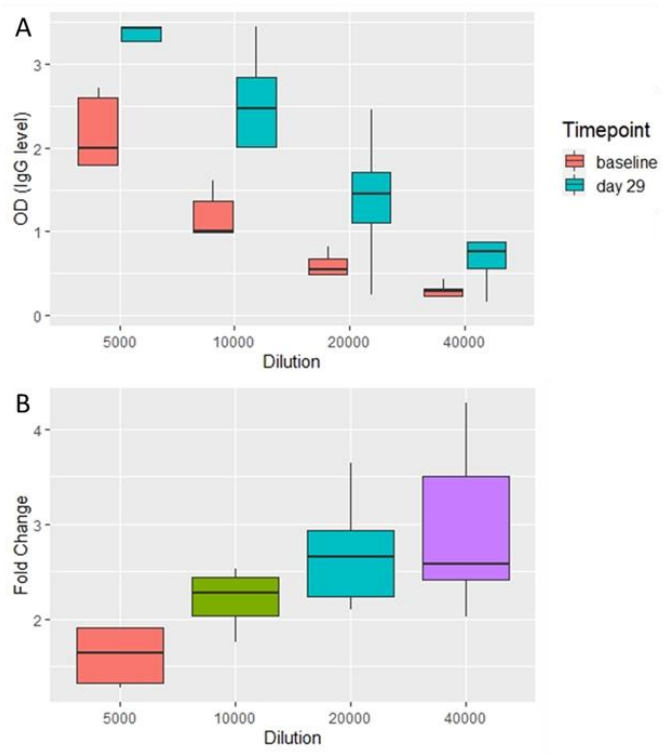


Figure 23: (A) Optical density (OD) for anti-PspA1 serum IgG at baseline and 29 days after experimental challenge with pneumococcus at 1:5000, 1:10,000, 1:20,000 and 1:40,000 serum concentration for 5 participants experimentally colonised with serotype 6B pneumococcus measured by ELISA. (B) Fold change in IgG levels from baseline at 29 days after colonisation (n = 5) measured by ELISA. Bars indicate inter-quartile range.

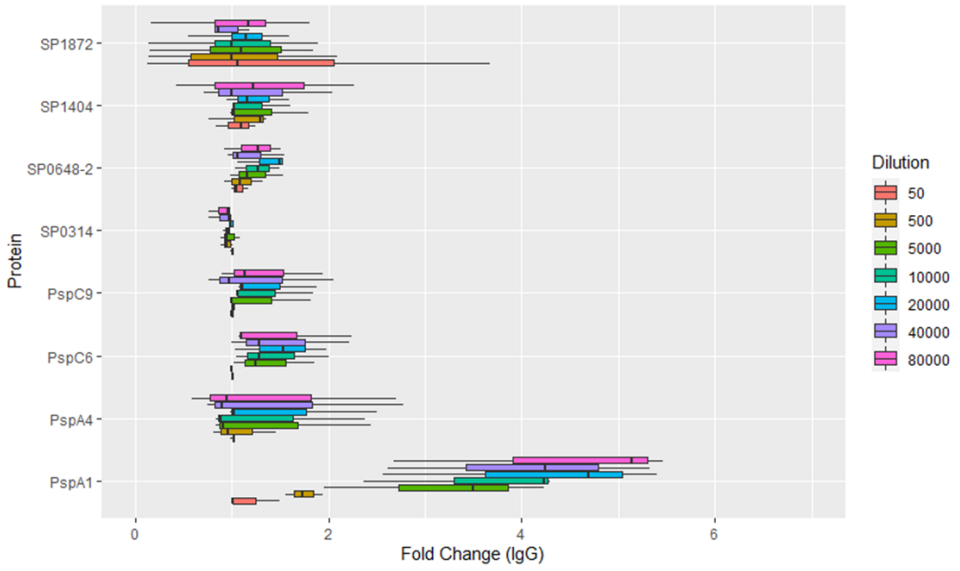


Figure 24: Fold change in protein-specific serum IgG response from baseline at 29 days post-experimental colonisation with *S. pneumoniae* serotype 6B (Spn6B) in 3 health adults aged 18-50 with serum concentrations 1:50, 1:500, 1:5000, 1:10,000, 1:20,000, 1:40,0000, 1:80,000 measured by Luminex assay. A serum dilution of 1:20,000 was optimal to detect changes in IgG levels, particularly in the most immunogenic protein, PspA1. Bars indicate inter-quartile range.

2.4.2.8 Re-evaluation of humoral response in healthy adults inoculated with serotype 6B pneumococcus

Following the re-validation of the Luminex assay, serum samples from volunteers inoculated with Spn6B were re-analysed at 1:20,000 serum concentration. Humoral response before experimental challenge to the panel of proteins was measured for 39 individuals. No analytes were removed from analysis as %CV of replicates did not exceed 25% for any analyte.

There was no significant difference in IgG levels at baseline between naturally protected individuals (n = 18) and those susceptible to pneumococcal colonisation (n = 21) (Figure 25). Similarly, there was no significant change in protein-specific IgG levels between baseline and day 29 in carriage negative individuals (Figure 26). However, a significant increase in IgG response to PspC6 (p = 0.015), PspC9 (p = 0.005) and PspA1 (p = 0.0006) was observed after established pneumococcal colonisation (Wilcoxon test, p-values corrected using the Benjamini-Hochberg procedure). PspA1 elicited the most prominent IgG response with a greater than two-fold median increase in protein-specific IgG after experimental carriage (Figure 27). Furthermore, it was interesting to find that if a strong immune response was elicited for any given protein, it was also observed for the other proteins in the panel (Figure 28).

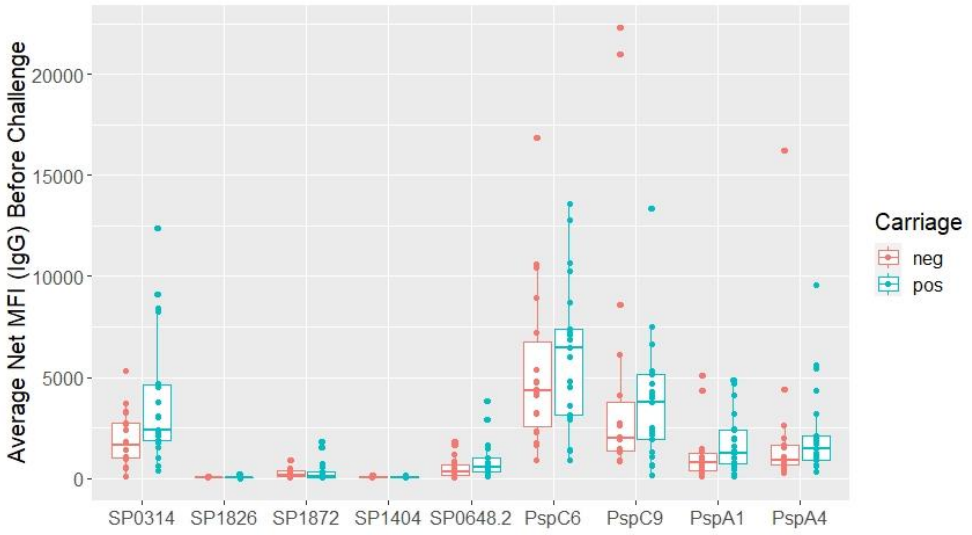


Figure 25: Protein-specific serum IgG response, measured as average net median fluorescence intensity (MFI), at baseline in a cohort of health adults aged 18-50 before inoculation with *S. pneumoniae* serotype 6B. Volunteers were intranasally inoculated with Spn6B in a human challenge model. Serum samples were taken before challenge and 29 days post-challenge. No significant difference in levels of baseline protein-specific IgG was detected between participants protected against experimental colonisation with Spn6B and those who developed carriage following inoculation with the bacteria (Mann-Whitney test followed by Benjamini Hochberg correction). MFI = median fluorescence intensity. Bars indicate interquartile range. Carriage negative n = 18, carriage positive n = 21.

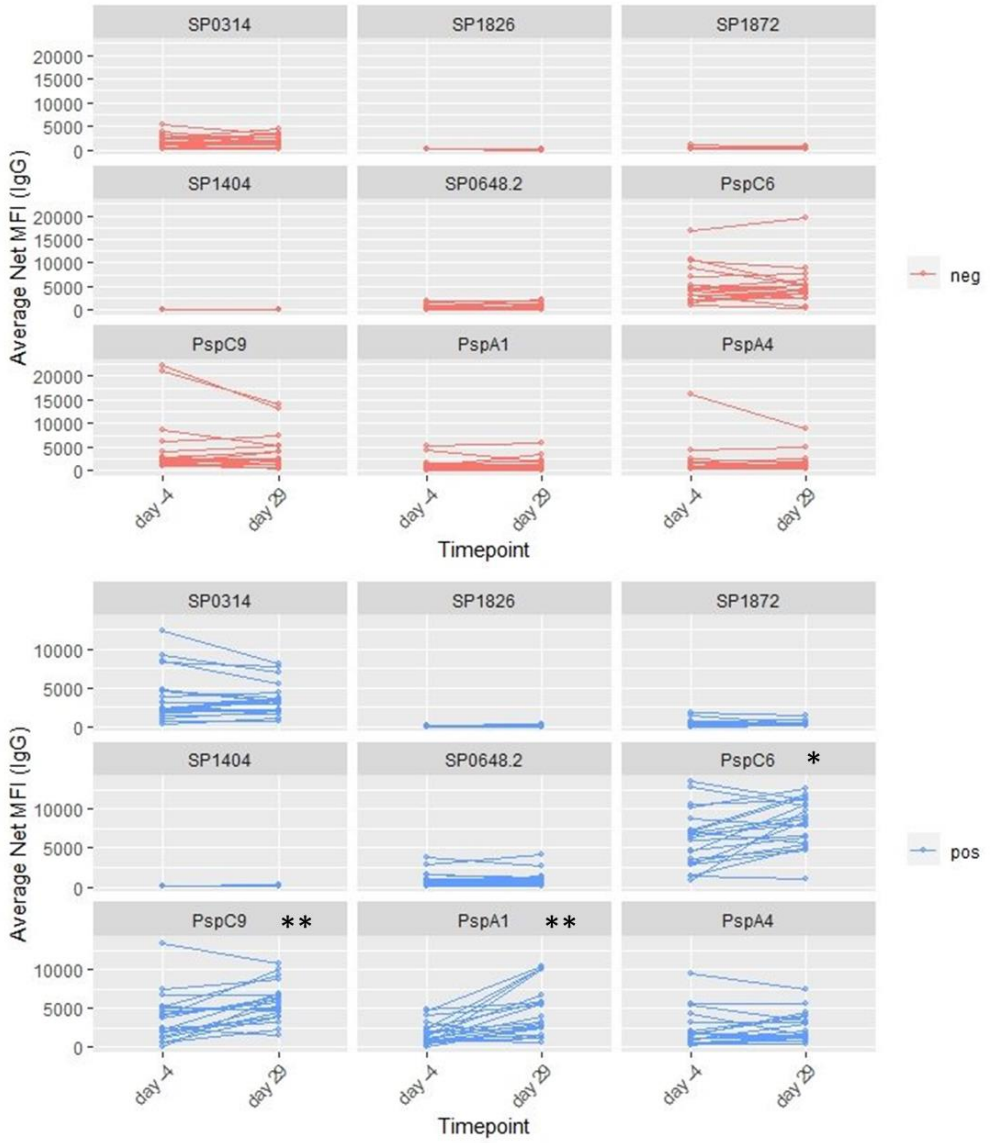


Figure 26: Protein-specific serum IgG response at baseline (day -4) and 29 days after experimental challenge with *S. pneumoniae* serotype 6B (Spn6B) in a cohort of health adults aged 18-50. Volunteers were intranasally inoculated with Spn6B in a human challenge model. Serum samples were taken before challenge and 29 days post-challenge. A significant increase in anti-protein IgG, measured as average net MFI, from baseline (Wilcoxon paired test followed by Benjamini Hochberg correction) was observed for PspA1, PspC9 and PspC6 in participants who were experimentally colonised with Spn6B following inoculation. No significant change in protein-specific IgG levels were observed after challenge in the carriage negative cohort. MFI = median fluorescence intensity. Carriage negative (top) n = 18, carriage positive (bottom) n = 21. * = $p < 0.05$, ** = $p < 0.005$.

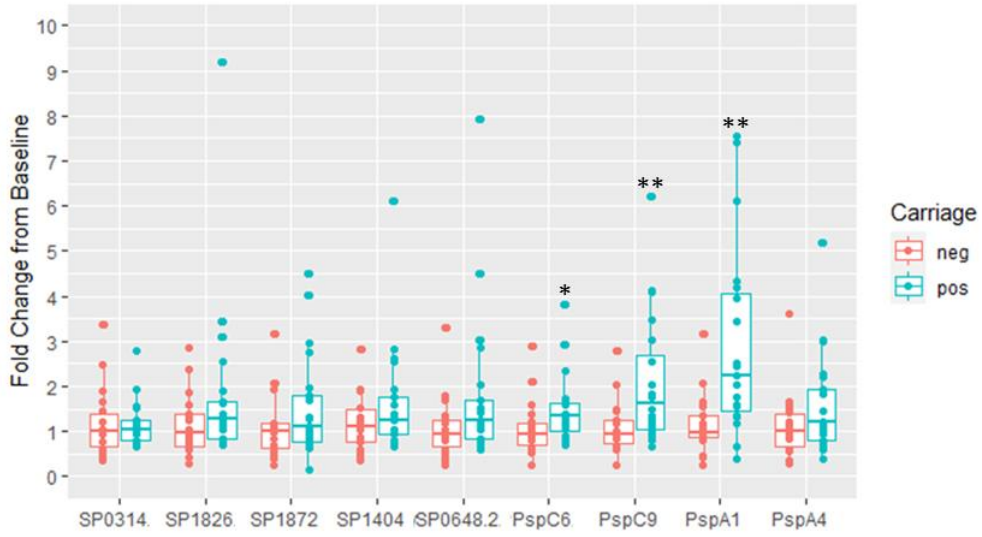


Figure 27: Fold change in protein-specific serum IgG response, measured as average net median fluorescence intensity (MFI), between baseline and 29 days after experimental challenge with *S. pneumoniae* serotype 6B (Spn6B) in a cohort of healthy adults aged 18-50. Volunteers were intranasally inoculated with Spn6B in a human challenge model. Serum samples were taken before challenge and 29 days post-challenge. A significant increase in anti-protein IgG from baseline (Wilcoxon paired test followed by Benjamini Hochberg correction) was observed for PspA1, PspC9 and PspC6 in participants who developed experimental colonisation with Spn6B following inoculation. Bars indicate interquartile range. Outliers were removed from graphs. Carriage negative n = 18, carriage positive n = 21. * = $p < 0.05$, ** = $p < 0.005$.

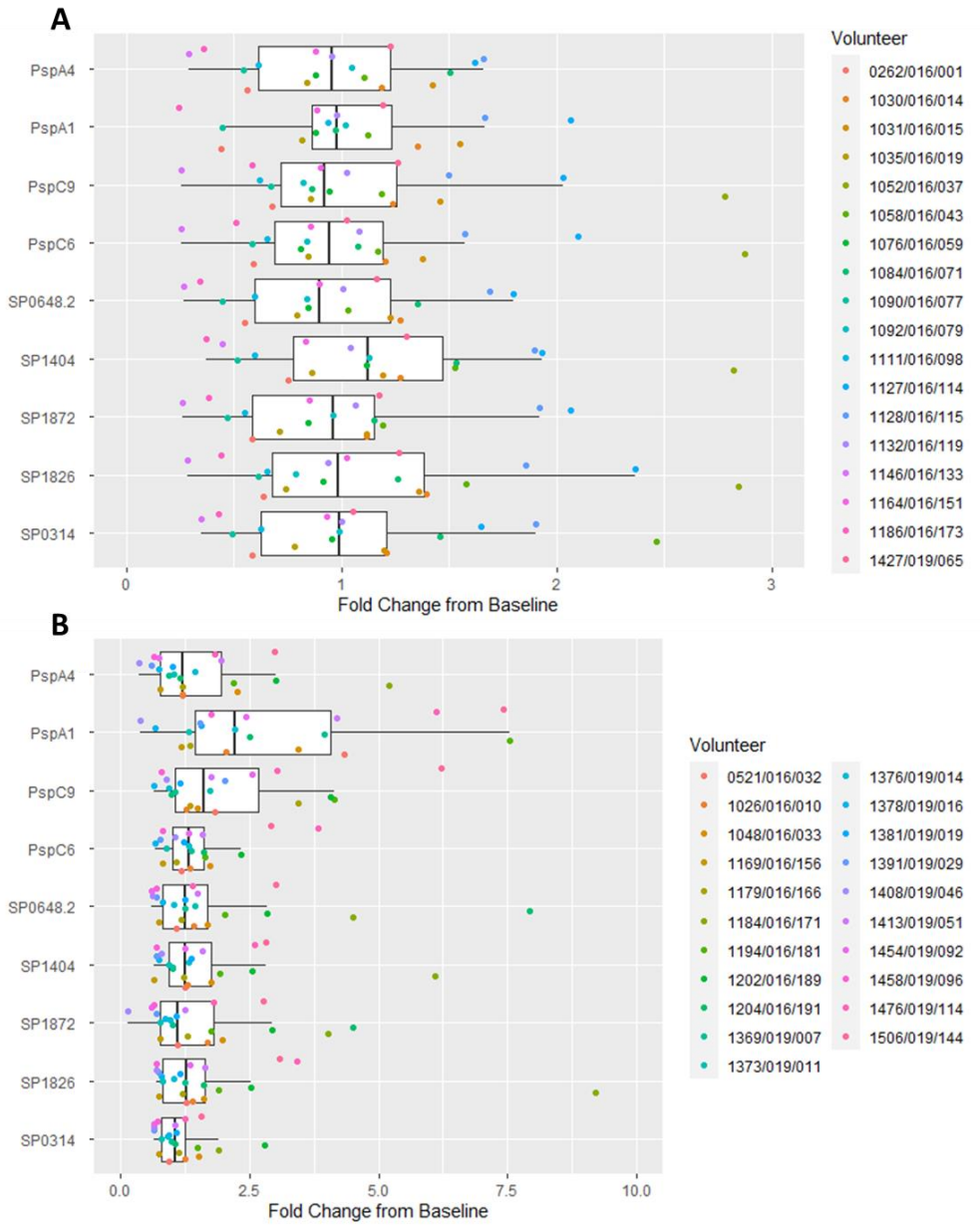


Figure 28: Fold change in protein-specific serum IgG levels, measured as average net median fluorescence intensity (MFI), between baseline and 29 days after experimental challenge with *S. pneumoniae* serotype 6B (Spn6B) in a cohort of healthy volunteers aged 18-50 in (A) carriage negative individuals (n = 18) and (B) carriage positive individuals (n = 21). Volunteers were intranasally inoculated with Spn6B. Serum samples were taken before challenge and 29 days post-challenge. A similar response to each protein was elicited in individuals, irrespective of experimental colonisation status, i.e., if a high level of response was generated towards one protein, a high level of response was generated against all proteins. Bars indicate interquartile range.

2.4.2.9 Validation of full protein library

To confirm that immunodetection was achieved at 1:20,000 serum dilution for all proteins in the library, baseline and day 29 sera from three volunteers experimentally colonised with Spn6B were analysed (Figure 29). Due to high CV values between replicates at baseline and day 29 across all three volunteers' samples, SP0084 and SP2070 were excluded. Immunodetection was achieved for all other proteins. Whilst MFI values were decreased for the less immunogenic proteins at 1:20,000 serum dilution as compared to 1:50, the lower serum concentration enabled the detection of a much more distinct change in anti-protein IgG levels post-pneumococcal colonisation as compared to baseline.

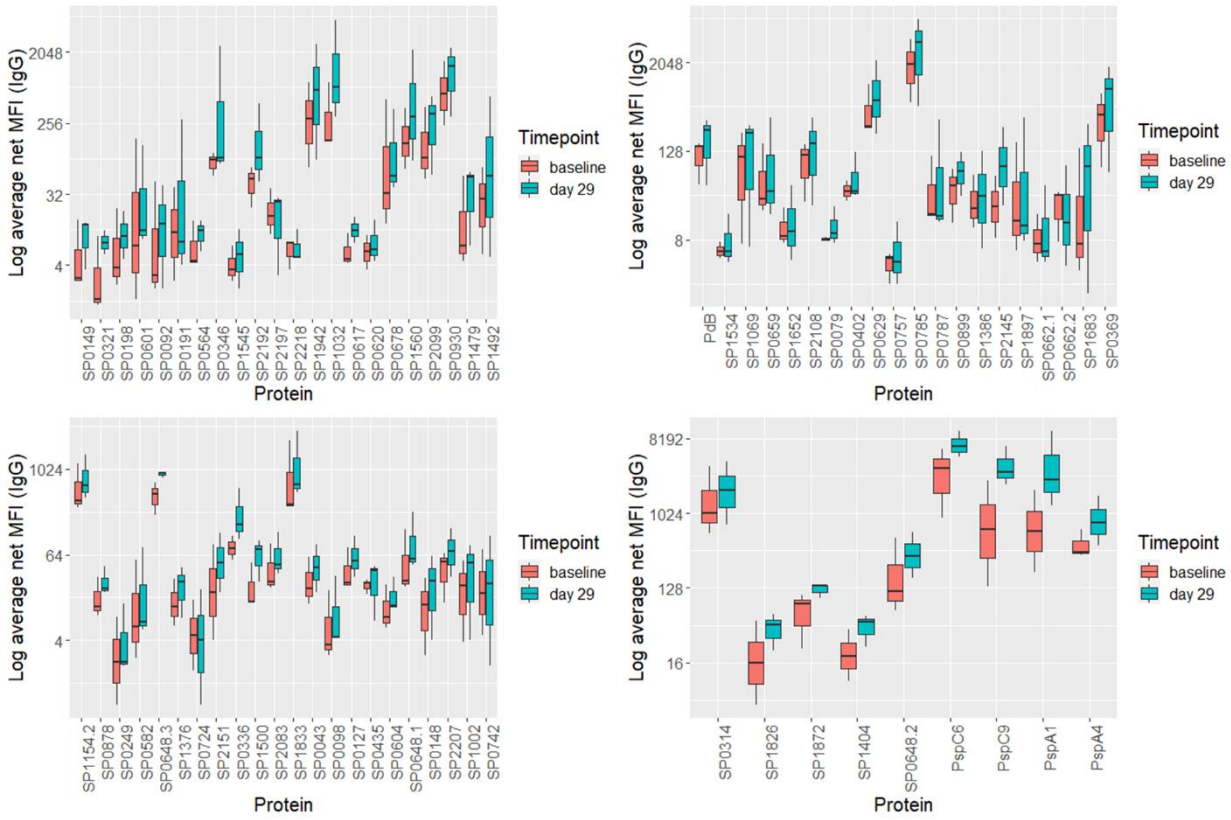


Figure 29: Detection of serum IgG (measured as median fluorescence intensity, MFI) against 73 pneumococcal proteins using a 22-plex Luminex assay at baseline and 29 days after experimental challenge with *S. pneumoniae* serotype 6B (Spn6B) in a cohort of healthy volunteers aged 18-50. Three volunteers were intranasally inoculated and subsequently colonised with Spn6B. Serum samples were taken before and 29 days post-colonisation and diluted 1:20,000 in assay buffer. 75 pneumococcal proteins were coupled to MagPlex microspheres and incubated with sera, however, two proteins (SP0084 and SP2070) elicited high percentage coefficient of variation between sample replicates and were thus excluded. Bars indicate interquartile range. Scales of graphs vary.

2.4.3 Final assay optimised and validated conditions

Overall, the best conditions to take forward for the assay were as follows:

1. 2µg/ml of biotinylated antibody and SA-PE for detection of antibody (IgG) binding to antigens

2. Overnight incubation at 4°C
3. Protein coupling concentration of 5µg protein per million microspheres
4. 1:20,000 serum dilution is optimal for detecting change in antigen-specific IgG binding before and after pneumococcal challenge

2.5 Discussion

Overall, here we have described the development, optimisation, and validation of a multiplex assay utilising Luminex xMAP technology for the detection of serum IgG to a vast library of highly conserved pneumococcal proteins. Initially, optimisation indicated that a 1:50 serum dilution was best for the detection of anti-protein serum IgG. However, we subsequently found that at this dilution, the assay was over-saturated and changes in serum IgG levels against immunogenic proteins post-colonisation with Spn6B as compared to baseline were not being detected. This likely occurred because high antibody levels against immunogenic proteins were present in both baseline and post-challenge sera and so all antigens on bead surfaces were occupied by antibodies at both timepoints. Subsequent validation and comparison with results obtained using an ELISA showed that at 1:20,000 serum dilution, we can detect a more distinct immune response to pneumococcal proteins after experimental colonisation. What was not tested was whether reducing the coupling concentration of proteins would have influenced ability to detect change in response. All microspheres had already been coupled with proteins at a coupling concentration of 5µg per million microspheres. We observed that for the highly immunogenic protein PspA1, a higher coupling concentration (10µg protein per million beads) resulted in reduced MFI values (IgG detection) at serum concentration above 1:400. We hypothesise that this was due to the well-documented prozone or "hook" effect first observed by Neisser & Wechsberg in 1901, which results in falsely low levels of fluorescence and has formerly been shown to occur in Luminex assays developed for detection of HLA (human leukocyte antigen) antibodies (Schnaidt *et al.*, 2011; Weinstock and Schnaidt, 2013; Jain *et al.*, 2018).

Weinstock and Schnaidt (Schnaidt *et al.*, 2011; Weinstock and Schnaidt, 2013) previously hypothesised that the prozone effect occurs in solid-phase assays when high levels of antigen-specific antibodies present in serum land in close proximity as they bind antigens on bead surfaces, resulting in the binding of C1 complement protein between IgG antibodies and thus blocking binding of PE-conjugated anti-human IgG detection antibodies. The binding of C1 between IgG antibodies is prevented by diluting serum, which reduces both the amount of C1 and IgG. Reduced serum IgG titres leads to increased distances between antibodies bound to protein antigens on the surface of microspheres and hence less C1 complement protein binding. Detection antibodies are thereby more able to bind primary IgG

antibodies and the prozone effect is eliminated. Although, whether the interference of complement in bead-based assays is the same as the prozone effect is debated in the literature (Berth, 2016; Jain *et al.*, 2018). We observed that MFI values plateaued at saturation point but did not decline with increasing serum concentration at protein coupling concentration of 5µg protein per million microspheres. A decline in MFI values with increasing serum concentration above 1:400 was only observed for PspA1 when the protein coupling concentration was increased to 10µg per million microspheres and was not observed with lower protein coupling concentrations. Therefore, the combination of high IgG titres in more concentrated serum with high antigen density on microspheres is most likely to have contributed to the prozone phenomenon in the Luminex assay described in this work. It is also plausible that at both high serum concentration and high antigen density, univalent antibody binding occurred, preventing the binding of detection antibodies to human IgG and contributing to prozone (Vos, Klasen and Haaijman, 1987).

Whilst MFI values were decreased for the less immunogenic proteins at 1:20,000 serum dilution as compared to 1:50, the lower serum concentration enables the detection of a much more distinct change in anti-protein IgG levels post-pneumococcal colonisation as compared to baseline. Moreover, successful vaccine candidates are likely to be highly immunogenic, generating high MFI readings, and it is therefore most important that the IgG response to those proteins is clearly identified, with the trade-off of perhaps detecting less IgG binding to the less immunogenic antigens. In other words, power to detect change in IgG response is increased at lower serum concentration. Alternatively, in future work, different dilutions could be used for different proteins, or a range of dilutions tested in general.

Notably, our assay showed very little non-specific heterophilic antibody binding to beads (Martins *et al.*, 2004), which varies depending on serum origin and sampling method (Waterboer, Sehr and Pawlita, 2006). Background values from assay buffer absent of any human sera incubated with protein-coated beads were also consistently low. Moreover, intra- and inter-assay CV values in our assay were comparable to those observed in previously developed Luminex assays (Lal *et al.*, 2005; Verkaik *et al.*, 2008; Jiménez-Munguía *et al.*, 2015). Some proteins may elicit less reproducible results due to higher levels of variation in MFI values between assays. Importantly, substantial variation between results from different laboratories using Luminex assays to quantify anti-pneumococcal antibody response has been observed (Zhang *et al.*, 2013), although qualitative agreement (“protected” or “non-protected”) was good. High inter-assay variability of a commercial Luminex assay to detect serum cytokine concentrations has also been reported (Chowdhury, Williams and Johnson, 2009). Previous studies have shown that

slightly reduced MFI values are observed after 7 and 12 months of initial coupling when protein-coupled carboxylated microspheres are stored at 4°C (Cham *et al.*, 2008; Crompton *et al.*, 2010). It is likely that absolute MFI values may not be wholly reproducible over time but good qualitative agreement across assays, i.e. relative change in immune response, will still be observed (Chowdhury, Williams and Johnson, 2009; Zhang *et al.*, 2013). CV of MFI values obtained for internal positive controls should always be assessed between plates of which results will be directly compared. Where inter-assay CV is high (>25%), experiments should be repeated to ensure comparability. Variation in MFI values for positive controls (day 29 pooled sera) between plates of which results would be pooled or directly compared was always assessed in our work (Chapters 2 and 3) to ensure suitable levels of comparability. Notably, in the current work, pooled sera aliquots were made after preparation of a single stock and so pooled sera never underwent more than one freeze-thaw cycle; increased rounds of freeze-thawing will potentially affect variability of results due to sample degradation.

Previous studies have found that protein coupling concentration is the most consistent factor influencing assay variability (Ondigo *et al.*, 2012; Wu *et al.*, 2019). Sub-optimal coating and low reactivity as well as over-coating and subsequent aggregation of microspheres (impairing antibody binding) should be avoided (Wu *et al.*, 2019). Notably, we aimed to limit aggregation of coupled microspheres on the bottom of plate wells in this work by constant agitation of plates on a plate-shaker. The optimal coupling concentration may vary based on antigen, and future work should validate the reproducibility of the coupling concentrations used in this work by monitoring the consistency of MFI values obtained between coupled-microsphere batches. Furthermore, proteins that are less suited to inclusion in bead-based assays may be identified (Ondigo *et al.*, 2012).

The lack of significant difference at baseline between volunteers naturally protected from and susceptible to experimental colonisation fits with previous findings that show baseline antibody concentrations are not associated with protection against experimental colonisation (Ferreira *et al.*, 2013; Adler *et al.*, 2021; Araujo *et al.*, 2021). The Luminex assay was also able to detect changes in IgG response against PspC and PspA, which are known to be immunogenic and generate a humoral response to colonisation (McCool *et al.*, 2003; Collins *et al.*, 2013; Ferreira *et al.*, 2013; Croucher *et al.*, 2017; Adler *et al.*, 2021). In an elderly cohort (50-84 years of age), experimental challenge with Spn6B resulted in significantly increased antibody titres against several pneumococcal proteins 29 days after pneumococcal colonisation, including PspC, PspA-UAB055, RrgA-TIGR4, PiuA, and PcpA, detected by MSD (Adler *et al.*, 2021). Yet, as is also seen in this work, there was no increase in anti-protein IgG post-challenge in those

volunteers who remained uncolonised. In a cohort of young (18-60 years of age), healthy adults, carriage induced a significant increase in the level of serum IgG to six antigens (out of 27 tested) at 14 days after inoculation (n = 21): PspC, PspAUAB0055, PcpA, PhtD, PiuA and RrgBT4 (Ferreira *et al.*, 2013). In this instance however, pneumococcal challenge also elicited increased levels of IgG antibodies against 14 antigens in the absence of carriage (n = 20): PspC, LytC, PspAUAB0055, PcsB, PhtD, Ply, PsaA, RrgBT4, SP2194, SP0057, SP0096, Spr1, Spr2021 and Stkp; suggesting that even in the absence of carriage, exposure can generate an immune boosting response. Nevertheless, the ratios of post-challenge-to-pre-challenge levels for each volunteer were generally lower in the carriage negative cohort as compared to the positive cohort. Moreover, five weeks-post inoculation, increased IgG levels against 13 antigens persisted in the carriage positive cohort only. These results thereby suggest that the kinetics of the IgG response differs in response to exposure compared to sustained colonisation with the bacteria, with a temporary and rapidly waning response in the carriage negatives that may not persist up to 29 days post-challenge. These results were also obtained via an MSD assay. The greater sensitivity of MSD technology as compared to Luminex likely explains why minimal increases in anti-protein IgG in the acute phase post-challenge, in the absence of colonisation, were detected in the previous study but not in the current study.

Comparatively, pre-existing serum IgG levels against PspA isolated from a Spn23F clinical isolate, P833 (measured using an ELISA), inversely correlated with propensity to experimental colonisation by Spn23F (McCool *et al.*, 2002). 6/14 volunteers experimentally challenged with the bacteria were colonised and all developed a significantly increased IgG response to the N-terminal region of PspA four weeks post-inoculation (average 8-fold increase). None of the carriage negative volunteers elicited an increased anti-PspA IgG response. However, no volunteers in a cohort challenged with Spn6B rather than 23F (6/7 of whom were colonised) mounted a significant anti-PspA IgG response. Thus, IgG elicited in response to PspA could be strain specific; this will be explored further in the next chapter of this work. Notably, increased IgG response against PspA did not temporally correlate with clearance of experimental carriage, which persisted for 122 days after inoculation. A subsequent study showed that experimental colonisation with Spn23F significantly increased IgG against both PspC isolated from P833 and TIGR4 (McCool *et al.*, 2003). This response was not observed in volunteers challenged with Spn6B (n = 8) rather than Spn23F. Again, this may be due to high variability in the N-terminal region of PspC across strains. A significant increase in anti-PspA IgG response was also observed in carriage negative volunteers post-challenge with Spn23F (n = 8).

It is noteworthy that previous work has shown assay choice is critical in determining biomarker assessment as quantitative differences may be found, although generally, relative differences are comparable (Chowdhury, Williams and Johnson, 2009; Günther *et al.*, 2020; Numis *et al.*, 2021). It should further be considered that Luminex assays have been deemed unreliable to evaluate antibody response to pneumococcal polysaccharide antigens for the assessment of humoral immunocompetence in clinical practice (Whaley *et al.*, 2010; Balloch, Licciardi and Tang, 2013; Zhang *et al.*, 2013; Hajjar *et al.*, 2018; Sorensen and Edgar, 2018). Moreover, substantial variation between results from different laboratories using Luminex assays to quantify anti-pneumococcal antibody response has been observed (Zhang *et al.*, 2013). Thus, discrepancies between antibody quantification using this Luminex assay and previous results determined by ELISA or other multiplexing methods such as MSD technology may be likely and could be explained by this known variability between testing methods. Nevertheless, overall, the results obtained using the Luminex assay described here are in line with those of previous studies analysing anti-protein humoral response to experimental challenge with Spn6B (measured by ELISA or MSD).

Importantly, the platform described here can detect not only the presence of, but changes in levels of circulating anti-protein IgG in response to recent exposure with pneumococcus under controlled conditions using the EHPC model. This platform can thus be used to investigate the kinetics of humoral response to a vast array of proteins from the point of pneumococcal challenge across different volunteer cohorts in the presence and absence of subsequent colonisation.

2.6 Conclusion

In summary, here is described a multiplex assay utilising Luminex xMAP technology, which enables the detection of a change in serum IgG from baseline in response to experimental challenge with serotype 6B pneumococcus for a vast library of conserved pneumococcal proteins. The assay provides a rapid means of detecting pneumococcal protein-specific IgG response, with minimal amount of sample, which can be used to inform pneumococcal vaccine development. Going forward, this will enable the selection of pneumococcal proteins which generate a humoral response and may thus provide protein-vaccine candidates. In the next chapter of this work, IgG response to the entire protein library will be analysed in two volunteer cohorts. The cohorts have been challenged with two different pneumococcal serotypes (Spn6B and Spn15B). Thus, we may determine if anti-protein response to challenge with different serotypes is comparable, indicating whether proteins may stimulate a serotype-independent response.

2.7 Acknowledgements

Thank you to Dr Simon Jochems, Dr Carla Solórzano, Dr Britta Urban and Professor Daniela Ferreira for their input in designing the Luminex-based assay.

Chapter 3

Analysis of protein-specific humoral response following experimental challenge with serotypes 6B and 15B pneumococcus

3.1 Abstract

The identification of immunogenic proteins, which confer protection against pneumococcal colonisation could enable the development of novel protein-based vaccines with serotype-independent coverage. Proteins which stimulate an increased IgG response following colonisation may also be promising vaccine candidates. As previously described, a multiplex assay was developed utilising Luminex xMAP technology to detect serum IgG response against a library of highly conserved pneumococcal proteins. IgG response before and after experimental challenge in a human model of infection with either *S. pneumoniae* serotype 6B (Spn6B) or serotype 15B (Spn15B) was measured. Baseline IgG responses to single protein antigens did not predict protection against experimental colonisation with either pneumococcal serotype. Five proteins elicited significantly higher antibody levels post-colonisation with Spn6B as compared to baseline, but this was not observed following experimental colonisation with Spn15B. Challenge in the absence of subsequent colonisation did not induce increased levels of anti-protein IgG. Further work is needed to determine whether increased anti-protein IgG following experimental pneumococcal colonisation is protective against re-acquisition of the bacteria.

3.2 Introduction

As previously outlined in Chapter 1.7.3.1.1, increased baseline levels of circulating anti-protein IgG has been associated with protection against pneumococcal colonisation in children (Holmlund *et al.*, 2006; Zhang, Bernatoniene, Bagrade, Pollard, *et al.*, 2006; Lebon *et al.*, 2011) and colonisation has an immune priming effect, stimulating the production of anti-protein IgG (Obaro *et al.*, 2000; Rapola *et al.*, 2000; Bogaert *et al.*, 2006; Simell *et al.*, 2006; Zhang, Bernatoniene, Bagrade, Pollard, *et al.*, 2006; Holmlund *et al.*, 2009). In adults experimentally inoculated with Spn6B, colonisation also induced elevated levels of circulating anti-protein IgG and 10/10 individuals were protected against homologous reacquisition up to 11 months post initial exposure (Ferreira *et al.*, 2013). Protection against re-colonisation was associated with significantly altered levels of both anti-capsular and anti-protein IgG. In an elderly cohort, antibody titres against several pneumococcal proteins were increased following colonisation but importantly, no increase in anti-capsular IgG was observed (Adler *et al.*, 2021).

In similarity, serum IgG against PspA isolated from an Spn23F clinical isolate correlated with resistance against experimental colonisation with Spn23F and experimental colonisation significantly induced anti-PspA IgG. However, no protection was conferred by baseline anti-PspA IgG against colonisation with Spn6B in a separate study, which points to the notion that protection conferred by anti-PspA IgG is strain specific (McCool *et al.*, 2002), as supported by studies in murine models (Darrieux *et al.*, 2007, 2008). In Chapter 2, it was found that experimental colonisation with Spn6B induced significantly elevated levels of IgG against PspA1, expressed by the inoculation strain, as compared to baseline but no significant increase in PspA4, cloned from a TIGR4 strain, was observed. In this chapter, protein response before and after experimental challenge with two different serotypes, Spn6B and Spn15B, will be analysed and compared. The data generated will help to discern whether anti-protein humoral responses confer protection against challenge with different serotypes to inform the development of the next generation of pneumococcal vaccines.

As detailed in Chapter 2, a multiplex assay using a library of highly conserved pneumococcal proteins was successfully developed, optimised, and validated. The aim of the work described in this chapter was to use the assay to measure levels of systemic antigen-specific IgG against the entire library of proteins in sera from two different cohorts. In this way, correlates of protection against pneumococcal colonisation as well as immunogenic protein antigens, which may confer protection against re-acquisition of the bacteria, may be identified. Comparison of responses to two pneumococcal serotypes will potentially reveal similarities in the immune response to both serotypes and thus highlight vaccine candidates that could confer serotype-independent protection. The full protein library was previously shown in Table 4 (Chapter 2.3.1) (with the addition of PdB) and Table 6 (Chapter 2.4.1.5).

3.3 Materials and Methods

3.3.1 Volunteer cohort inoculated with serotype 6B pneumococcus

As previously described in Chapter 2.3.3.1, serum samples were taken from healthy non-smoking volunteers between the ages of 18 and 50 who were intranasally inoculated with 80,000 CFU in 0.1ml solution of *S. pneumoniae* serotype 6B strain BHN418 per naris (full sequence available GenBank: ASHP00000000). Serum samples were taken prior to inoculation (baseline) and 29 days post inoculation. Volunteers were inoculated as part of a previous study (EudraCT 2014-004634-26) (split into 2 parts), which examined the effect of Live Attenuated Influenza Vaccine (LAIV) on colonisation with serotype 6B pneumococcus (Jochems *et al.*, 2018; Rylance *et al.*, 2019). The samples used in this work were from the control cohorts of these previous studies. Participants in the study control groups were given nasal

placebo of normal saline paired with intramuscular influenza vaccination (Fluarix Tetra; TIV; GlaxoSmithKline). In comparison, test groups received nasal LAIV paired with intramuscular placebo of normal saline (AstraZeneca). Participants either received inoculation with Spn6B 3 days after receiving an influenza vaccine (study part 1) or 3 days prior to receiving an influenza vaccine (study part 2). The order in which participants received TIV and inoculation with *S. pneumoniae* showed no effect on carriage rate or colonisation density. All participants gave written informed consent, with approval from the North West NHS Research Ethics Committee (14/NW/1460). Colonisation was determined by classical microbiology and the presence of Spn6B in nasal wash samples taken at any timepoint between inoculation and 29 days post-inoculation. Nasal wash samples were taken on days 2, 6, 9, 14 and 29 post-challenge with Spn6B. Complementary molecular methods (*LytA* qPCR) to detect the presence of bacteria were also used to confirm carriage results. No volunteers had previously received any pneumococcal vaccine. Full inclusion and exclusion criteria are given in Appendix D. Pre- and post-inoculation paired serum samples from 21 carriage positive and 18 carriage negative volunteers from this study were analysed.

3.3.2 Volunteer cohort inoculated with serotype 15B pneumococcus

Healthy, non-smoking volunteers aged 18-50 were were intranasally inoculated with 80,000 CFU *S. pneumoniae* serotype 15B per naris (clinical isolate 15B P1262. European Nucleotide Archive accession number: ERS2632437). Spn15B is included in the PPV23 formulation but not in the 13-valent PCV formulation. No volunteers had previously received any pneumococcal vaccine. All participants gave informed written consent, ethical approval was obtained from the National Health Service Research Ethics Committee, Liverpool East (15/NW/0931). Serum samples were taken at baseline and 14 days post inoculation (Trial identifier: ISRCTN68323432; 20815). Pneumococcal colonisation was assessed in nasal washes taken at days 2, 7 and 14 following first pneumococcal challenge using classical culture methods. Pre- and post-inoculation paired serum samples from 9 carriage positive and 15 carriage negative volunteers from this study were analysed.

3.3.3 Luminex assay

As previously described (Chapter 2.3.2.1), 75 pneumococcal protein antigens were conjugated to magnetic microspheres at a concentration of 5µg per million microspheres. Proteins were randomly allocated to different microsphere regions. As 22 regions were available, proteins were divided across 4 panels. A ‘master-mix’ of each panel was made containing 250 beads/µl of each protein-coupled bead population (stored in wash buffer). These stocks were protected from light and stored at 4°C. From this

stock solution, the required volume of stock microspheres was removed and diluted 1:5 in 1%BSA-PBS (assay buffer) to achieve 50 beads/µl of each protein-coupled bead population. 25µl of bead solution was added to each well on a 96 well plate. To this, 25µl of 1:20,000 serum diluted in assay buffer was added. The same stock of day 29 pooled sera as previously (diluted 1:50 in assay buffer) (Chapter 2.3.3.2) was used as an internal positive control across all plates and assay buffer was used as a negative control to detect background median fluorescence intensity (MFI) values. The plate was covered to protect microspheres from light and incubated overnight at 4°C on a plate shaker (300rpm). Following incubation, the plate was clipped into place on the Luminex Magnetic Plate Separator and rapidly and forcefully inverted over a biohazard receptacle to expel the liquid from the wells. Microspheres were washed three times with 200µl assay buffer. Biotinylated human IgG Fc antibody (Invitrogen™ Novex™, catalogue number: A18821, lot: 62, 129, 090718) was diluted to 2µg/ml in assay buffer and 50µl was then added to each well. The plate was then incubated in the dark for 30 minutes at room temperature on a plate shaker. Following incubation, liquid was expelled from wells and microspheres were washed three times as previously. Strepdavidin R-phycoerythrin conjugate (SA-PE) (Invitrogen, catalogue number: S866, lot: 1973501) was diluted idem as for the biotinylated secondary antibody and 50µl of SA-PE solution was added to each well. The plate was incubated as previously for 10 minutes. Liquid was expelled from wells and microspheres were then washed three times and re-suspended in 50µl assay buffer. The plate was incubated for five minutes before analysing the contents of each well on the Luminex analyser. All samples were run in duplicate. Serum samples from two volunteer cohorts, as described above, were analysed. All data reported are in units of average net MFI (average of replicates) with background MFI subtracted (average net MFI - blank wells).

It should be noted that the internal positive control both confirmed immunodetection as well as inter-assay variation of the panel of proteins against which serum IgG was being analysed. In the case of high variation between plates, experiments were repeated to ensure comparability of results (<25% CV for each analyte). Final inter-assay CV for all proteins as determined by the internal positive control was <20%. Thus, we deem results obtained across different assays to be comparable. Baseline and post-challenge samples for the same volunteer were always analysed on the same plate.

3.3.4 Data cleaning and statistical analysis

As described in Chapter 2.3.3.1.1, raw Luminex data was cleaned prior to analysis through the following workflow. The coefficient of variation (CV) of replicates was calculated for all analytes. If any analyte had greater than 25% CV and both replicates had greater than 50 microspheres per well, the

analyte was removed from analysis. If only one well had greater than 50 microspheres for a given analyte and the value for that well appeared to be comparable to the trend of results, the value of the analyte taken from the well with greater than 50 microspheres was taken as opposed to the average of replicates. If any paired samples had less than 50 microspheres of a given analyte in each well, the analyte was removed from analysis. The determinant of 50 microspheres is based on recommendations by the Luminex Corporation that a recovery of less than 50 microspheres renders a result unreliable as well as previous work showing that lower bead counts elicit higher CV values (Bjornstal *et al.*, 2011).

All statistical analysis was carried out in RStudio (version 1.0.153). Statistical significance was determined via a Mann-Whitney or Wilcoxon test for unpaired and paired groups, respectively, followed by Benjamini Hochberg correction for multiple comparisons.

3.3.5 Correlation analysis

To determine the correlation between carriage density in the Spn6B cohort and the degree of humoral response, a correlation analysis was carried out between log area under the curve (AUC) of density data (obtained by nasal wash samples at days 6, 9, 14 and 27 post-inoculation) and either baseline IgG or fold change in IgG levels between baseline and day 29 post-inoculation for each antigen using the cor.test function. Similarly, the same function was used to determine correlation between carriage duration and baseline IgG levels or fold change in IgG for each antigen.

3.4 Results

As >75% of samples in both the Spn6B and Spn15B cohorts exhibited >25% CV between replicates for SP0084, this protein was removed from analysis entirely. SP2070 was removed for carriage positive cohorts in the Spn6B cohort and removed entirely for the Spn15B cohort due to consistently high %CV between replicates for this protein across samples. In the Spn6B cohort, a total of 7% of remaining analytes were removed from the final dataset and in the Spn15B cohort, a total of 2.3% of remaining analytes were removed. Final sample sizes for all analytes are given in Appendix E.

3.4.1 Anti-protein IgG levels against 75 antigens at baseline did not associate with protection against experimental pneumococcal carriage acquisition with Spn6B or Spn15B

Protection against acquisition of pneumococcal carriage following experimental challenge was not significantly associated with baseline IgG against any of the proteins in the library. Following Benjamini Hochberg correction for multiple comparisons, no significant difference in baseline anti-protein IgG was observed between carriage positive and carriage negative cohorts in either the Spn6B or the Spn15B study

(Figure 30-parts A through D, Appendix F). Prior to multiple correction, participants protected against experimental colonisation with Spn6B (n = 18) had significantly higher levels of anti-SP0742 (p = 0.015) and anti-SP0620 (p = 0.049) IgG as compared to participants susceptible to colonisation (n = 21) (Table 12), however overall IgG titres for these proteins were low. Participants who were protected against experimental colonisation with Spn15B (n = 22) elicited higher baseline levels of IgG against PdB (p = 0.015), SP2099 (p = 0.042) and SP1833 (p = 0.044), as compared to those who developed carriage (n = 11); whilst the significance of this was lost following correction for multiple comparisons, the trend was clear (Figure 31, Table 12). Moreover, PspA1 also appeared to be higher in those resistant to colonisation with Spn15B, although this was not significant before Benjamini Hochberg correction (p = 0.063) (Figure 31, Table 12).

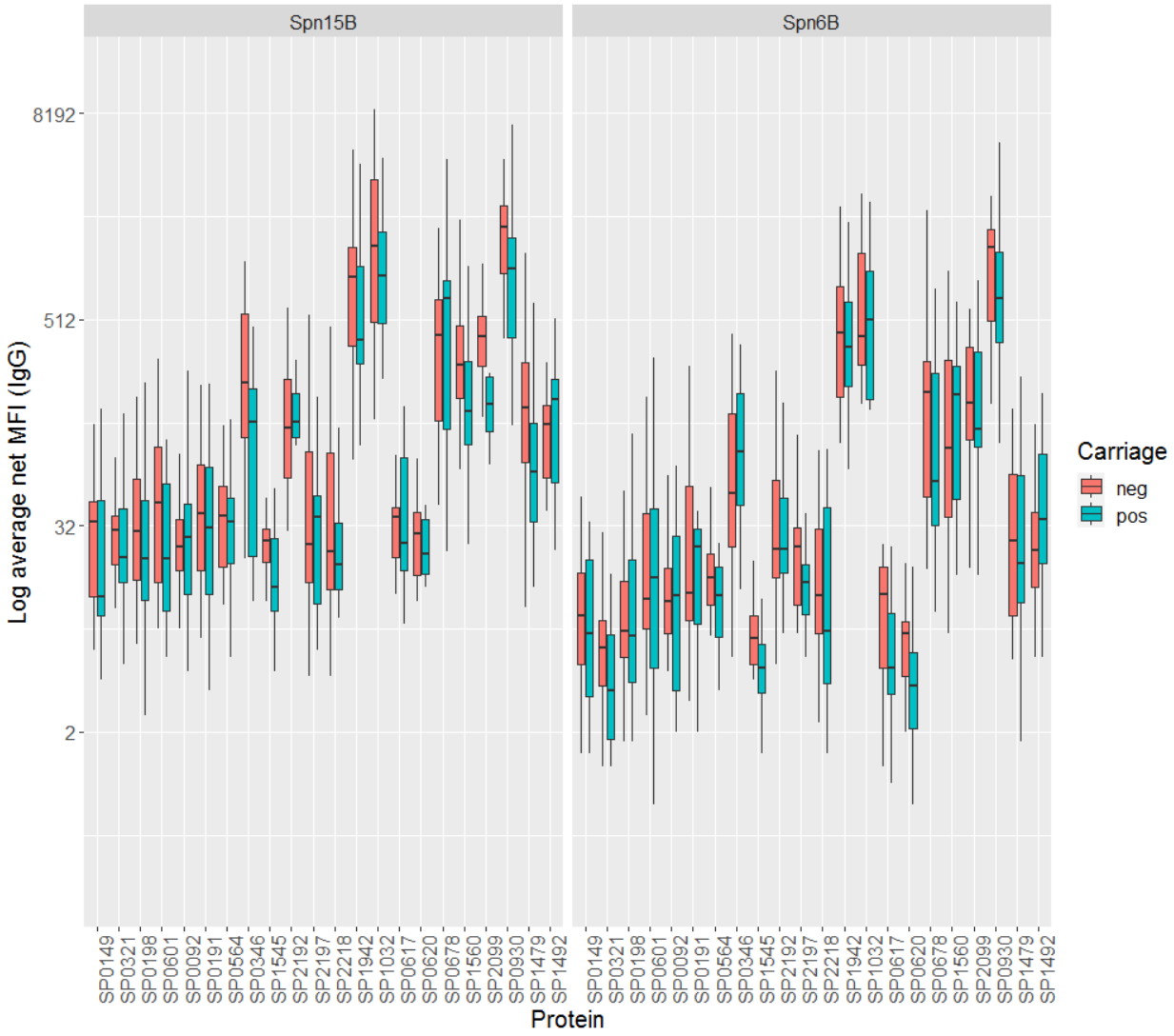


Figure 30-part A (Panel 1): Baseline levels of anti-protein serum IgG in healthy adults aged 18-50, measured as average net median fluorescence intensity (MFI). Following Benjamini Hochberg correction for multiple comparisons, there was no significant difference between baseline levels of protein-specific IgG in volunteers susceptible to experimental colonisation with either S. pneumoniae serotype 6B or 15B (carriage positive, pos) following experimental challenge and those who were protected against colonisation following inoculation (carriage negative, neg) in an experimental human challenge model. Bars indicate interquartile range. Spn15B carriage negative n = 15, Spn15B carriage positive n = 9; Spn6B carriage negative n = 18, Spn6B carriage positive n = 21.

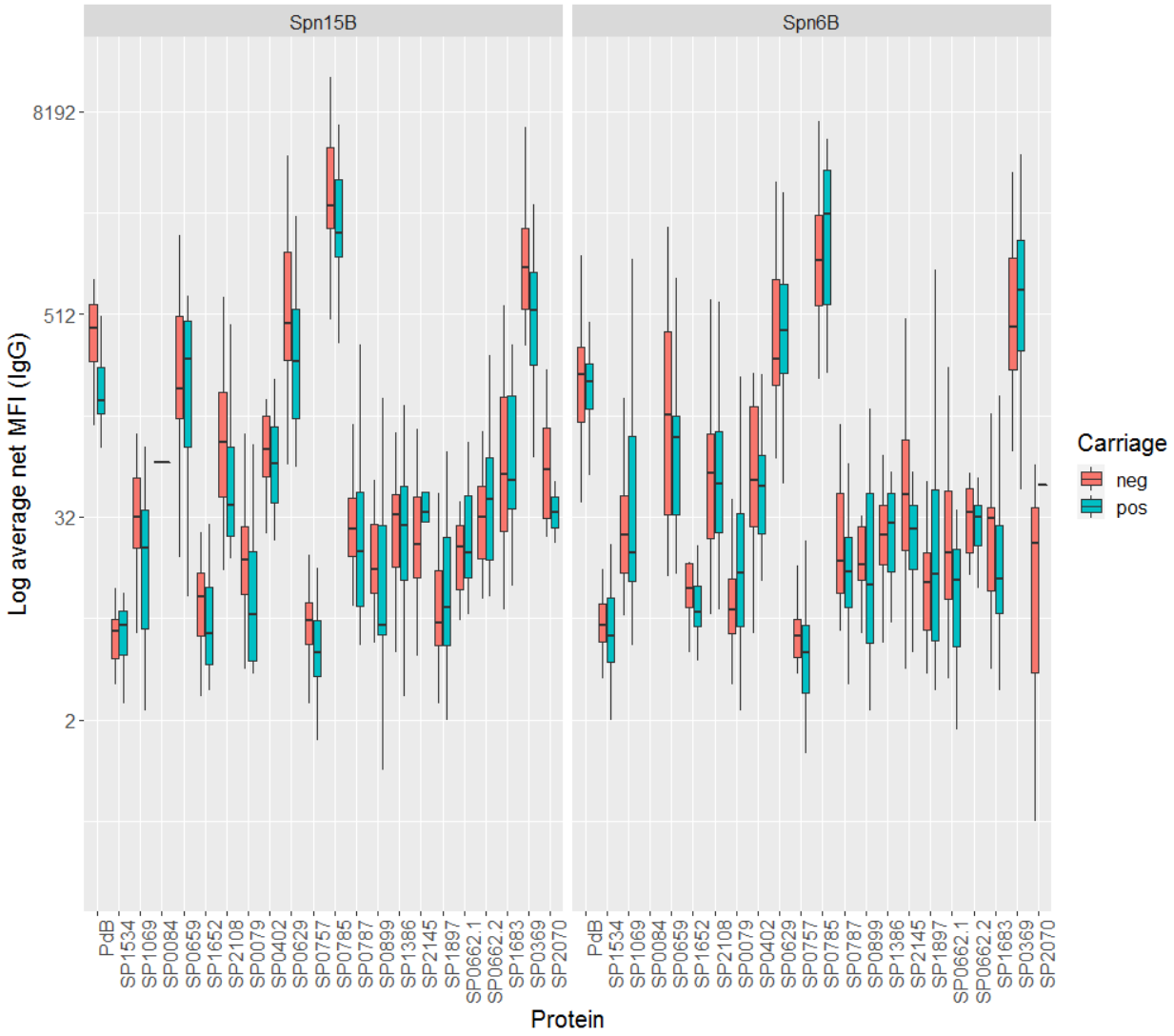


Figure 30-part B (Panel 2): Baseline levels of anti-protein serum IgG in healthy adults aged 18-50, measured as average net median fluorescence intensity (MFI). Following Benjamini Hochberg correction for multiple comparisons, there was no significant difference between baseline levels of protein-specific IgG in volunteers susceptible to experimental colonisation with either S. pneumoniae serotype 6B or 15B (carriage positive, pos) following experimental challenge and those who were protected against colonisation following inoculation (carriage negative, neg) in an experimental human challenge model. Bars indicate interquartile range. Spn15B carriage negative n = 15, Spn15B carriage positive n = 9; Spn6B carriage negative n = 18, Spn6B carriage positive n = 21.

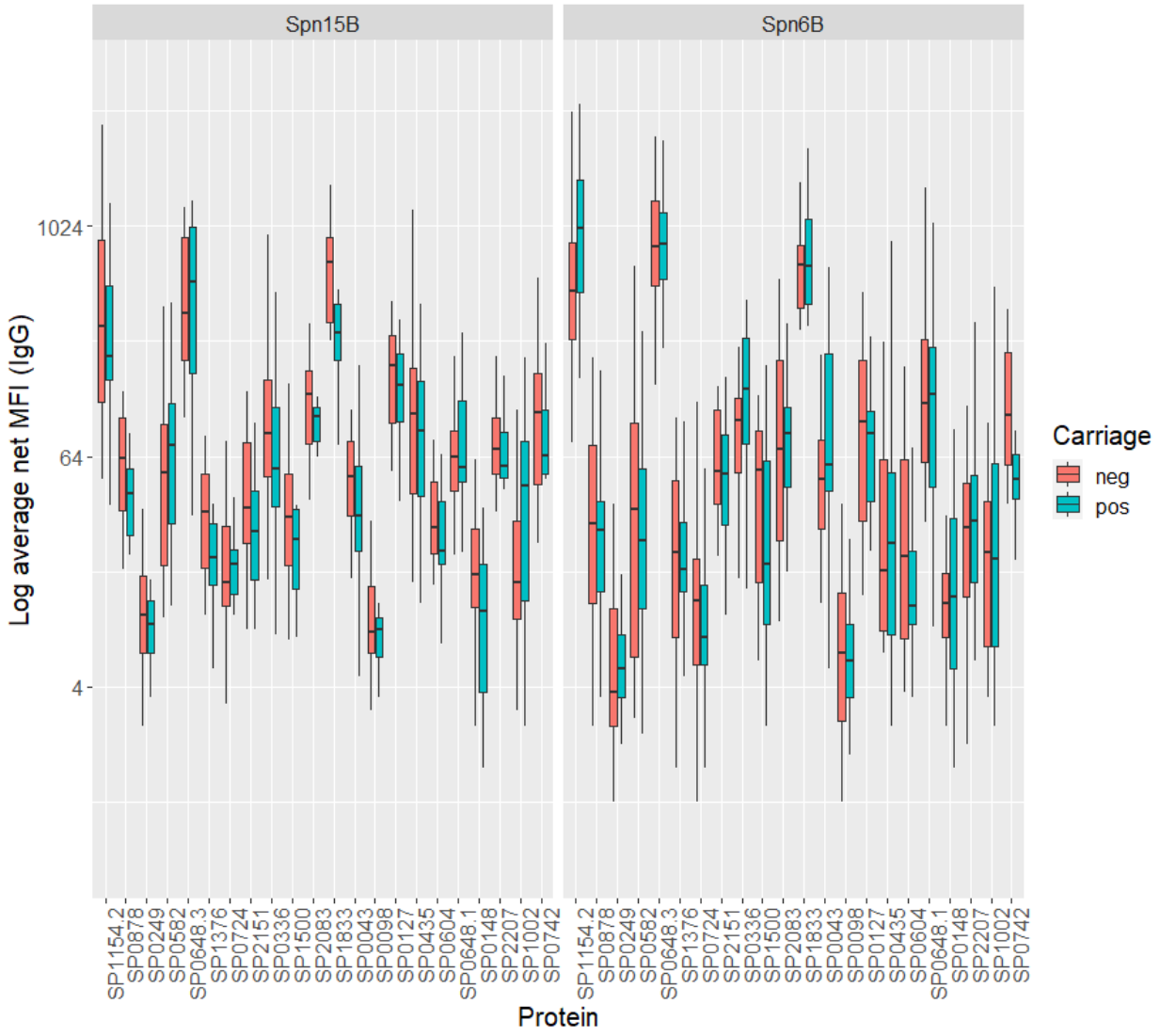


Figure 30-part C (Panel 3): Baseline levels of anti-protein serum IgG in healthy adults aged 18-50, measured as average net median fluorescence intensity (MFI). Following Benjamini Hochberg correction for multiple comparisons, there was no significant difference between baseline levels of protein-specific IgG in volunteers susceptible to experimental colonisation with either S. pneumoniae serotype 6B or 15B (carriage positive, pos) following experimental challenge and those who were protected against colonisation following inoculation (carriage negative, neg) in an experimental human challenge model. Bars indicate interquartile range. Spn15B carriage negative n = 15, Spn15B carriage positive n = 9; Spn6B carriage negative n = 18, Spn6B carriage positive n = 21.

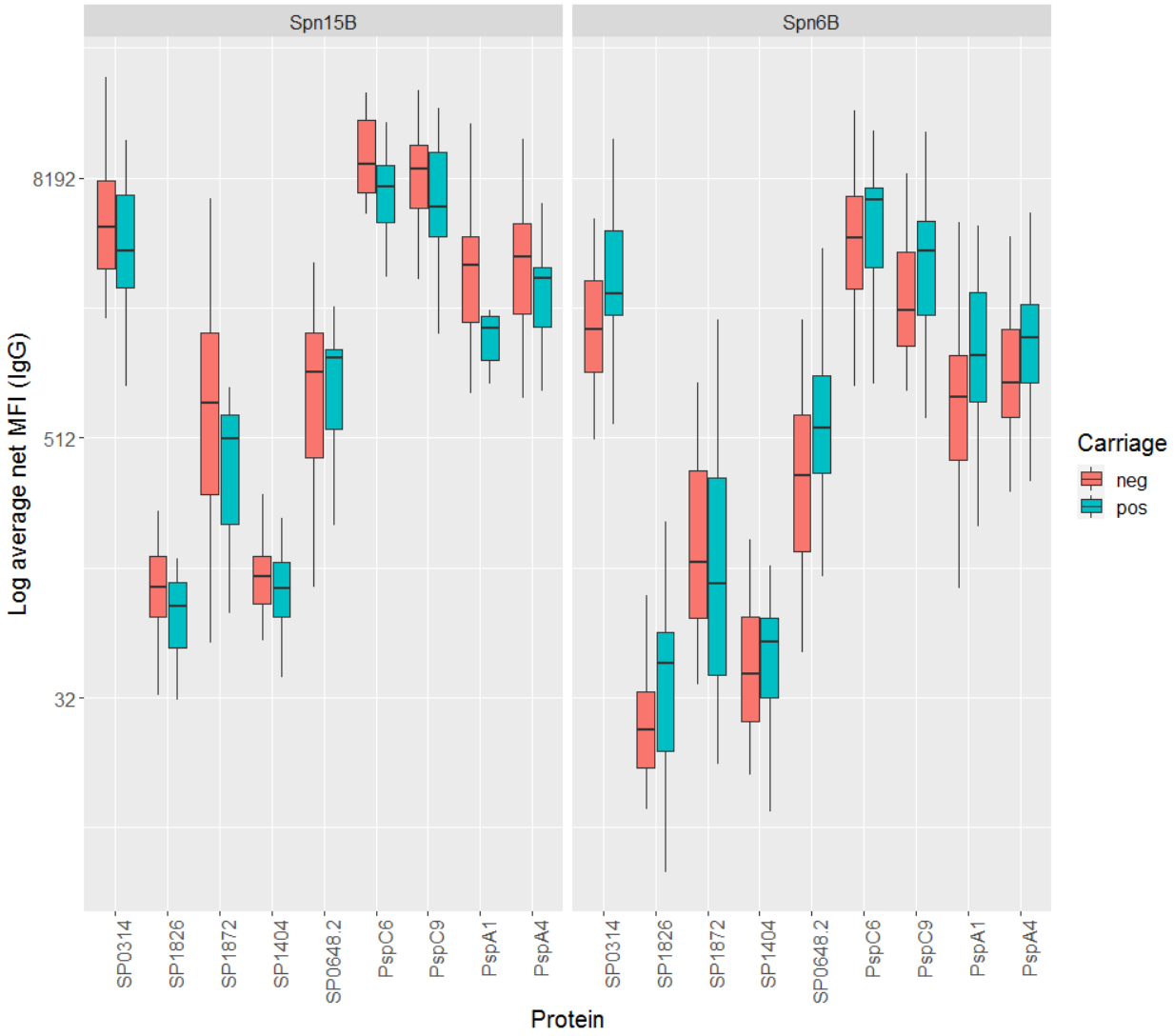


Figure 30-part D (Panel 4): Baseline levels of anti-protein serum IgG in healthy adults aged 18-50, measured as average net median fluorescence intensity (MFI). Following Benjamini Hochberg correction for multiple comparisons, there was no significant difference between baseline levels of protein-specific IgG in volunteers susceptible to experimental colonisation with either *S. pneumoniae* serotype 6B or 15B (carriage positive, pos) following experimental challenge and those who were protected against colonisation following inoculation (carriage negative, neg) in an experimental human challenge model. Bars indicate interquartile range. Spn15B carriage negative n = 15, Spn15B carriage positive n = 9; Spn6B carriage negative n = 18, Spn6B carriage positive n = 21.

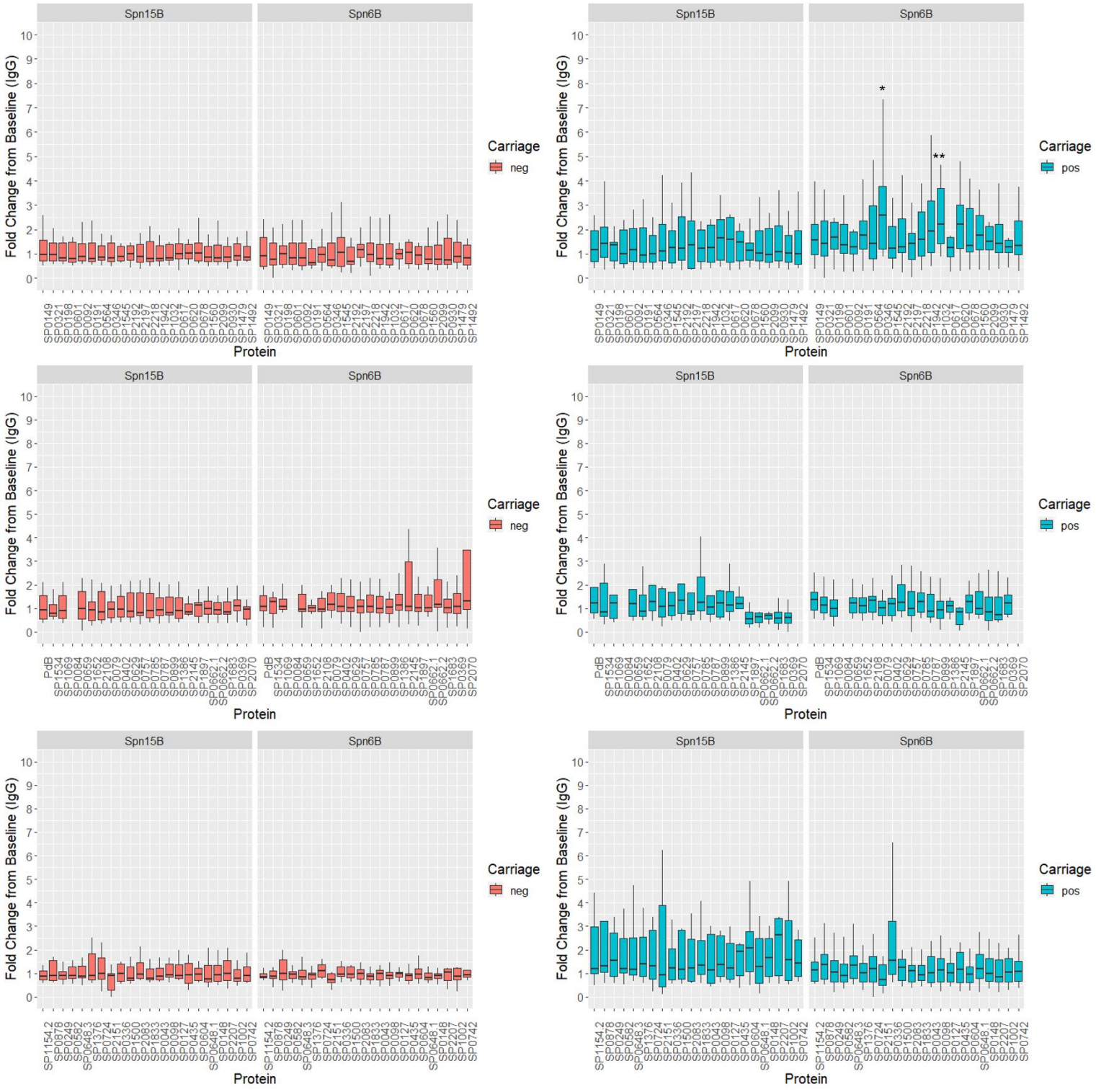
Figure 31: Baseline levels of anti-protein serum IgG in healthy adults aged 18-50 inoculated with *S. pneumoniae* in a human challenge model, measured as average net median fluorescence intensity (MFI). Healthy adults aged 18-50 were inoculated with either serotype 15B (Spn15B) or serotype 6B (Spn6B) in a human challenge model. Participants protected against experimental colonisation with Spn6B had higher levels of anti-SP0742 and anti-SP0620 IgG as compared to participants susceptible to colonisation. Participants who were non-susceptible to experimental colonisation with Spn15B elicited higher baseline levels of IgG against PdB, SP2099 and SP1833. However, following Benjamini Hochberg correction for multiple comparisons, there was no significant difference between baseline levels of protein-specific IgG in volunteers susceptible to experimental colonisation with Spn and those who were protected against colonisation. Spn15B carriage negative n = 22, Spn15 carriage positive n = 9; Spn6B carriage negative n = 18, Spn6B carriage positive n = 21. * = $p < 0.05$ prior to Benjamini Hochberg correction; all significance was lost following correction.

Table 12: Significance values before and after Benjamini Hochberg correction for the association between protein-specific IgG at baseline and protection against subsequent experimental colonisation with either *S. pneumoniae* serotype 6B or 15B.

	***S. pneumoniae* serotype 6B**		***S. pneumoniae* serotype 15B**	
Protein	*p*-value before correction	*p*-value after correction	*p*-value before correction	*p*-value after correction
PspA1	0.094	0.775	0.063	0.690
SP0620	**0.049**	0.775	0.812	0.898
SP2099	0.510	0.910	**0.042**	0.690
PdB	0.762	0.911	**0.015**	0.690
SP1833	0.789	0.911	**0.044**	0.690
SP0742	**0.015**	0.775	0.593	0.801

3.4.2 Experimental colonisation with Spn6B but not Spn15B induces significant protein-specific humoral response

Fold change induction of protein-specific IgG before and after challenge with either *S. pneumoniae* serotype for all proteins are shown in Figure 32 (split across pages 120-121).


Spn15B
Spn6B
Fold Change from Baseline (IgG)
Protein
Carriage
neg
pos
*
**

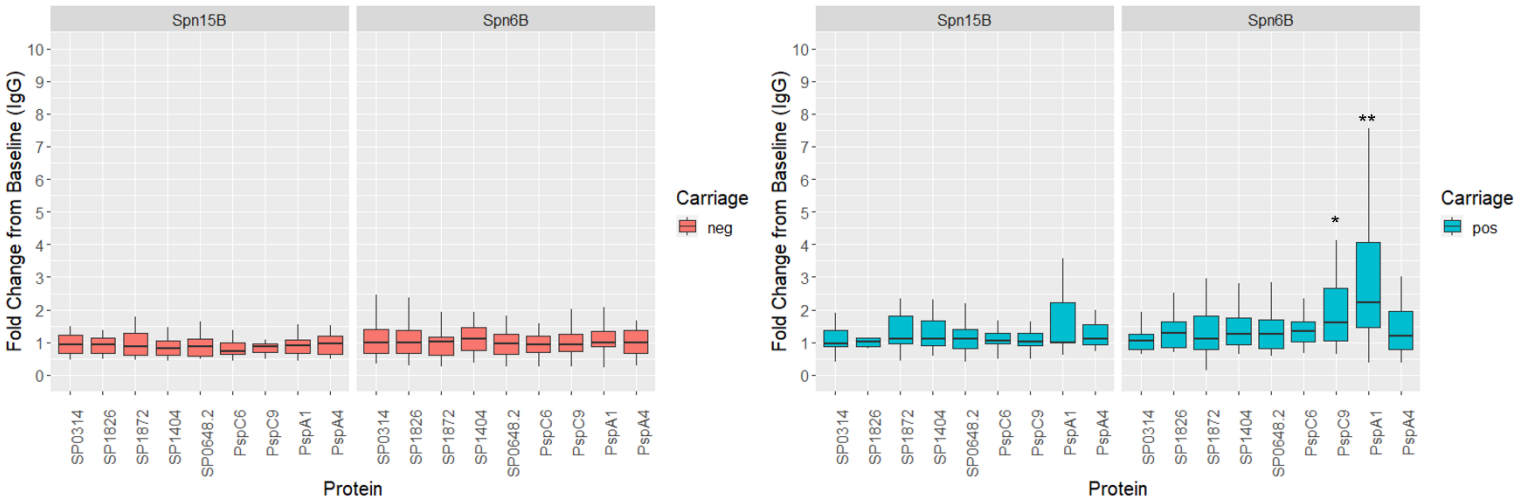


Figure 32: Fold change in anti-protein serum IgG (measured as average net median fluorescence intensity, MFI) from baseline in carriage positive and carriage negative volunteers (aged 18-50) following experimental colonisation with either *S. pneumoniae* (Spn) serotype 15B or 6B. Healthy volunteers were inoculated with either serotype 15B or serotype 6B *S. pneumoniae* as part of a human challenge model. In volunteers colonised with Spn15B, post-challenge samples were taken 14 days after initial challenge (n = 9). Post-challenge samples were taken 29 days after colonisation with Spn6B (n = 21). No significant increase between baseline and post-challenge levels of serum anti-protein IgG was detected in volunteers colonised with Spn15B. A significant increase in serum IgG response (determined by Wilcoxon paired test followed by Benjamini Hochberg correction) to PspA1, PspC9, SP1032, SP0346 was detected in volunteers colonised with Spn6B 29 days post-inoculation. The increase in anti-PspC6 IgG post-colonisation showed borderline significance (p = 0.056). * = $p < 0.05$, ** = $p < 0.005$. Bars indicate interquartile range.

A significant increase in IgG response to the following proteins was detected 29 days post-inoculation in the cohort colonised with Spn6B: PspA1 (p = 0.002), PspC9 (p = 0.016), SP1032 (p =0.002), SP0346 (p = 0.004) (Figure 33). The increase in anti-PspC6 post-colonisation showed borderline significance (p = 0.056). No significant change in anti-protein IgG was observed in the carriage negative cohort 29 days post-challenge.

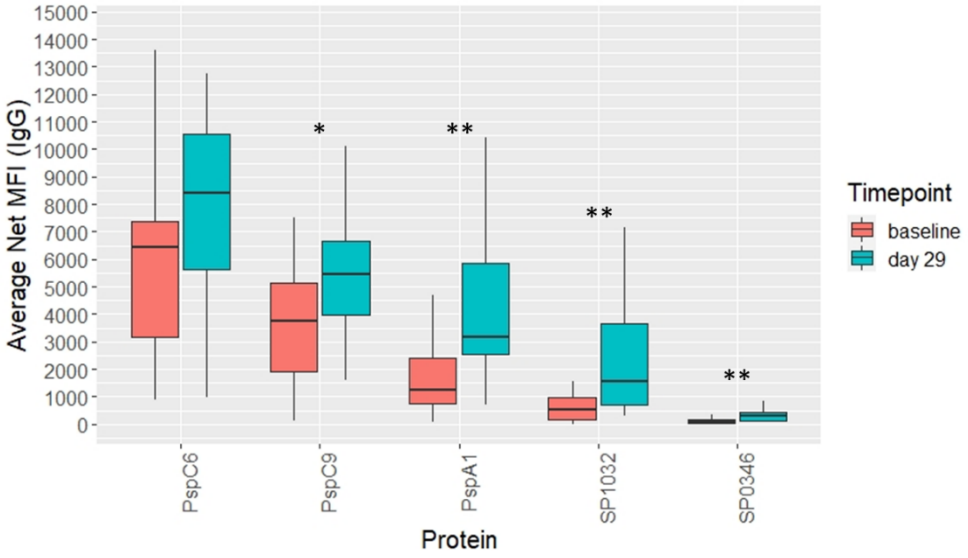


Figure 33: Levels of anti-protein IgG (measured as average net median fluorescence intensity, MFI) before challenge with *S. pneumoniae* serotype 6B (Spn6B) and 29 days post-challenge in colonised individuals. 21 healthy adults aged 18-50 were inoculated with Spn6B in a human challenge model. A significant increase in serum IgG response (determined by Wilcoxon paired test followed by Benjamini Hochberg correction) to PspA1, PspC9, SP1032, SP0346 was detected in volunteers colonised with Spn6B 29 days post-inoculation. The increase in anti-PspC6 IgG post-colonisation showed borderline significance ($p = 0.056$). * = $p < 0.05$, ** = $p < 0.005$.

No significant difference was observed in anti-protein IgG response 14 days post-challenge with Spn15B in either the carriage positive or carriage negative cohorts. Although not statistically significant following Benjamini Hochberg correction, a median fold change ≥2 from baseline in levels of anti-SP0435, anti-SP0604 and anti-SP2207 IgG was observed following experimental colonisation with Spn15B (Figure 34, Table 13). No induction in IgG against these proteins was observed in the cohort protected from colonisation with Spn15B, nor was there a notable induction of IgG for these proteins following experimental colonisation with Spn6B. The same was observed in the case of proteins SP1942 and SP0620, which induced a ≥2-fold median change in IgG from baseline in the cohort experimentally colonised with Spn6B but in the absence of carriage, no increase in IgG was observed and no change in IgG against these proteins was observed following colonisation with Spn15B (Figure 34, Table 13). It is important to note however that levels of IgG against SP0435, SP0604, SP2207 and SP0620 were overall very low. SP1942 elicited higher levels of IgG and the increase in anti-SP1942 IgG following colonisation with Spn6B was significant prior to correction ($p = 0.005$).

The median fold change and percentage of volunteers with a ≥2-fold increase in protein-specific IgG following experimental challenge with either *S. pneumoniae* serotype is given in Table 13.

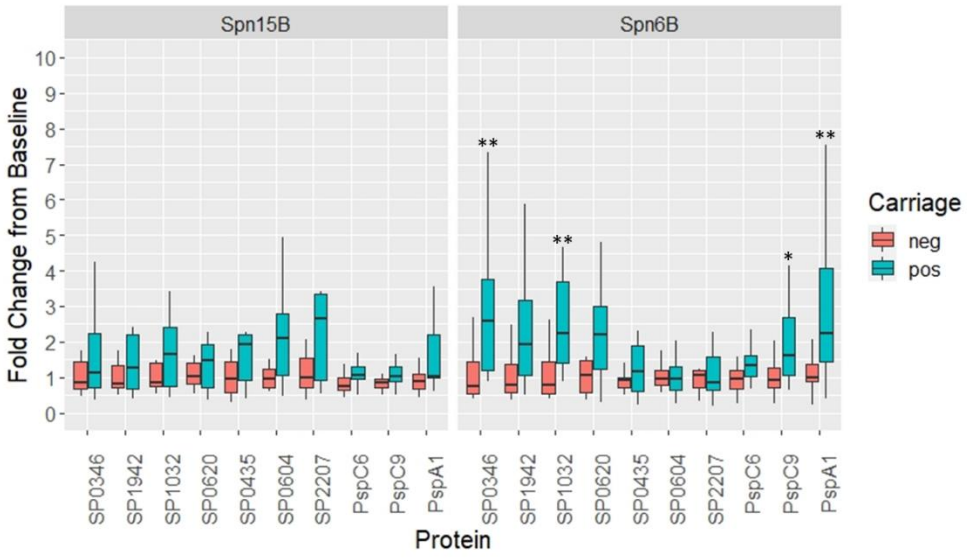


Figure 34: Fold change in anti-protein serum IgG (measured as average net median fluorescence intensity, MFI) from baseline in carriage positive and negative volunteers (aged 18-50) following experimental challenge with either *S. pneumoniae* (Spn) serotype 15B or 6B. Healthy volunteers were inoculated with S. pneumoniae in a human challenge model. For volunteers inoculated with Spn15B, post-challenge samples were taken 14 days after initial challenge, whereas post-challenge samples were taken 29 days after inoculation with Spn6B. A significant increase in anti-protein IgG from baseline (Wilcoxon paired test followed by Benjamini Hochberg correction) was observed for PspA1, PspC9, SP1032, SP0346 in participants colonised with Spn6B. The increase in anti-PspC6 IgG in this cohort was of borderline significance (p = 0.056). Although not statistically significant following Benjamini Hochberg correction, a median fold change from baseline in levels of anti-SP0435, anti-SP0604 and anti-SP2207 IgG ≥2 was observed following experimental colonisation with Spn15B. No induction in IgG against these proteins was observed in the cohort protected from colonisation with Spn15B, nor was there a notable change in anti-SP0435, anti-SP0604 or anti-SP2207 IgG following experimental colonisation with Spn6B. SP1942 and SP0620 induced a ≥2-fold median change in IgG from baseline in the cohort experimentally colonised with Spn6B but in the absence of carriage, no increase in IgG was observed and no change in IgG against these proteins was observed following colonisation with Spn15B. Spn15B carriage negative n = 15, Spn15B carriage positive n = 9; Spn6B carriage negative n = 18, Spn6B carriage positive n = 21. * = $p < 0.05$, ** = $p < 0.005$.

Table 13: Median fold change and percentage of responders with ≥2-fold increase in protein-specific IgG following experimental challenge with either *S. pneumoniae* serotype 6B or 15B. Proteins for which greater than 50% of volunteers elicited a ≥2-fold increase in protein-specific IgG challenge with the bacteria are highlighted in yellow. Spn15B carriage negative n = 15, Spn15B carriage positive n = 9; Spn6B carriage negative n = 18, Spn6B carriage positive n = 21.

	Inoculated with Spn6B				**Inoculated with Spn15B**			
	Carriage positive		**Carriage negative**		**Carriage positive**		**Carriage negative**	
	% Volunteers with ≥2-fold increase in protein-specific IgG following experimental challenge	Median fold change in protein-specific IgG	% Volunteers with ≥2-fold increase in protein-specific IgG following experimental challenge	Median fold change in protein-specific IgG	% Volunteers with ≥2-fold increase in protein-specific IgG following experimental challenge	Median fold change in protein-specific IgG	% Volunteers with ≥2-fold increase in protein-specific IgG following experimental challenge	Median fold change in protein-specific IgG
SP0314	4.76%	1.05	11.11%	0.99	0.00%	0.96	0.00%	0.95
SP1826	19.05%	1.28	11.11%	0.98	14.29%	1.01	0.00%	0.94
SP1872	19.05%	1.11	11.11%	1.01	22.22%	1.11	0.00%	0.89
SP1404	19.05%	1.25	5.56%	1.12	11.11%	1.11	0.00%	0.82
SP0648-2	23.81%	1.25	5.56%	0.95	12.50%	1.10	0.00%	0.88
PspC6	19.05%	1.34	11.11%	0.94	12.50%	1.04	0.00%	0.73
PspC9	38.10%	1.75	11.11%	0.92	0.00%	1.02	0.00%	0.86
PspA1	61.90%	2.44	11.11%	0.97	33.33%	1.01	0.00%	0.90
PspA4	23.81%	1.21	5.56%	1.00	12.50%	1.12	0.00%	0.95
SP0149	42.11%	1.73	23.53%	0.92	22.22%	1.16	7.14%	0.98
SP0321	44.44%	1.47	23.53%	0.80	44.44%	1.42	7.14%	0.98
SP0198	31.58%	1.68	17.65%	1.00	11.11%	1.38	7.14%	0.84
SP0601	36.84%	1.50	17.65%	0.83	22.22%	1.00	7.14%	0.81
SP0092	26.32%	1.27	25.00%	0.91	33.33%	1.18	7.14%	0.88
SP0191	36.84%	1.86	17.65%	0.68	33.33%	0.95	7.14%	0.81
SP0564	36.84%	1.42	17.65%	1.00	22.22%	0.99	7.14%	0.86
SP0346	63.16%	2.80	23.53%	0.80	33.33%	1.11	7.14%	0.84
SP1545	36.84%	1.25	17.65%	1.05	22.22%	1.25	7.14%	0.88
SP2192	42.11%	1.30	23.53%	0.73	44.44%	1.23	7.14%	1.01
SP2197	25.00%	1.44	17.65%	1.17	33.33%	1.38	7.14%	0.87
SP2218	36.84%	1.64	23.53%	0.97	33.33%	1.23	7.14%	0.81
SP1942	52.63%	2.00	23.53%	0.79	33.33%	1.26	7.14%	0.80
SP1032	63.16%	2.59	25.00%	0.91	44.44%	1.65	7.14%	0.84
SP0617	20.00%	1.25	16.67%	1.01	33.33%	1.58	7.14%	1.00
SP0620	55.56%	2.27	18.75%	1.06	22.22%	1.47	7.14%	1.04
SP0678	31.58%	1.35	23.53%	1.02	22.22%	1.14	7.14%	1.02
SP1560	44.44%	1.81	23.53%	0.81	22.22%	1.02	7.14%	0.86
SP2099	36.84%	1.59	23.53%	0.78	33.33%	0.97	7.14%	0.83
SP0930	36.84%	1.45	23.53%	0.75	33.33%	1.07	7.14%	0.85

SP1479	22.22%	1.29	25.00%	1.10	22.22%	1.02	7.14%	0.92
SP1492	36.84%	1.36	23.53%	0.87	22.22%	0.99	7.14%	0.85
PdB	15.79%	1.36	0.00%	1.08	22.22%	1.24	13.33%	0.92
SP1534	21.05%	1.13	5.88%	1.29	33.33%	0.84	0.00%	0.80
SP1069	22.22%	1.00	12.50%	1.08	22.22%	1.22	0.00%	0.90
SP0659	15.79%	1.22	11.76%	0.97	22.22%	1.19	6.67%	0.98
SP1652	21.05%	1.12	6.25%	1.02	22.22%	0.88	13.33%	0.92
SP2108	15.79%	1.36	11.76%	0.95	22.22%	1.27	13.33%	0.86
SP0079	7.14%	1.02	18.75%	1.18	12.50%	1.09	0.00%	0.95
SP0402	10.53%	1.21	5.88%	1.08	22.22%	1.11	6.67%	0.95
SP0629	26.32%	1.26	11.76%	1.02	33.33%	1.33	13.33%	0.86
SP0757	21.05%	1.00	5.88%	1.09	22.22%	0.87	13.33%	0.82
SP0785	10.53%	1.28	11.76%	1.07	33.33%	1.26	13.33%	0.90
SP0787	16.67%	0.87	5.88%	0.98	22.22%	1.04	6.67%	0.94
SP0899	12.50%	0.93	18.75%	1.16	28.57%	1.63	6.67%	0.82
SP1386	11.76%	1.10	6.25%	1.15	22.22%	1.13	6.67%	0.92
SP2145	23.08%	0.89	28.57%	1.09	0.00%	1.21	0.00%	0.86
SP1897	10.53%	1.28	5.88%	1.02	0.00%	0.55	0.00%	1.15
SP0662-1	21.05%	1.00	6.25%	1.02	0.00%	0.65	0.00%	1.00
SP0662-2	11.11%	0.83	33.33%	1.16	0.00%	0.71	7.14%	1.04
SP1683	21.05%	0.79	11.76%	1.03	0.00%	0.58	6.67%	0.86
SP0369	15.79%	1.22	11.76%	1.08	0.00%	0.60	13.33%	1.10
SP1154-2	16.67%	1.13	5.88%	0.85	44.44%	1.21	0.00%	0.87
SP0878	21.05%	1.37	5.88%	0.86	44.44%	1.30	6.67%	0.91
SP0249	9.52%	1.04	11.76%	1.00	33.33%	1.56	13.33%	0.94
SP0582	10.00%	0.92	5.88%	0.97	33.33%	1.21	6.67%	0.91
SP0648-3	16.67%	1.33	5.88%	0.85	44.44%	1.27	6.67%	0.92
SP1376	14.29%	1.03	5.88%	0.95	33.33%	1.39	6.67%	0.90
SP0724	17.65%	1.24	14.29%	1.15	33.33%	1.27	6.67%	0.99
SP2151	10.00%	0.74	11.11%	0.73	33.33%	0.57	7.14%	0.84
SP0336	27.78%	1.54	11.76%	0.97	33.33%	1.22	7.14%	0.98
SP1500	14.29%	1.25	11.76%	0.96	44.44%	1.17	7.14%	0.82
SP2083	10.00%	1.12	5.88%	0.98	33.33%	1.22	7.14%	0.96
SP1833	14.29%	0.94	5.88%	0.88	33.33%	1.34	7.14%	0.78
SP0043	19.05%	1.02	5.88%	0.98	33.33%	1.13	7.14%	0.91
SP0098	14.29%	1.15	5.88%	0.89	44.44%	1.38	7.14%	0.92
SP0127	11.11%	1.03	5.88%	0.98	33.33%	1.22	7.14%	0.91
SP0435	23.81%	1.18	11.76%	0.90	55.56%	2.16	7.14%	0.94
SP0604	9.52%	0.96	5.88%	0.96	55.56%	2.08	7.14%	0.96
SP0648-1	26.32%	1.19	5.88%	0.82	44.44%	1.30	7.14%	0.76
SP0148	21.05%	1.00	5.88%	0.91	44.44%	1.67	14.29%	0.93
SP2207	19.05%	0.84	5.88%	1.04	55.56%	2.64	7.14%	0.99
SP1002	14.29%	1.06	5.88%	0.88	44.44%	1.57	7.14%	0.89

SP0742	8.33%	1.07	10.00%	0.95	33.33%	1.44	14.29%	0.93

3.4.3 Colonisation density of Spn6B did not significantly correlate with anti-protein IgG response

An external statistician generated area under the curve data for the volunteer cohort experimentally colonised with Spn6B, denoting colonisation density throughout the study period. No significant correlation was found between anti-protein IgG at baseline and total colonisation density across the study duration (area under the curve) or duration of carriage in volunteers colonised with Spn6B for any of the proteins (significance was not found even prior to Bonferroni correction and all correlations coefficients, except for SP0757, were <0.4). SP0757 showed a weak association with decreased carriage duration (-0.42, p = 0.068 prior to Bonferroni correction). Following Bonferroni correction for multiple comparisons, the analysis also did not find any significant correlation between density of Spn6B in the nasopharynx of volunteers and fold change in anti-protein serum IgG 29 days post-challenge. Prior to Bonferroni correction, 8 proteins (PspA1, PspA4, PspC9, SP0314, SP0662-1, SP0757, SP1404 and SP1534) did elicit significant correlation between increased colonisation density and increased protein-specific IgG response, although this was generally modest. These proteins, with correlation coefficients and unadjusted p-values, are given in Table 14. Appendix G shows correlation coefficients for all proteins with unadjusted and adjusted p-values. No proteins showed a significant correlation between increased protein-specific IgG and decreased colonisation density, even before multiple correction. However, SP0742 did elicit a weak association with decreased colonisation density (-0.43; p = 0.16). Figure 35 shows correlation of log fold change in protein-specific IgG with total colonising density; proteins with a correlation coefficient ≥0.4 or ≤-0.4 are highlighted in the volcano plot.

Table 14: Significant correlation prior to Bonferroni correction between colonising density of Spn6B (n = 21) in the nasopharynx of volunteers intranasally inoculated with the bacteria and fold change in protein-specific serum IgG (between baseline and 29 days post-inoculation) was observed for PspA1, PspA4, PspC9, SP0314, SP0662-1, SP0757, SP1404 and SP1534. Significance was lost following adjustment of *p*-values and correlation was generally modest. CI denotes confidence interval.

Protein	Lower CI	Upper CI	Estimate	p-value (unadjusted)
PspA1	0.06	0.75	0.48	0.02
PspA4	-0.004	0.73	0.43	0.05
PspC9	0.03	0.74	0.45	0.04
SP0314	0.01	0.73	0.44	0.05
SP0662-1	0.03	0.77	0.48	0.04
SP0757	0.06	0.78	0.50	0.03
SP1404	0.08	0.76	0.49	0.02
SP1534	0.20	0.83	0.60	0.01

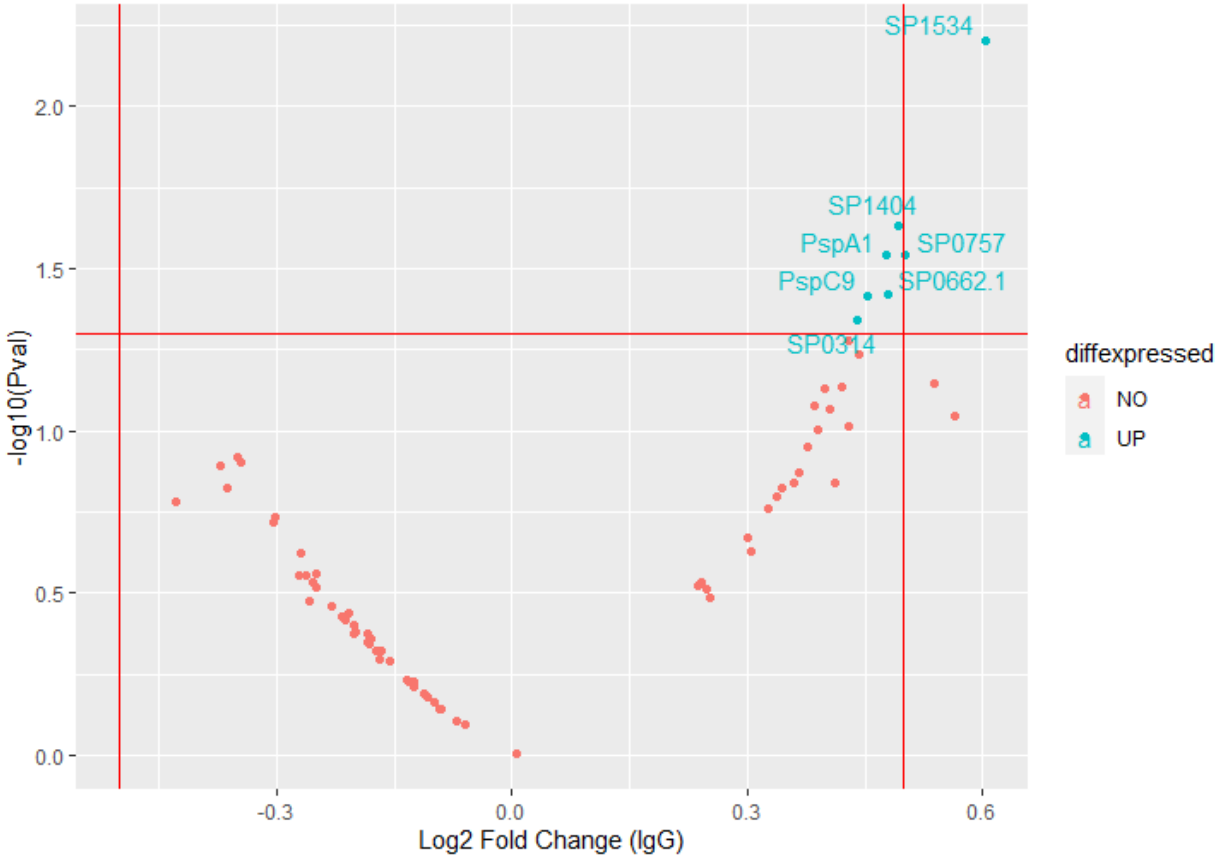


Figure 35: Correlation of log fold change in protein-specific serum IgG for a library of pneumococcal proteins following experimental colonisation with *S. pneumoniae* serotype 6B and colonisation density of the bacteria. Fold change in circulating anti-protein IgG (between baseline and 29 days post-inoculation) is correlated with total pneumococcal carriage density (area under the curve) in the nasopharynx of healthy volunteers (aged 18-50)

experimentally inoculated with *S. pneumoniae* serotype 6B, determined by nasal wash and classical microbiology at days 6, 9, 14 and 27 post-inoculation. Proteins with a correlation coefficient ≥0.4 or ≤-0.4 and a *p*-value <0.05 prior to Bonferroni correction are highlighted in blue. Red lines indicate a correlation coefficient of 0.5 or -0.5 and a *p*-value of <0.05 prior to Bonferroni correction for multiple comparisons. No significant correlation following Bonferroni correction between carriage density and increased anti-protein IgG for any protein was observed.

In similarity, following Bonferroni correction there was no significant correlation between carriage duration and increase in anti-protein serum IgG. Prior to multiple correction, increased IgG against 13 proteins, shown in Table 15, significantly correlated with longer carriage duration. Again, no proteins showed a significant correlation between increased protein-specific IgG and decreased carriage duration, even before multiple correction. However, SP0742 also elicited a weak association with decreased carriage duration (-0.52; $p = 0.08$). Figure 36 shows correlation of log fold change in protein-specific IgG with carriage duration; proteins with a correlation coefficient ≥0.4 or ≤-0.4 and a *p*-value <0.05 prior to Bonferroni correction are highlighted in the volcano plot. Appendix H shows correlation coefficients for all proteins with unadjusted and adjusted *p*-values.

Table 15: Significant correlation prior to Bonferroni correction between nasopharyngeal carriage duration of Spn6B (n = 21) in the nasopharynx of volunteers intranasally inoculated with the bacteria and fold change in protein-specific serum IgG (between baseline and 29 days post-inoculation) was observed for listed proteins). Significance was lost following adjustment of *p*-values and correlation was generally modest. CI denotes confidence interval

Antigen	Lower CI	Upper CI	Estimate	*p*-value	Adjusted *p*-value
PspA1	0.07	0.76	0.49	0.03	1
PspA4	0.11	0.77	0.51	0.02	1
SP0314	0.26	0.83	0.62	0.008	0.19
SP0648.2	0.20	0.81	0.58	0.01	0.38
SP0659	0.12	0.80	0.54	0.02	1
SP0757	0.05	0.77	0.49	0.03	1
SP0899	0.01	0.80	0.50	0.05	1
SP1404	0.01	0.73	0.44	0.05	1
SP1534	0.15	0.81	0.57	0.01	0.80
SP1683	0.04	0.77	0.49	0.03	1
SP1826	0.02	0.74	0.45	0.04	1
SP1872	0.23	0.82	0.60	0.004	0.29
SP1897	0.01	0.76	0.46	0.05	1

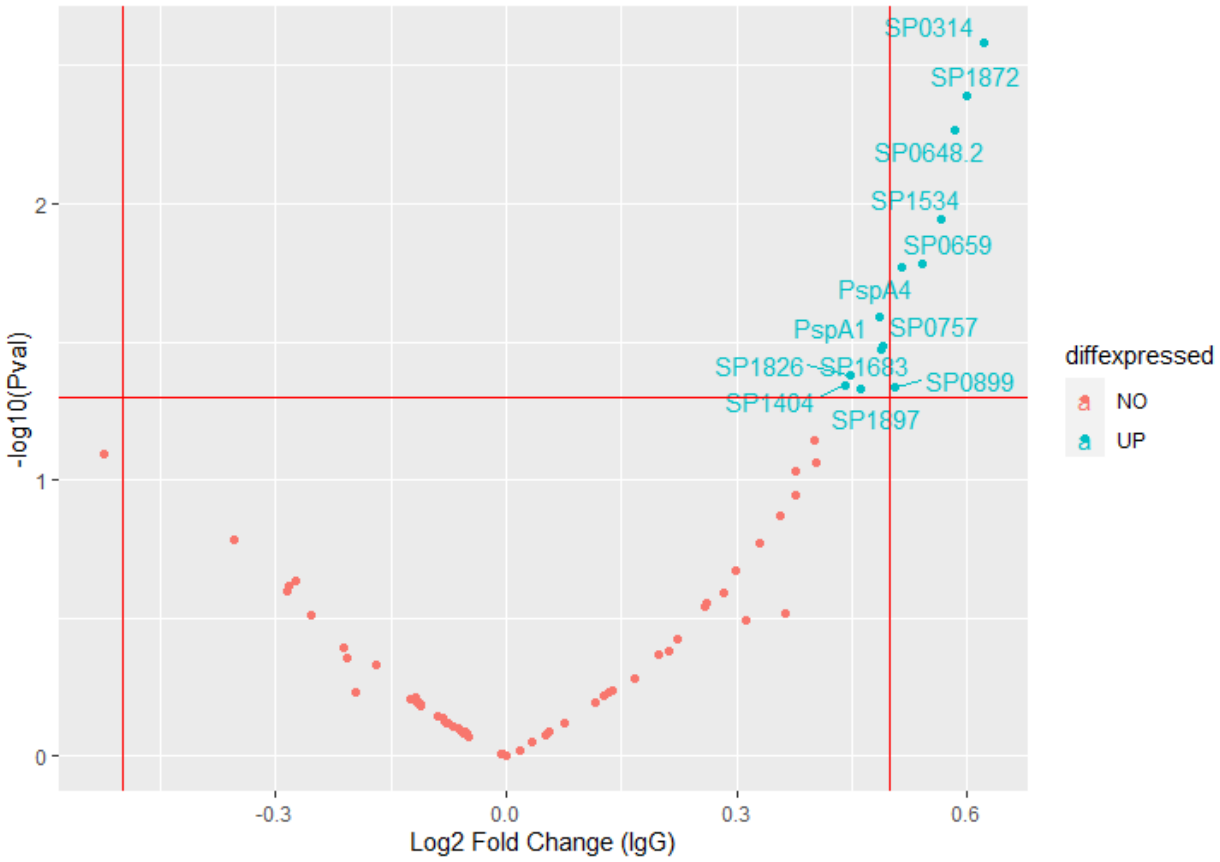


Figure 36: Correlation of log fold change in serum protein-specific IgG for a library of pneumococcal proteins following experimental colonisation with *S. pneumoniae* serotype 6B and pneumococcal carriage duration. Fold change in circulating anti-protein IgG (between baseline and 29 days post-inoculation) is correlated with pneumococcal carriage duration in the nasopharynx of healthy volunteers (aged 18-50) experimentally inoculated with *S. pneumoniae* serotype 6B, determined by nasal wash and classical microbiology at days 6, 9, 14 and 27 post-inoculation. Proteins with a correlation coefficient ≥0.4 or ≤-0.4 and a p-value <0.05 prior to Bonferroni correction are highlighted in blue. Red lines indicate a correlation coefficient of 0.5 or -0.5 and a p-value of <0.05 prior to Bonferroni correction for multiple comparisons. No significant correlation following Bonferroni correction between carriage durations and increased anti-protein IgG for any protein was observed.

3.5 Discussion

Baseline circulating IgG against any of the pneumococcal proteins examined did not correlate with protection against experimental colonisation with Spn6B or Spn15B. This observation is consistent with previous finding that anti-protein antibodies at baseline do not correlate with protection against carriage following experimental exposure to serotype 6B pneumococcus in young or older adults (Ferreira *et al.*, 2013; Adler *et al.*, 2021; Araujo *et al.*, 2021).

Previous studies using an experimental human model of colonisation have shown that higher levels of circulating anti-PspA IgG correlate with protection against acquisition of Spn23 pneumococcus (the strain from which the PspA was isolated) but not against acquisition of Spn6B pneumococcus (McCool

et al., 2002). It is postulated that the high variability of the N-terminal region of PspA renders the correlation of preexisting anti-PspA IgG and susceptibility to carriage strain-specific. However, in agreement with the findings of our study, a study conducted by Araujo and colleagues, in which levels of serum IgG against PspA and PspC variants were measured before and after experimental challenge with Spn6B in volunteers aged 18–30 and 50–70+, found no protection was conferred by baseline levels of serum IgG against PspA clades expressed by the challenge strain (Araujo *et al.*, 2021). This was also the case for PspC, which has been shown to confer protection against pneumococcal colonisation in infants (Zhang, Bernatoniene, Bagrade, Pollard, *et al.*, 2006; Lebon *et al.*, 2011; Mendy *et al.*, 2017). However, in human challenge studies, baseline serum antibodies against PspC expressed by the challenge strain of pneumococcus have been shown to confer no protection against experimental colonisation, in corroboration with the findings of the current study (McCool *et al.*, 2003; Araujo *et al.*, 2021).

Whilst there was a tentative, non-significant trend that participants protected against experimental colonisation with Spn6B had higher baseline levels of anti-SP0742 and anti-SP0620 IgG as compared to participants susceptible to colonisation, both these proteins displayed low IgG titres. SP0742, a putative lipid binding protein, which might be involved in lipid transport and fatty acid metabolism, has previously been shown to correlate with IL-17A-dependent protection against nasopharyngeal pneumococcal colonisation in mice and induce an IL-17A response in human adenoidal mucosal cells (Lu *et al.*, 2018). SP0742 was also the only protein in the library that noticeably correlated with both decreased carriage duration and density in carriage positive volunteers colonised with Spn6B, although this was not significant. SP0620 is an amino acid ABC transporter, a class of proteins known to be promising targets for the development of antibacterial vaccines due to their vital role in nutrient uptake (Garmory and Titball, 2004). Other pneumococcal ABC transporters, such as those involved in the transport of iron (PiaA and PiuA) or manganese (PsaA), are established pneumococcal protein vaccine candidates (Brown *et al.*, 2001; Gor *et al.*, 2005; Entwisle *et al.*, 2017). Although not significant, IgG against PdB, SP2099, SP1833 and PspA1 appeared to be higher in volunteers protected against experimental colonisation with Spn15B; there was no trend for these proteins in those protected against colonisation by Spn6B. SP2099 is a penicillin binding protein and SP1833 is a calcium-binding cell wall surface anchor protein. Pneumolysin (Ply) has been shown to confer antibody-mediated protection against pneumococcal acquisition in infants (Holmlund *et al.*, 2006; Zhang, Bernatoniene, Bagrade, Pollard, *et al.*, 2006), although there is contrasting evidence surrounding the protective effect of anti-pneumolysin IgG (Mendy *et al.*, 2017). Pneumolysin is also a component of multiple trial vaccine formulations (Kaur *et al.*, 2014; Entwisle *et al.*, 2017; Chan *et al.*, 2019). However, as previously mentioned, in a phase II trial of a

trivalent vaccine comprising recombinant PcpA, PhtD, and pneumolysin, low levels of protection were conferred against nasopharyngeal pneumococcal carriage in Gambian infants, despite immunogenicity (Odutola *et al.*, 2017).

Work in mouse models found that intraperitoneal or subcutaneous immunisation with PdB in alum adjuvant, although inducing functional anti-PdB IgG response, had no effect on mouse survival in subsequent invasive pneumonia or bacterial density in the lungs, but did significantly reduce colonisation density in the nasopharynx after challenge (Neill *et al.*, 2013). Immunised mice also elicited a significant increase in IgG-producing B-cell numbers in the draining cervical lymph nodes 7 days post-challenge as compared to control mice. However, most intriguingly, subcutaneous immunisation with PdB/alum followed by establishment of carriage 14 days post-immunisation and subsequent intravenous challenge 21 days post-colonisation generated 80% survival in PdB-immunised mice compared to 50% survival in non-immunised, colonised mice. Strikingly, PdB-immunised but non-colonised mice, along with naïve mice, showed lowest survival (30%). PdB immunisation also conferred significant protection against sepsis, defined as reduced bacterial numbers in the blood, as compared to colonisation alone. PdB-induced protection was shown to be mediated by cellular immune response. Thus, PdB immunisation elicited significant protection against invasive disease in the context of low-level nasopharyngeal carriage, which augmented the immunising effect of PdB. The authors postulated that either immunisation with PdB primes the immune response, which is boosted by nasopharyngeal pneumococcal carriage or, more likely, carriage and PdB-immunisation induce different immune responses (humoral and cellular) that combine to confer protection against invasive disease. Importantly, these data suggest that elimination of nasopharyngeal carriage by vaccination might not be the most desirable outcome, but instead, maintenance of nasopharyngeal carriage with low density after vaccination might serve to reinforce vaccine-induced immunity.

As previously discussed, the multiple colonising events that occur in childhood, resulting in a protein-mediated adaptive immune response (Wilson *et al.*, 2015; Ramos-Sevillano, Ercoli and Brown, 2019), suggest that immune response against several proteins across various serotypes is developed over time rather than being serotype specific. Thus, it is likely that to observe a protective effect of anti-protein IgG against colonisation, a response against multiple proteins would be needed to confer serotype-independent protection.

A recent study in mice found that protection against pneumococcal colonisation conferred by IgG response to pneumococcal proteins may be blocked by the capsule, even regarding surface exposed

proteins (Zangari *et al.*, 2021). In mice, no protection against pneumococcal colonisation following immunisation with five pneumococcal proteins was observed, despite induction of effective anti-protein IgG. However, protection was conferred in the absence of a capsule, in which increased antibody binding to the pneumococcal surface was observed. Thus, it is possible that the lack of protection against experimental carriage acquisition conferred by preexisting anti-protein IgG in human models could be due to interference of the capsule. Higher titres of anti-protein IgG induced by immunisation with vaccines may overcome this issue, although this is unclear. It could be hypothesised that variations in capsule thickness of different serotypes interferes with the immunising effect of preexisting anti-protein IgG during experimental colonisation. Further work is needed to ascertain the effect, if any, of the different pneumococcal capsules on protein-mediated immune response against colonisation or infection in humans.

It is highly important to note that carriage is a measurable endpoint that can be ethically tested for in human models but in practice, protection against carriage may not be the desired endpoint for a protein-based vaccine. Instead, protection against severe disease, particularly pneumonia in this instance, may be a more likely primary goal. Rather than a categorical outcome of carriage positive or negative, a vaccine might aim to reduce colonisation density and/or duration of carriage, which as previously discussed, might augment immune response to a protein-based vaccine (Neill *et al.*, 2013). In this work, we also did not find any significant correlation between increased anti-protein IgG and decreased carriage duration or density. Despite a lack of strong protein correlates of protection against carriage acquisition, duration, or density, we cannot ascertain from our human challenge model whether anti-protein response is protective against disease. Higher antibody levels may be required for mucosal protection against *S. pneumoniae* colonisation as compared to protection against systemic infection, pneumonia or mucosal disease (Pelton *et al.*, 2003; Bogaert *et al.*, 2004). Indeed, it has been shown that higher mucosal IgG titres against PcpA, and IgA to PhtD, PcpA and PlyD1 correlate with reduced risk of acute otitis media infection but not with reduced risk of nasopharyngeal colonisation in young children (Xu *et al.*, 2017). In addition, at the time of pneumococcal exposure in the EHPC model, levels of naturally-acquired circulating or mucosal anti-capsular IgG were also not associated with protection against pneumococcal acquisition (Pennington *et al.*, 2016). However, it is known that high levels of capsular antibodies at the nasal mucosa, such as those induced by vaccination with PCVs, are responsible for pneumococcal agglutination and control of carriage (Roche *et al.*, 2015; Mitsi *et al.*, 2016) and may be responsible for the protection conferred by PCVs against pneumococcal carriage acquisition as well as the effect on reducing bacterial density in the nasopharynx (Collins *et al.*, 2015; German *et al.*, 2019). It could be hypothesised that if

higher levels of systemic and/or mucosal anti-protein IgG are induced by pneumococcal carriage in the nasopharynx or mucosal vaccination, those antibodies may provide protection against a subsequent acquisition episode or result in reduced carriage density and duration due to antibody-mediated agglutination of the bacteria and subsequent mucociliary clearance. Future work may involve optimising the Luminex assay described here for detection of IgG in nasal wash samples, which could enable measurement of anti-protein IgG at the nasal mucosa and the potential discovery of correlates of protection against colonisation in this niche (Wright *et al.*, 2012; Xu, Casey and Pichichero, 2015).

A significant increase in IgG response to PspA1, PspC9, SP1032 and SP0346 was detected in the cohort colonised with Spn6B 29 days post-inoculation. The increase in anti-PspC6 post-colonisation showed borderline significance. A ≥2-fold increase in median IgG was also elicited against SP1942 (a putative transcription regulator) and SP0620 (ABC transporter) following colonisation with Spn6B, although anti-SP0620 IgG titres were low overall. No significant change in anti-protein IgG was observed in carriage negative volunteers 29 days post-challenge in the cohort challenged with Spn6B. No significant difference was observed in anti-protein IgG response 14 days post-challenge with Spn15B in either the carriage positive or carriage negative cohorts. A ≥2-fold increase in median IgG against SP0435 (translation elongation factor P), SP0604 (histidine kinase sensor) and SP2207 (a putative competence protein) was generated in response to Spn15B colonisation, but again, overall IgG levels were low. Notably, SP0435 has been shown to elicit a robust IL-17A response in mice and mice intranasally immunised with a mixture of 4μg SP0435, SP1534 and SP2070 with 1μg cholera toxin as adjuvant were significantly protected against colonisation compared to mice that received adjuvant alone (Moffitt, Malley and Lu, 2012). Findings that challenge in the absence of carriage does not generate a systemic IgG response are consistent with previous findings that, while mucosal exposure without subsequent development of carriage may generate anti-protein antibody response at mucosal surfaces, mucosal exposure alone does not generate the systemic IgG response elicited by experimental colonisation (Wright *et al.*, 2012).

Increases in anti-PspA and anti-PspC titres following experimental colonisation are consistent with previous findings that these proteins are immunogenic (McCool *et al.*, 2003; Holmlund *et al.*, 2009; Collins *et al.*, 2013; Ferreira *et al.*, 2013). It is important that a significant increase in anti-PspA and anti-PspC IgG was only observed following colonisation with Spn6B but not Spn15B. As Luminex microspheres were coated with PspA and PspC expressed by the Spn6B strain used in the human challenge model, these results might suggest that IgG induced in response to these proteins may be strain-specific. Colonisation with Spn6B elicited an increase in IgG against PspA1, expressed by the colonising strain, but antibodies

were not cross reactive with PspA4, which is consistent with reports that antibodies against family 1 PspA are only effective against family 1 bearing strains but not family 2 bearing strains (Darrieux *et al.*, 2008; Moreno *et al.*, 2010). These data further substantiate that any protein-based vaccine comprising PspA, should contain functional fragments from PspA families 1 and 2 to induce humoral response with broad coverage (Darrieux *et al.*, 2007, 2008). However, in animal models, immunisation with a DNA vaccine expressing the complete N-terminal regions of PspA fragments from families 1 and 2 did not correlate with cross-protection against intraperitoneal challenge (Miyaji *et al.*, 2002). Protection was restricted to immunisation with a DNA vaccine expressing a complete N-terminal PspA fragment belonging to the same clade as the strain used for the challenge. Contrastingly, passive transfer of human antibodies elicited by a family 1 PspA to mice protected against fatal infection with *S. pneumoniae* expressing either family 1 or 2 PspA clades (Briles *et al.*, 2000). Protection against fatal challenge with six pneumococcal strains expressing three different capsular serotypes was also conferred. Examination of potential vaccine combinations of five PspA clades found that a vaccine containing PspA3 + PspA2 conferred increased protection than did a combination of PspA2 + PspA4 and PspA2 + PspA5 in terms of a broad range of cross-reactivity with clinical isolates from adult IPD patients and cross-protection against pneumococcal challenge in mice (Piao *et al.*, 2014). It has been suggested that pneumococcal exposure primes the human immune system to generate anti-PspA antibodies with broad cross-reactivity (Nabors *et al.*, 2000). Previously, immunisation of humans with recombinant PspA2 induced increased binding of post immune sera to 37 pneumococcal strains expressing a variety of PspA clades (Nabors *et al.*, 2000). A PspA hybrid protein composed of the B region (or clade defining region) from all clades in addition to conserved domains within the entire N-terminal region of clades 1-4, could provide enhanced protection against *S. pneumoniae* by increasing cross-reactivity (Darrieux *et al.*, 2008; Vadesilho *et al.*, 2014; Akbari *et al.*, 2019). A limitation of this study is that the PspA and PspC clades expressed by the Spn15B were not used in this study, thus it cannot be ascertained whether lack of induction in anti-PspA and anti-PspC IgG response by Spn15B colonisation is due to expression of different protein families than were screened against or reduced immunogenicity of the strain.

SP1032, also known as PiaA (pneumococcal iron acquisition ABC transporter), has been shown to confer protection against pneumococcal nasopharyngeal colonisation and systemic infection in mice and induce IL-17A production in adenoidal cells of children (Brown *et al.*, 2001; Lu *et al.*, 2018). Furthermore, the protein is immunogenic from early infancy and has been shown to be clinically relevant, inducing IgG in acute and convalescent-phase patients with pneumococcal septicemia (Whalan *et al.*, 2005). Even more importantly, the induction of anti-SP1032 IgG in patients with pneumococcal septicemia is serotype-

independent as it was detected in patients with invasive disease caused by 13 different serotypes. SP1032 is thus a highly promising protein-vaccine candidate. Nevertheless, further work is needed to assess the level of protection conferred by the protein. Moreover, the SP1032 gene has been found to be required for invasive *S. pneumoniae* infection in wild-type mice but dispensable in mouse models of sickle cell disease (Carter *et al.*, 2014). The protein was also found to be extremely divergent in isolates from paediatric patients with sickle cell disease. Thus, although potentially protective in the general population, SP1032 might be ineffective at protecting high-risk groups such as those with sickle cell disease. PiaA is a component of a novel protein-based vaccine, PnuBioVax, that has been shown to be safe and immunogenic in Phase I trials and will soon undergo Phase II testing (Entwisle *et al.*, 2017). A ≥2-fold increase in antibody titres against PiaA was observed for most participants following immunisation with the vaccine.

SP0346, also known as Cps4A (Capsular polysaccharide biosynthesis protein), plays a vital role in capsule synthesis (Shainheit, Mulé and Camilli, 2014; Nakamya *et al.*, 2018) and the *Cps4A* gene has been found to be one of the most highly expressed pneumococcal genes in nasopharyngeal samples of healthy, colonised children in Peru (Sakai *et al.*, 2013). In a murine model of infection, SP0346 transcription was significantly upregulated during pneumonia and bacteraemia, suggesting that this protein plays a role in disease pathogenesis and thus may be particularly relevant in designing a novel vaccine against pneumonia (Gupta, Shah and Swiatlo, 2009). Cps4A expression is down-regulated in response to exposure to penicillin, which may elicit protection against antibiotics (Rogers *et al.*, 2007).

Using the PneumoExpress tool (https://veeninglab.com/pneumoexpress) developed by the Veening Lab (Aprianto *et al.*, 2018), the level of gene expression for the proteins PspA, PspC, SP1032, SP0346, SP1942, SP0620, SP0435, SP2207 in various conditions mimicking relevant biological environments was investigated. Of these proteins, PspA, PspC, SP0620 and SP1032 showed highest normalized expression values. All proteins except for SP2207 and SP1942, which together with SP0435 and SP0346 showed low levels of expression in comparison to the other proteins, had increased expression at competence timepoints, particularly 20 minutes after the exogenous addition of competence stimulating peptide-1 (CSP-1) in C+Y medium. As might be expected, the expression of both PspA and PspC increased during incubation with a human epithelial cell line, mimicking interaction with the host. PspA expression increased from 30 minutes post-incubation with epithelial cells and PspC expression increased immediately upon incubation (with expression of both proteins remaining elevated thereafter). PspC expression also rose in conditions mimicking the lung and migration from the nose to

cerebrospinal fluid (CSF). SP0620 expression increased in conditions mimicking translocation from the nose to the lung and the nose to the blood, potentially suggesting a role in invasion. SP0346 expression was also increased in conditions mimicking the nose (colonisation) and lung (pneumonia) as well as migration from the nose to the lung and blood. The different expression levels of proteins at different timepoints during infection and in various niches are an important consideration when designing vaccines to protect against pneumococcal disease, particularly if protection against carriage is difficult to achieve. Proteins which are known to play a role in pathogenesis and generate a robust systemic IgG response following colonisation are desirable vaccine candidates. On the other hand, proteins with lower levels of expression during colonisation but higher levels of expression during disease may not have induced a robust systemic IgG response in EHPC volunteers following colonisation in our model. Systemic anti-protein IgG profiles following a pneumonia episode for example may provide a different perspective on which proteins are immunogenic during disease and potentially protective against a subsequent disease episode. Whilst this may be beyond the scope of the EHPC model to determine, optimisation of the Luminex assay to detect anti-protein IgG in bronchoalveolar lavage of colonised EHPC participants could enable the detection of relevant immunogenic proteins within the lung environment prior to lung infection following microaspiration of nasopharyngeal pneumococci into the lung (Mitsi *et al.*, 2020).

It is of note that no increase in anti-pneumolysin (PdB) IgG was detected following colonisation, despite previous findings that this protein is immunogenic during colonisation or infection in children (Rapola *et al.*, 2000). Antibodies against pneumolysin and various other proteins in the library were detected at baseline in our volunteers, thus response to these proteins has likely been generated by past exposure. The lack of immunogenicity following experimental colonisation detected for the less variable proteins in the library, such as pneumolysin, is unlikely to be explained by strain-to-strain diversity as is the case with PspA and PspC. IgG response to experimental pneumococcal challenge in adults who may have had multiple past exposure events could be less prominent for certain proteins than that which may be observed in children with fewer exposure events. Notably, in a previous study in adults it was found that no increase in circulating PdB-specific plasmablasts occurred in colonised individuals 10 days post-experimental challenge with Spn6B in the presence of carriage (Jochems *et al.*, 2019).

Another limitation of this study is that post-challenge serum samples from volunteers inoculated with Spn15B were taken 14 days post-challenge compared to 29 days-post challenge in the Spn6B cohort. It is unclear whether a more robust anti-protein IgG response may have been detected at a later timepoint in volunteers colonised with Spn15B, although significant systemic anti-protein IgG response to

colonisation has previously been detected at 14 days post initial challenge (Ferreira *et al.*, 2013). Mucosal anti-PspA IgG response to colonisation with Spn6B however has been shown to reach significantly higher levels as compared to baseline only at 28 days post-challenge but not at 14 days post-challenge (Ferreira *et al.*, 2013). Previous work by Ferreira and colleagues also found that challenge in the absence of subsequent carriage mounted an increase in IgG against several proteins at 14 days post-challenge, though this was much less marked than response induced in the presence of carriage. Whilst this response did not persist much beyond 14 days post-challenge in carriage negatives as it did in carriage positives (up to five weeks post-challenge), these results suggest that challenge alone might have a minor immune boosting effect in the short-term, whereas no immune boosting effect was observed in response to challenge with Spn15B 14 days post-challenge in this study. Nevertheless, as previously discussed in Chapter 2, these previous results were obtained using MSD technology, which is more sensitive than Luminex and may have detected more minor increases in IgG that Luminex was unable to detect. Indeed, the fold-change in IgG levels from baseline found to be significant at 14 days post-challenge in the study conducted by Ferreira and colleagues was less pronounced than that found to be significant in the current study at 29 days post-challenge. Short-term IgG boosting in carriage negative volunteers is likely to be generated by differentiation of memory B-cells into short-lived plasma cells (Tangye *et al.*, 2003; Pennington *et al.*, 2016; Jochems *et al.*, 2019). The increase in antibody titres is rapidly waning, persisting only in the acute phase post-challenge. Furthermore, previous results were obtained in response to experimental inoculation with Spn6B and so it is unclear whether similar results would be observed with a different serotype. A possible explanation for differences between IgG response to Spn15B and Spn6B in this work is that the sample size of the cohort inoculated with Spn15B was smaller than the Spn6B cohort; a larger sample size may have found a more significant change in anti-protein IgG response to Spn15B. However, it remains to be determined why Spn15B elicited a less marked protein-based humoral response than did Spn6B.

No significant correlation was found between anti-protein IgG at baseline and total colonisation density across the study or duration of carriage in volunteers colonised with Spn6B for any of the proteins. Higher levels of anti-SP0757 IgG at baseline showed a weak association with decreased carriage duration. Increased anti-SP0757 IgG post-colonisation was associated with prolonged carriage duration. It has previously been hypothesised that pneumococcal colonisation is necessary for the development of a sustained serotype-specific plasma cell response in circulation and the continued immune stimulation and antibody production elicited by carriage is what leads to the detection of elevated serum capsule-specific IgG as bacteria are cleared from the nasopharynx (Pennington *et al.*, 2016). Longer carriage duration and

higher carriage density may provide sustained immune stimulation, leading to elevated systemic anti-protein IgG, which would explain this seemingly confounding observation that elevated anti-protein IgG correlates with longer duration and increased density of carriage. An increase in levels of anti-SP0742 IgG in response to carriage weakly associated with both decreased carriage duration and bacterial density in the nasopharynx. It is plausible that clusters of proteins, rather than single proteins, may together correlate with control of carriage, however this is yet to be determined. Future machine learning work within our group aims to assess whether hierarchical clustering of proteins shows a clearer correlation between anti-protein IgG and decreased duration and density of carriage.

Further work is needed to ascertain the level of protection conferred by immunogenic proteins against re-acquisition of *S. pneumoniae* or pneumococcal disease. It is conceivable that a cumulative effect of multiple antibodies to several pneumococcal proteins may confer protection against carriage.

3.6 Conclusion

In conclusion, baseline IgG responses to single protein antigens did not predict protection against experimental colonisation in this study. Five proteins elicited significantly higher antibody levels 29 days post-colonisation with Spn6B as compared to before challenge, but this was not observed 14 days post-colonisation with Spn15B. Challenge is the absence of subsequent colonisation did not induce increased levels of anti-protein IgG in both cohorts. A significant decrease in pneumococcal carriage and density was not elicited by increased anti-protein IgG for any single protein. Further work is needed to determine whether increased anti-protein IgG following experimental pneumococcal colonisation is protective against re-acquisition of the bacteria.

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Chapter 4

Investigating protein-mediated memory B-cell response

4.1 Abstract

Induction of memory B-cell response may be a better predictor of long-term protection against nasopharyngeal acquisition of pneumococcus compared with serological measurements. Peripheral blood mononuclear cells (PBMCs) of volunteers taken before and 29 days after experimental challenge with serotype 6B pneumococcus were cultured and used to evaluate IgG^+ memory B-cell responses to six selected proteins and the PnuBioVax vaccine (ImmunoBiology Ltd) by ELISpot. Baseline B-cell responses to any single antigen did not correlate with protection against colonisation. A trend was detected between PnuBioVax and protection against experimental colonisation, although this was not significant. Colonisation associated with significantly increased B-cell responses to PnuBioVax and SP0346 29 days post-challenge, whereas there were no significant increases in B-cell responses in non-colonised volunteers. This is one of the first studies investigating potential serotype-independent memory B-cell response to pneumococcus. Further work is needed to ascertain what level of protection is conferred against homologous or heterologous challenge with pneumococcus following the induction of protein-specific memory B-cell response.

4.2 Introduction

Following the identification of immunogenic proteins (Chapter 3), which stimulate significantly increased systemic anti-protein IgG 29 days post colonisation with Spn6B, a cultured B-cell ELISpot assay was set up to assess memory B-cell response to selected proteins (PspA1, SP1032, SP0346) before and after challenge with Spn6B. In addition, three further proteins (SP1683, SP2099, SP1154-2) were selected based on other work carried out within the EHPC team at the Liverpool School of Tropical Medicine investigating cellular responses to pneumococcal protein antigens. It is conceivable that induction of memory B-cell response is a better predictor of long-term protection against nasopharyngeal acquisition of pneumococcus compared with serological measurements (Pennington *et al.*, 2016; Papadatou, Tzovara and Licciardi, 2019).

B-cell responses against the PnuBioVax™ (PBV) vaccine developed by ImmunoBiology Ltd (ImmBio), Cambridge, UK were also tested. The PBV vaccine is being developed as a multi-protein antigen, serotype-independent prophylactic vaccine against *S. pneumoniae* disease. The vaccine is produced as

detailed in (Cecchini *et al.*, 2015). The source strain (TIGR4 B7.1) used to produce PBV has been genetically modified to express a non-toxic Ply (PlyD6). TIGR4 B7.1 was grown in Hoeprich medium. The bacteria are stressed during fermentation, induced by a temperature shift from 30°C to 37°C, which is designed to mimic the translocation of *S. pneumoniae* from the nasopharynx to the circulatory system and thereby promote upregulation of proteins that may stimulate protective immune responses during infection and transition from a commensal to an invasive phenotype (Cecchini *et al.*, 2015; Entwisle *et al.*, 2017). Following detergent extraction, ion exchange chromatography is used to process the filtrate, enriching for surface exposed antigens. The manufacturing process was designed to generate a multi-antigen vaccine including key *S. pneumoniae* antigens as well as families of heat shock proteins, which act as natural adjuvants and facilitate the uptake of immunogenic peptides by dendritic cells for presentation to $CD4^+$ and $CD8^+$ T-cells (De Nagel and Pierce, 1991; Cao *et al.*, 2013; Colaco *et al.*, 2013; Chionh *et al.*, 2014). PBV was analysed using SDS-PAGE, Western blot and mass spectrometry. This vaccine manufacturing approach has multiple advantages. The presentation of multiple antigens facilitates a high level of efficacy against strain variants, antibiotic resistant strains, and newly emergent strains and proteins are appropriately presented to address human and pathogen diversity. Furthermore, adjuvants, or the addition of DC-targeting vehicles, are not required. Importantly, production costs are also low, essential for widespread accessibility (Cecchini *et al.*, 2015). The vaccine contains protein antigens PspA2, Ply, PsaA, PiaA (SP1032), PiuA, RrgB and RrgA, phosphoglycerate kinase, translation elongation factor G, translation elongation factor Tu, enolase, pyruvate oxidase, pyruvate kinase, glyceraldehyde 3-phosphate dehydrogenase, endopeptidase O, aminopeptidase N and lipoprotein as well as others (Cecchini *et al.*, 2015; Hill *et al.*, 2018).

PBV was recently licensed to the largest Chinese vaccine manufacturer, CNBG, part of Sinopharm, covering co-development and commercialisation of the vaccine in China (European Commission, 2020b). The vaccine has been tested in a phase I double-blind trial (trial registration number: NCT02572635) of 36 participants, in which no serious adverse events and only common systemic vaccine-related adverse events, such as mild injection site reaction, were observed. Trial participants were randomized to receive three doses of PBV, 28 days apart, at one of three dose levels (50µg, 200µg, 500µg or placebo). A statistically significant fold-increase in IgG titres compared to the 50µg starting dose and placebo was observed at each time-point post immunisation with 200µg or 500µg of PBV. A ≥2-fold increase in antibody titres against Ply, PsaA, PiaA, PspA and pilus proteins RrgB and RrgA was observed for most participants (Entwisle *et al.*, 2017). Strong responses were also elicited against the heterologous non-vaccine type pilus protein RrgB6B (serotype 6B protein sequence). Additionally, a robust antibody

response was elicited in rabbits in response to the PBV vaccine against PspA, Ply, PsaA and PiuA and the vaccine promoted killing of the vaccine pneumococcal strain, TIGR4, as well as heterologous strains (Spn6B, Spn19F and Spn15B) in an opsonophagocytic killing assay (Hill *et al.*, 2018). Agglutination of several pneumococcal strains was observed after incubation with sera from immunised rabbits and bacterial invasion of lung cells *in vitro* was inhibited. It is anticipated that manufacturing costs for PBV will be <€1 per dose (European Commission, 2020a). Should phase II and III clinical trials prove promising, this could mean an extremely cheap new pneumococcal vaccine is introduced to the market, greatly benefitting low- and middle-income countries in particular.

Peripheral blood mononuclear cells (PBMCs) of volunteers that were challenged with Spn6B as part of the aforementioned study (Chapter 2.3.3.1) in which volunteers were inoculated with 80,000 CFU Spn6B per naris (EudraCT number: 2014-004634-26) were used for this body of work. PBMCs were collected prior to intranasal inoculation and 29 days post-inoculation.

4.3 Materials and methods

4.3.1 Thawing and stimulation of peripheral blood mononuclear cells (PBMCs)

Previously frozen PBMCs were thawed as follows. Each cryovial containing 1 million donor PBMCs in 1ml CTL-CRYOABC medium was placed at 37°C for 10 minutes and inverted twice. Cells from each vial were transferred to a 15ml Falcon tube. Cells remaining in the cryovial were washed dropwise with 1ml pre-warmed (37°C) complete RPMI 1640 medium (RPMI 1640, catalogue number: 52440-041 + 10% foetal bovine serum (ThermoFisher Scientific) + 1% PNS) containing 1% deoxyribonuclease (DNase) (Sigma Aldrich) and added dropwise to the rest of the cells. 8ml of RPMI containing 1% DNase was added dropwise to the cells, which were then centrifuged at 400 x g for 10 minutes at room temperature. Cell supernatant was removed. A further wash with 10ml RPMI-DNase and centrifugation step was carried out and supernatant removed. Cells were then resuspended in complete RPMI 1640 medium to achieve a final concentration of 1 million cells per 1ml medium. 1 million cells were seeded per well of a 24-well plate (cell culture treated). Cells were then stimulated with a cocktail of 1µg/ml *Staphylococcus aureus* Cowan (SAC), 0.1µg/ml pokeweed mitogen and 3µg/ml class B CpG oligodeoxynucleotide (ODN) TLR-9 agonist for 7 days (incubated at 37^{o}C, 5% CO_2).

4.3.2 Preparation of Enzyme-linked Immunospot (ELISpot) Assay Plates

As previously mentioned, the protein antigens PspA1, SP1032, SP0346 were selected for this work because they stimulate significantly increased systemic anti-protein IgG 29 days post colonisation with

Spn6B (Chapter 3), SP1683, SP2099, SP1154-2 were selected based on other work carried out within the EHPC team at Liverpool School of Tropical Medicine investigating cellular responses to pneumococcal protein antigens (unpublished data). Sterile 96-well Multiscreen-IP filter plates with PVDF (Millipore) were pre-treated with 20µl of 35% ethanol for 1 minute followed by two washes with 200µl sterile H_2O (room temperature) as per manufacturer's instructions. Water was discarded and plate wells were coated (as depicted in Figure 37A) in triplicate (except for anti-human Ig, which was coated in quadruplet) with 100µl PBS containing either:

a) PBS only (negative control)
b) 10µg/ml of anti-human Ig polyvalent (positive control)
c) 2µg/ml PspA1
d) 5µg/ml PnuBioVax
e) 2µg/ml SP1683
f) 5µg/ml SP2099
g) 2µg/ml SP1032
h) 2µg/ml SP0346
i) 5µg/ml SP1154-2

Protein concentrations were determined following titration experiments to find which concentration elicited more immunospots. Plates were incubated at room temperature for 1 hour then overnight at 4°C. Cells were seeded onto plates following overnight incubation.

4.3.3 Blocking of ELISpot plates

Prior to cell seeding, ELISpot plates were blocked as follows. Coating solution was flicked off and plates were washed three times with 200µl sterile PBS. 200µl of RPMI media (serum and antibiotic free) containing 2% BSA was then added to plates, which were then incubated for at least 2 hours (maximum 6 hours) at room temperature. Following the incubation, liquid was discarded from plates, which were washed four times with 200µl PBS-0.05% tween and then three times with 200µl PBS. 200µl PBS was left on plates until seeding of cells to prevent plate membranes drying out.

4.3.4 Memory B-cell harvesting

Cells were harvested from the 24-well plates and each well was washed up to three times with 1ml complete RPMI to recover maximum number of cells. Cell suspensions were then centrifuged at 1500rpm for 10 minutes. Supernatant was discarded and cells were resuspended in 1-2ml complete RPMI.

Cell concentrations were adjusted to attain 5 x10^6 cells/ml. A further 1:10 and 1:100 dilution of a fraction of cells was performed to achieve 5 x10^5 cells/ml and 5 x10^4 cells/ml for positive control wells. 100µl of the appropriate cell suspension was added per well and plates were incubated for 16-24 hours at 37°C, CO_2.

4.3.5 Detection of IgG^+ memory B-cells

Cells were discarded from plates, which were then washed four times with 200µl PBS-0.05% tween and the three times with 200µl PBS. Anti–human IgG alkaline phosphatase (B5655, Sigma Aldrich) was prepared at 1:2000 dilution in 2% BSA-PBS. 100µl of antibody was added to corresponding wells and plates were incubated for 2 hours at room temperature. BCIP/NBT substrate (Sigma Aldrich) was dissolved in dH_2O as per manufacturer's instructions. Plates were washed four times with 200µl PBS-0.05% tween and then three times with 200µl PBS to remove unbound antibody and 50µl of BCIP/NBT substrate solution was added per well. Plates were left to develop for 15 minutes in the dark (room temperature). Following incubation, plates were washed under running water and left to dry for 1-2 days in the dark. Plates were analysed using an AID ELISpot Reader System (ELR07) and ELISpot Reader software (Version 7.0, Cadama Medical, Oxford, UK). Background spots (as determined by the negative control) were subtracted from total spot numbers. In the case of no spots being detected in positive control wells, plates were not included in analysis. Thus, 2 carriage positive and 2 carriage negative volunteers were excluded.

After development with the BCIP/NBT substrate, spots appear dark purple in color. Figure 37B shows typical results and some false positive results (negative control wells). The negative control wells should contain very few spots, while the total IgG (positive control) wells should have spots covering the entire surface of the membrane. Two cell dilutions are used for the positive control wells as often spots saturate the membrane to a point where no individual spots can be counted. The spots should be uniform in shape and slightly darker in color at the center than on the outer edges, which have a "halo-esque" appearance. False positive responses may occur due to substrate aggregates within the well.

A

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS					**PBS**						
B	Ig dil1		Ig dil2			Igdil 1		Ig dil 2				
C	PspA (pre)					PspA (post)						
D	PnuBioVax (pre)					PnuBioVax (post)						
E	SP1683 (pre)					SP1683 (post)						
F	SP2099 (pre)					SP2099 (post)				SP1154-2 (pre)		
G	SP1032 (pre)					SP1032 (post)						
H	SP0346 (pre)					SP0346 (post)				SP1154-2 (post)		

B

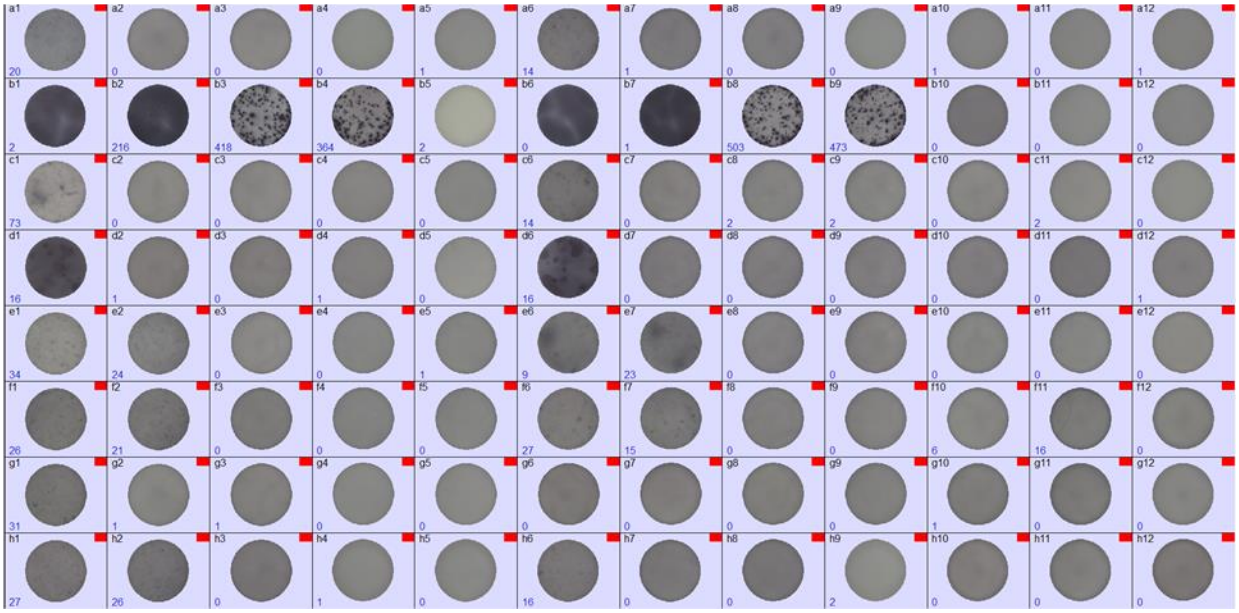

Figure 37: (A) Example plate layout of ELISpot plate, (B) Example of developed ELISpot plate as read by an AID ELISpot Reader System ELR07 and ELISpot Reader software (Version 7.0). 500,000 PBMCs taken pre- or 29 days post-inoculation with *S. pneumoniae* serotype 6B (Spn6B) were added to negative control wells (PBS) and protein-coated wells (PspA, PnuBioVax, Sp1683, SP2099, SP1032 and SP0346). 200,000 PBMCs were added to Ig dilution 1 wells and 20,000 PBMCs were added to Ig dilution 2 wells. Negative control wells should contain no spots. Positive control wells should be covered in spots; complete saturation may prevent counting of individual spots for wells seeded with 200,000 PBMCs as compared to 20,000 PBMCs.

4.3.6 Statistical analysis

All data analysis was carried out using GraphPad Prism (Version 9.3.1, GraphPad Software, La Jolla, CA). Statistical significance was determined via a Mann-Whitney or Wilcoxon test for unpaired and paired groups, respectively.

4.4 Results

As sample availability was limited due to the preciousness of biobank samples, preliminary experiments were carried out with pre- and post-challenge samples from five carriage positive and five carriage negative volunteers to determine which proteins showed an association with either protection against carriage or boosted immune response after challenge. These proteins would then be tested with PBMCs from additional volunteers. Due to varying cell numbers upon recovery of cultured PBMCs, actual sample size for each protein is given in Table 16.

Table 16: Sample size per protein for memory B-cell ELISpot preliminary experiments.

	PspA1	PnuBioVax	SP1683	SP2099	SP1032	SP0346	SP1154-2
Carriage positive paired	4	4	3	4	4	3	4
Carriage negative paired	5	5	3	4	2	4	2

As shown in Figure 38, an increased response to PspA1 was observed following experimental colonisation and there was a tentative suggestion that a higher frequency of memory B-cells specific for SP1683, SP0346 and PnuBioVax was observed at baseline in volunteers protected against experimental colonisation. Thus, these four candidates were taken further and B-cell responses from a larger volunteer sample size (total n = 20; 10 carriage positive and 10 carriage negative, including those previously tested) were examined. Actual sample sizes are given in Table 17.

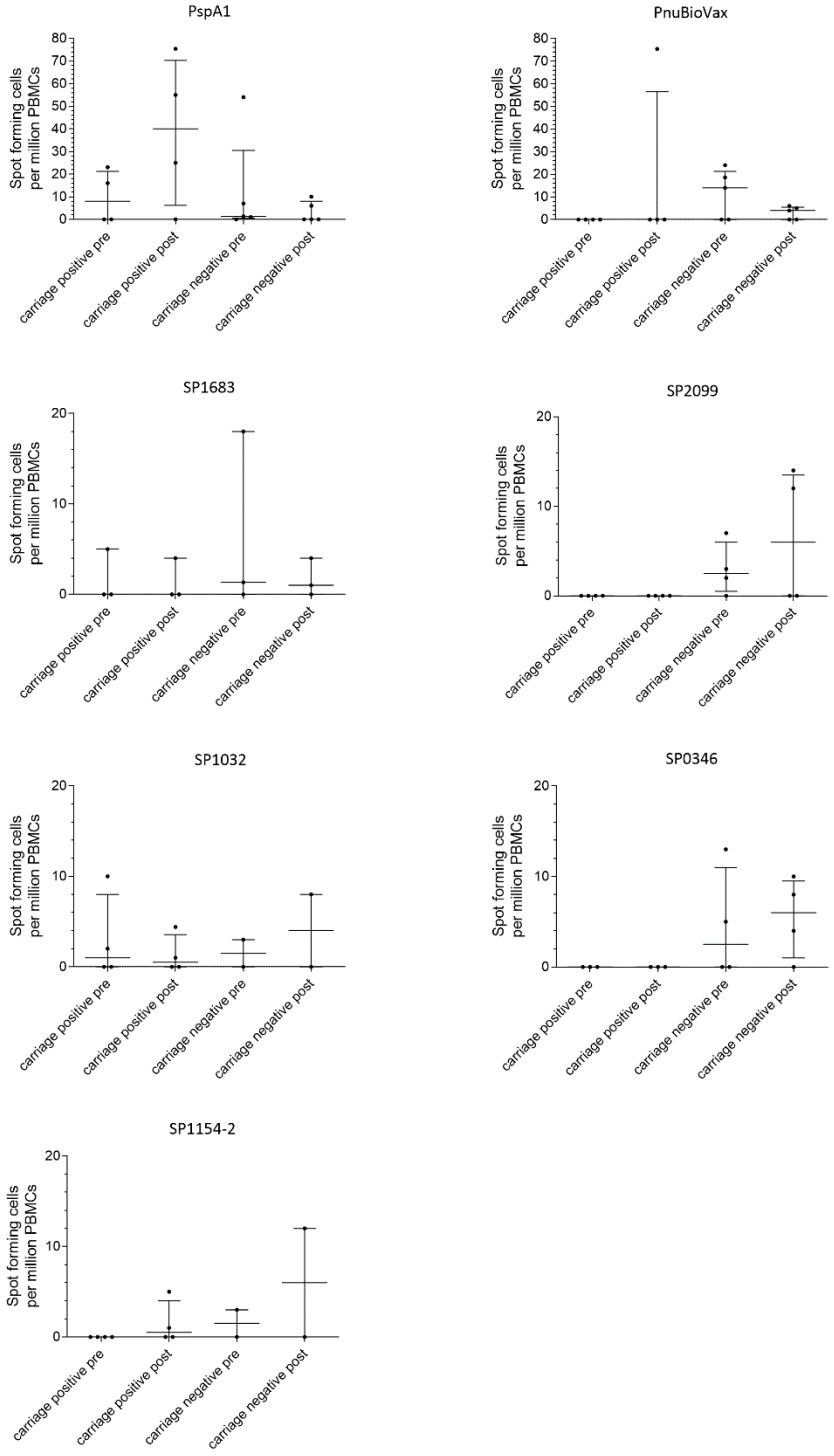

Figure 38: Spot forming cells (IgG$^+$ memory B-cells) per million PBMCs for pneumococcal proteins before and after experimental challenge with *S. pneumoniae* serotype 6B. Peripheral blood mononuclear cells were isolated before and after experimental challenge with serotype 6B pneumococcus in healthy adults and memory B-cell responses to selected proteins, as well as the PnuBioVax vaccine, were analysed by ELISpot. Error bars indicate median value with interquartile range. Note, a different y-axis scale is used for PspA and PnuBioVax graphs compared to the others.

Table 17: Sample size per protein for memory B-cell ELISpot experiments.

	PspA1	PnuBioVax	SP1683	SP0346
Carriage positive paired	8	9	7	7
Carriage negative paired	9	10	8	9

A significant difference in the median number of IgG^+ memory B-cells per million PBMCs before and after challenge with Spn6B was found in the carriage positive cohort in response to PspA1 ($p = 0.031$) and PnuBioVax ($p = 0.031$) (Figure 39). Whilst not significant, a greater response to SP0346 post colonisation in the carriage positive cohort as compared to baseline was observed. Furthermore, results showed a tentatively higher response to PnuBioVax at baseline in the carriage negative cohort as compared to the carriage positive cohort ($p = 0.285$), indicating that the vaccine may associate with protection against colonisation. However, this difference was not statistically significant. It is possible that significance may be observed with a larger sample size.

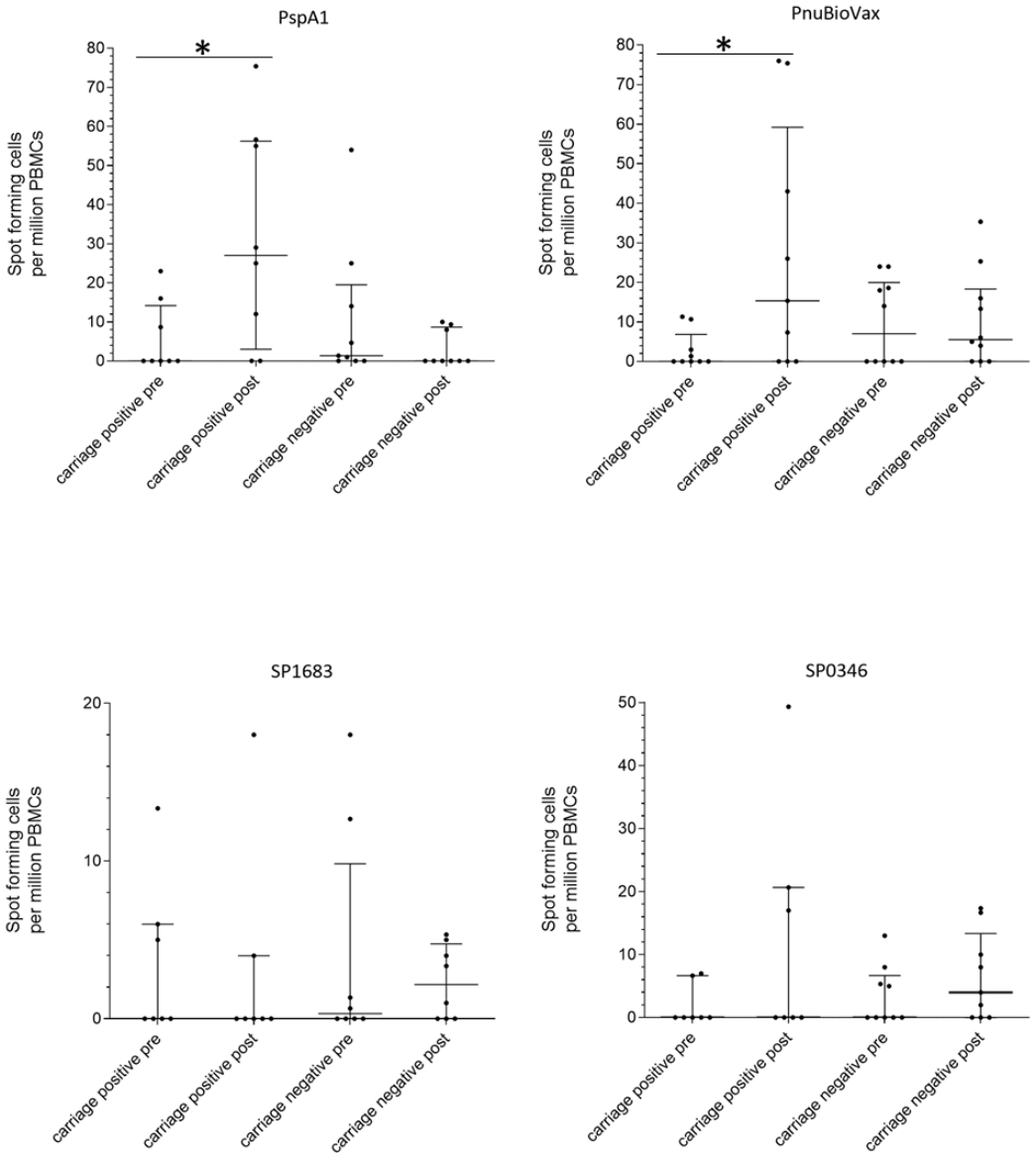

Figure 39: Spot forming cells (IgG$^+$ memory B-cells) per million PBMCs for selected proteins before and after experimental challenge with *S. pneumoniae* serotype 6B (Spn6B). Peripheral blood mononuclear cells were isolated before and after experimental challenge with serotype 6B pneumococcus in healthy adults and memory B-cell responses to selected proteins, as well as the PnuBioVax vaccine, were analysed by ELISpot. Error bars indicate median value with interquartile range. A Wilcoxon paired test was used to determine significant difference between carriage positive response at baseline compared to post-colonisation and a Mann-Witney U test was used to compared differences between the median values at baseline between the carriage positive and negative cohorts. A significant difference in the median values before and after challenge with Spn6B was found in the carriage positive cohort in response to PspA1 ($p = 0.0313$) and PnuBioVax ($p = 0.0313$). Note, a different y-axis scale is used for each graph.

4.5 Discussion

The finding that none of the single protein antigens tested in this work significantly correlated with higher numbers of protein-specific memory B-cells at baseline is consistent with previous findings that no protection against experimental colonisation with Spn6B was conferred by circulating PspA- or PspC-specific memory B-cells (Pennington *et al.*, 2016). In the previous study by Pennington and colleagues no increase in circulating PspA-specific IgG secreting memory B-cells was observed up to 35 days post-inoculation in the absence or presence of colonisation. No increase in protein-specific B-cells from baseline was detected in carriage negative individuals. However, it is not clear from the published data whether the PspA used in the study conducted by Pennington and colleagues was homologous to Spn6B, which might explain differences between the findings of their work and that reported here, where the PspA protein used is homologous to the challenge strain. In this current study, it was found that PspA1 generated a significant increase in the number of protein-specific memory B-cells 29 days post-inoculation in the presence of experimental carriage. So too did PBV, which is promising for this novel protein vaccine. Whilst not significant, there was a trend that PBV-specific memory B-cells also conferred protection against experimental pneumococcal colonisation, evidenced by higher levels at baseline in those protected. It is plausible that a larger sample size might have identified a significant level of protection against colonisation, however this memory B-cell ELISpot assay was optimised for the detection of B-cell responses against single antigens and further optimisation of the assay is necessary to determine response to multiple antigens simultaneously. PnuBioVax contains antigens PspA2, Ply, PsaA, PiaA (SP1032), PiuA, RrgB and RrgA as well as others (Cecchini *et al.*, 2015; Hill *et al.*, 2018). Further optimisation is necessary to elucidate how response is distributed across all proteins. Nevertheless, our results indicate that a response to this protein pool is associated with protection against carriage. Further experiments need to be carried out to determine if this is the case. Whilst PspA1 and SP1032 did not correlate with memory B-cell-associated protection against carriage alone, the combination of these proteins with other immunogenic proteins might be needed to confer protection. No induction of memory B-cell response to SP2099, SP1154-2 or SP1683 following experimental challenge was detected, nor did these proteins correlate with protection against colonisation.

It is important to consider that a combination of proteins might confer protection at different stages of pneumococcal infection. Significantly lower percentages of memory B-cells to three pneumococcal protein antigens (PhtD, PhtE, and Ply) and reduced antigen-specific IgG is associated with increased propensity to otitis media in children (Sharma, Casey and Pichichero, 2012). *In vitro s*timulation

of freshly isolated adenoidal cells from children (n = 20) showed a significant increase in proliferation of PspC, PsaA and PspA specific IgG-secreting cells, likely to be derived from resident memory B-cells, suggesting that prior carriage induces a protein-specific memory response. The numbers of PspC- and PsaA-specific IgG antibody-secreting cells were significantly greater than those of PspA- and PdB-specific cells, suggesting that different proteins may be superior antigens for the induction of mucosal immunity and corroborating the findings of Briles *et al.* (2000) that PsaA and PspC were the most efficacious of the four antigens in conferring protection against pneumococcal colonisation in mice.

Previously, it was shown that at the time of pneumococcal exposure in a human model of infection, naturally acquired capsular polysaccharide-specific memory B cells associated with protection against pneumococcal acquisition (Pennington *et al.*, 2016). In volunteers protected against experimental colonisation, frequency of circulating capsule-specific IgG secreting memory B-cells fell over the 35 days post-inoculation with Spn6B, reaching significance at day 35. On day 7 post-inoculation, frequency of capsule-specific IgG secreting plasma cells was significantly increased as compared to pre-challenge, demonstrating that exposure to pneumococcus induces activation of B-cells; frequency of plasma cells returned to levels comparable with baseline by day 14. However, increased numbers of circulating capsule-specific IgG secreting plasma cells did not correlate with increased levels of mucosal or systemic capsule-specific IgG, suggesting that antibodies produced by plasma cells may migrate to the nasopharynx and sequester bacterial colonisation, thus providing protection against carriage acquisition (Nurkka *et al.*, 2001; Roche *et al.*, 2015). A limitation of this current work is that memory B-cell response in the acute period following bacterial challenge was not investigated and so it is unknown whether there might have been a temporary depletion in circulating protein-specific memory B-cells in the short-term, implicating them in the control of carriage. Moreover, sampling of the nasal mucosa before and after experimental pneumococcal colonisation in a previous study suggested that upon colonisation, B-cells at the nasal mucosa become activated and recirculate (Jochems *et al.*, 2019). Furthermore, lung resident memory B-cells have been implicated in protection against pneumococcal pneumonia (Barker *et al.*, 2021). Thus, it must be considered that tissue-resident memory B-cells may protect against pneumococcal infection in a way that is not detected by our investigations into circulating memory B-cell response. It has been demonstrated that booster vaccination with PCV induces the appearance of memory B-cells at the nasal mucosa but this reflects the recirculation of systemic memory cells rather than the generation of tissue-resident cells, which would likely be stimulated upon colonisation and prevent subsequent infection (Clarke *et al.*, 2012). Development of mucosal vaccines capable of inducing tissue-resident memory B-cell responses may provide a greater level of protection against carriage acquisition than circulating memory

B-cell responses. Intranasal immunisation of mice with a non-adjuvanted γ-irradiated pneumococcal whole-cell vaccine elicited serotype-independent protection against lethal pneumonia and sepsis, which was shown to be dependent on B-cells and IL-17A responses from innate immune cells (Babb *et al.*, 2016). Ideally, the present study would have obtained nasal biopsies from volunteers to investigate local protein-specific memory B-cell response to challenge with pneumococcus at the nasal mucosa to compare with systemic response. However, the Covid-19 pandemic prevented this from being carried out.

Colonisation with pneumococcus has been shown to enhance memory B-cell response to subsequent vaccination with PCV in mice (Rabquer *et al.*, 2007) and evidence suggests that pre-vaccination levels of both antibodies and memory B-cells might predict response to immunisation with a protein-conjugated polysaccharide vaccine against serogroup C meningococcus in humans (Rohner *et al.*, 2008), although some conflicting evidence also exists and suggests that this may not be suitable for all antigens in young children (Perrett *et al.*, 2012; Blanchard-Rohner *et al.*, 2013). Moreover, a booster dose of PCV10 following priming with PCV13 in early infancy does not induce detectable peripheral blood memory B-cell responses but a PCV13 booster does induce robust responses, suggesting booster responses to PCVs may be dependent on homologous carrier protein priming (Trück *et al.*, 2016).

Previous studies into the immune response to West Nile or influenza viruses have shown that whilst humoral responses mediate protection against homologous re-challenge, it is memory B-cell responses that are responsible for protection against antigenically distinct virus that may escape humoral immunity (Purtha *et al.*, 2011; Adachi *et al.*, 2015). To our knowledge, along with the previously mentioned work conducted by Pennington and colleagues investigating PspA- and PspC-specific memory B-cell frequencies before and after experimental challenge with Spn6B, this is one of the only studies investigating potential serotype-independent B-cell response to pneumococcus. Further work is needed to ascertain what level of protection is conferred against homologous or heterologous challenge with pneumococcus following the induction of protein-specific memory B-cell response. This response should also be evaluated in different age-groups, particularly the elderly, for whom further work is needed to elucidate age-related changes in immunological memory but for whom we know the overall B-cell populations, including switched memory B-cells, are reduced (Shi *et al.*, 2005; Colonna-Romano *et al.*, 2008; Ademokun, Wu and Dunn-Walters, 2011; Frasca *et al.*, 2011; Scholz *et al.*, 2013).

4.6 Conclusion

To conclude, whilst no single antigen tested in the current study correlated with protection against pneumococcal colonisation, the study did identify protein-specific induction of memory B-cell

responses post experimental colonisation and a trend suggests that a cluster of proteins, such as those included in the novel PnuBioVax formulation, may correlate with protection against experimental colonisation. Further optimisation is needed to assess the protective effect elicited by multiple protein antigens and substantiate the level of protection conferred by protein-mediated memory response against re-colonisation with the bacteria.

4.7 Acknowledgements

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Chapter 5

Testing novel nanoparticle-based vaccine formulations for induction of adaptive immune response *in vitro*

5.1 Abstract

Nanoparticles provide an exciting method of delivery of immunogenic protein antigens to mucosal sites for immunisation. Activation of the adaptive immune system involves the internalization of nanoparticle-based vaccines by dendritic cells for antigen presentation to naïve T-cells. An assay was developed to test nanoparticle induction of human monocyte-derived dendritic cell maturation *in vitro.* Further, co-culture of nanoparticle-exposed dendritic cells with peripheral blood mononuclear cells enabled evaluation of subsequent support of T-cell activation *in vitro.* Five novel nanoparticle-based vaccines were investigated. A PLGA nanoparticle coated with water-soluble chitosan was found to induce dendritic cell maturation, results around the support of T-cell activation were inconclusive. Further work is needed to establish the true effect of nanoparticle-based vaccines on T-cells and the adaptive arm of the immune system. Nevertheless, a platform for these investigations has been successfully developed.

5.2 Introduction

As previously outlined in Chapter 1.7.3.1.4.1, nanoparticles provide a promising method of mucosal pneumococcal vaccine delivery (Kunda *et al.*, 2014; Kunda, Alfagih, Dennison, *et al.*, 2015; Kunda, Alfagih, Miyaji, *et al.*, 2015; Gonçalves *et al.*, 2019; Kaneko *et al.*, 2021). The nanoparticles themselves can harbor immunostimulatory properties and the conjugation of pneumococcal protein antigens to the nanoparticles can induce an enhanced specific anti-pneumococcal response. Furthermore, nanoparticles in dry powder form eliminate the need for cold chain and improve stability of protein antigens as well as the nanoparticles themselves (Kunda *et al.*, 2012). Dry powder formulations also offer the potential for inhalation for pulmonary delivery with controllable antigen release (Scherließ and Janke, 2021). For nanoparticles to be an effective means of mucosal vaccine delivery, they must initiate a local immune response. Activation of the adaptive arm of the immune system involves the internalization of nanoparticle-based vaccines by dendritic cells for antigen presentation on major histocompatibility complex (MHC) II receptor molecules to naïve T-cells. Support of T-cell activation by dendritic cells exposed to nanoparticles would indicate the ability of nanoparticles to support differentiation of naïve T-

cells into antigen-specific effector or memory cells, which may confer protection against pulmonary infection.

As previously outlined in Chapter 1.7.3.1.4, deposition of particles along the respiratory tract is size-dependent (Table 3) and determined by various mechanisms including inertial impaction, Brownian diffusion, gravitational sedimentation and electrostatic effects (Yang, Peters and Williams, 2008). Larger particles (>8µm) have greater impact on the upper respiratory tract, depositing from nasal passages to larger bronchioles in a size-dependent manner, whilst fine particles (<1–3µm) penetrate lung tissue and deposit in the alveoli (Thomas, 2013). Importantly, particles should be large enough in size to avoid clearance by lymphatic drainage (>100nm) but small enough to avoid clearance by alveolar macrophages (<500nm) (Fifis *et al.*, 2004; Scherließ and Janke, 2021).

Dendritic cells are the most efficient and potent antigen-presenting cells and play an integral role in the initiation of various T-cell mediated responses inducing memory and protection (Théry and Amigorena, 2001; Sallusto and Lanzavecchia, 2002). Immature dendritic cells (DCs) reside in peripheral tissues and the blood acting as sentinels for invading pathogens and foreign antigens. The recognition of PAMPs and DAMPs by pattern recognition receptors, particularly Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), triggers DC activation (Lundberg *et al.*, 2014). Invading pathogens and foreign antigens are phagocytosed by immature DCs and degraded into small fragments in endosomal vesicles for presentation on major histocompatibility complex (MHC) II receptor molecules to naïve $CD4^+$ T-cells or MHC class I receptor molecules to $CD8^+$ T-cells. DCs undergo a maturation process in response to foreign antigens, which enhances their ability to efficiently prime naïve T-cells. During maturation, DCs cease to uptake pathogens or foreign antigens and begin to upregulate expression of MHC II and co-stimulatory molecules, such as CD40, CD80, CD86 and the chemokine receptor CCR7, on the cell surface. Migration to T-cell rich zones in secondary lymphoid organs in induced by chemokines, in particular CCL19 and CCL21 (Tiberio *et al.*, 2018). The DCs further begin to release cytokines to stimulate the differentiation of naïve antigen-specific T-cells into effector cells, as well as the activation of various other types of immune cells.

There are four main subsets of DCs, all arising from haematopoeitic stem cells (Collin, McGovern and Haniffa, 2013) (Figure 40). The subsets of DCs are separated by their location and primarily by whether they are migratory, i.e. trafficked through tissues, or resident, i.e. arising in lymph nodes directly from the blood (Shortman and Naik, 2006). Respiratory tract DCs with antigen-presenting capability have been identified in the airway epithelium, lung parenchyma and visceral pleura of rodents and humans (Sertl *et al.*, 1986), where they play different roles to maintain immunological homeostasis and coordinate

immune response to respiratory pathogens (Stumbles, Upham and Holt, 2003). DCs are also present on the alveolar surface, in the nasal mucosa and nasal turbinates, and in the vasculature of the lung (Stumbles, Upham and Holt, 2003). DCs in the respiratory tract are constantly replenished from bone marrow output (Holt *et al.*, 1994) and degree of DC maturation along the respiratory tract appears to be associated with proximity to the external environment (Vermaelen and Pauwels, 2012). Furthermore, influx of DCs to the airways in rats in response to inhaled antigens has been shown to precede neutrophil influx (McWilliam *et al.*, 1994), highlighting the integral role of DCs in rapid innate immune response in the respiratory tract. DCs, or their precursors, are constantly recruited to the lungs from the blood. In the lungs, they act as sentinels and are able to present inhaled antigens, which they transport to the draining pulmonary lymph nodes (Xia, Pinto and Kradin, 1995; Vermaelen and Pauwels, 2012).

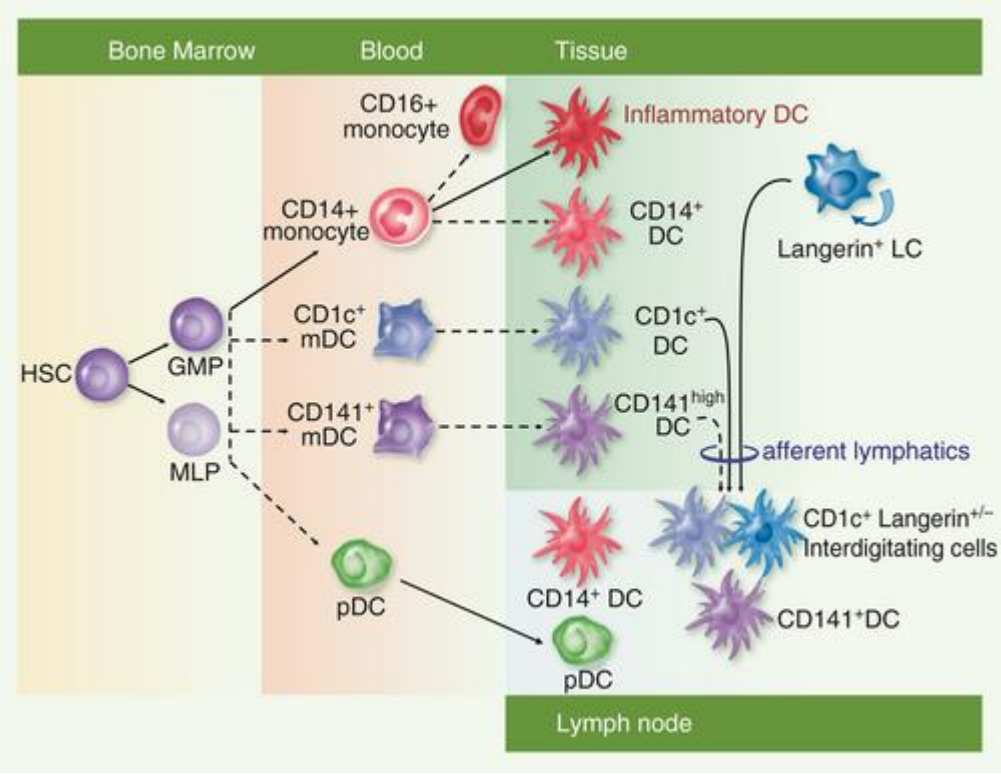

Figure 40: The distribution of major human dendritic cell (DC) subsets in blood, epithelial tissues, and lymph nodes. Broken arrows indicate relationships that require further confirmation in humans. Human DCs can be generated either from granulocyte–macrophage progenitors (GMP) or multi-lymphoid progenitors (MLP) both of which ultimately arise from haematopoietic stem cells (HSC). Classical monocytes, blood myeloid DC (mDC) and plasmacytoid DC (pDC) are putative precursors of tissue and lymphoid DCs. Non-classical monocytes are reported to arise by conversion of classical monocytes in the mouse. Inflammatory DCs and $CD14^+$ DCs have transcriptional profiles suggesting that they arise from monocytes; likewise, tissue $CD1c^+$ DCs and $CD141^+$ DCs are related to their blood counterparts. Myeloid DCs and Langerhans cells (LCs) both form interdigitating cells in skin-draining lymph nodes. $CD14^+$ DCs and pDCS are also found in nodes but may arise directly from the blood rather than by migration from tissues. Image source: Collin, McGovern and Haniffa, 2013.

There is evidence that regional activation of tissue-resident memory T-cells by antigen presenting DCs occurs in the airway submucosa, and is not restricted to activation in the lymph nodes (Constant *et al.*, 2002; Blank, Stumbles and Von Garnier, 2011; Thornton *et al.*, 2012). It has been suggested that inducible bronchus-associated lymphoid tissue (iBALT), which promotes the recruitment, priming and expansion of antigen-specific lymphocytes *in situ* and the sustained presence of which is dependent upon DCs, may be the location of such respiratory tract DC/T-cell interactions (Moyron-Quiroz *et al.*, 2004; Geurtsvankessel *et al.*, 2009; Halle *et al.*, 2009). iBALT may also provide a niche for the maintenance of memory cells at the local level as well as the production of long-lived antibody secreting plasma cells (Moyron-Quiroz *et al.*, 2006; Tan *et al.*, 2019). In fact, secondary lymphoid organs have been shown to be dispensable for mounting a T-cell mediated immune response to pulmonary pathogens such as *Mycobacterium tuberculosis* (Day *et al.*, 2010; Kashino *et al.*, 2010) or influenza (Moyron-Quiroz *et al.*, 2004). Importantly, the induction of infection-independent iBALT by a protein cage nanoparticle in mice was found to enhance protection against respiratory viruses whilst ameliorating inflammation normally associated with the recruitment of immune responses into the lung (Wiley *et al.*, 2009). Moreover, a silver nanoparticle-adjuvanted vaccine induced antigen-specific IgA production against influenza through the promotion of iBALT neogenesis (Sanchez-Guzman *et al.*, 2019). In additional, intranasal vaccination and challenge in a mouse model of tularemia showed that iBALT formation following challenge with *Francisella tularensis* in vaccinated mice was associated with protection against mortality (Chiavolini *et al.*, 2010). Thus, the induction of iBALT in either an antigen-independent or antigen-specific manner by nanoparticles may provide a novel vaccination strategy against pulmonary pathogens. Nevertheless, it is important that the potential role played by iBALT in lung immunopathology is repressed while protective iBALT is promoted (Marin *et al.*, 2019).

It is well established that functional DCs can be isolated from classical $CD14^+$ blood monocytes (Collin, McGovern and Haniffa, 2013), these specific DCs are termed monocyte-derived dendritic cells or Mo-DCs. Mo-DCs are capable of stimulating naïve $CD4^+$ T-cells and cross-presenting antigens to $CD8^+$ T-cells. A modest population of $CD14^+$ dendritic cells reside in most non-lymphoid epithelial and interstitial tissues, where they acquire antigen and migrate to lymph nodes. It remains unclear whether $CD14^+$ DCs are migratory, particularly due to their low expression of CCR7 which is known to be highly involved in the migratory process (Angel *et al.*, 2006; Haniffa *et al.*, 2009).

Dendritic cells uptake nanoparticles via endocytic processes such as receptor-mediated endocytosis, phagocytosis, and micropinocytosis. The cellular uptake of nanoparticles is facilitated by

particle size, shape, hydrophobicity, elasticity, surface modification and surface charge (Foroozandeh and Aziz, 2018; Sousa De Almeida *et al.*, 2021). For example, the size of particles plays a major role in determining how the particles are internalized by cells (Figure 41). Nanoparticles ranging from a few to several hundred nanometers are internalized by pino- or micropinocytosis, whilst larger particles (250nm - 3µm) have been shown to be phagocytosed. Nanoparticles ranging from 120-200nm enter the cell via clathrin- or caveolin-mediated endocytosis. Cellular uptake of nanoparticles has been shown to decrease for smaller and larger particles, with 30-50nm seemingly optimal size for efficient interaction with the cell membrane and subsequent internalization via receptor-mediated endocytosis (Foroozandeh and Aziz, 2018). Particles should be large enough in size to avoid clearance by lymphatic drainage (>100nm) but small enough to avoid clearance by alveolar macrophages (<500nm) (Fifis *et al.*, 2004; Scherließ and Janke, 2021). It has been shown that the optimal particle size for fast and efficient uptake by human blood-derived DCs is ≤500nm in diameter (Foged *et al.*, 2005). Whilst positively charged particles are optimal for cellular uptake compared to negatively or neutrally charged particles, positively charged particles have been shown to induce cell death (Dawson, Salvati and Lynch, 2009; Foroozandeh and Aziz, 2018).

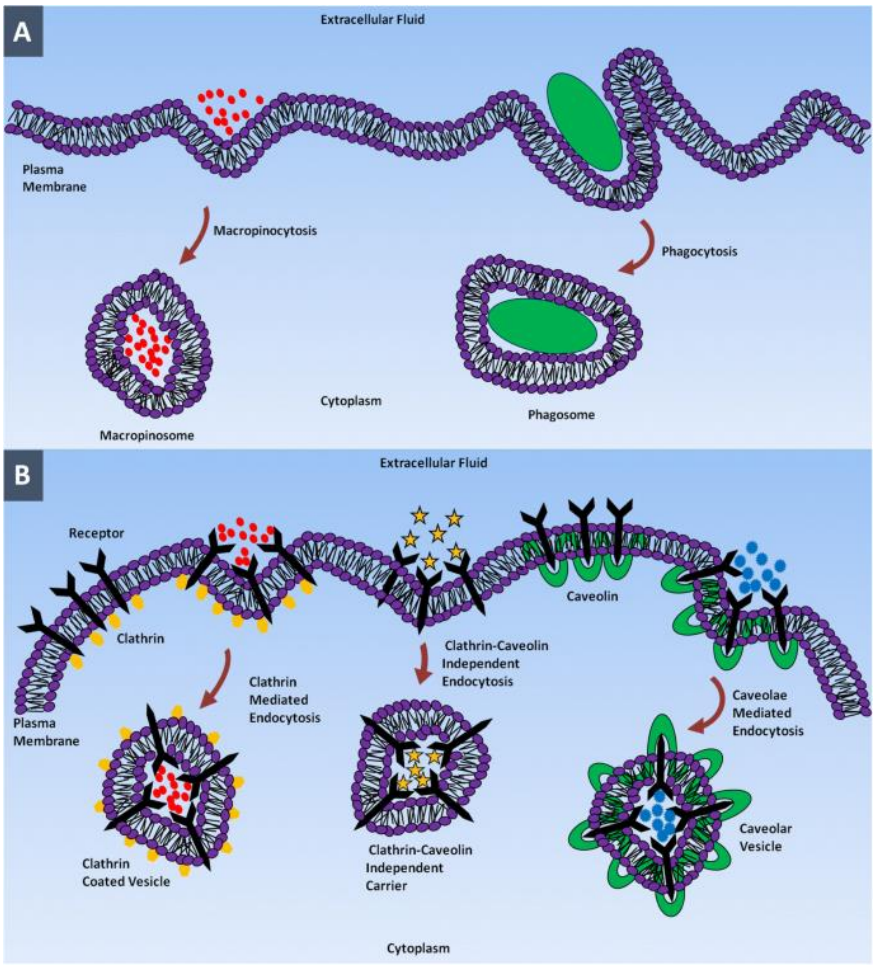

Figure 41: Entry of NPs into cell using different endocytotic pathways. A) Macropinocytosis and phagocytosis. **B)** Clathrin-mediated endocytosis, clathrin-caveolin independent endocytosis and caveolae-mediated endocytosis. Image source: Foroozandeh and Aziz, 2018.

Polymeric nanoparticles can be formulated by either the polymerization of monomers or the dispersion of preformed polymers (Zielinska *et al.*, 2020). Irrespective of the production method employed, products are generally obtained as aqueous colloidal suspensions (Jawahar and Meyyanathan, 2012). Polymeric nanoparticles can be loaded with active compounds entrapped within the nanoparticle or adsorbed onto the nanoparticle surface. Several encapsulation techniques have been used to transport active molecules that are either chemistry based or physiochemical based, such as multiple emulsion techniques and spray drying. Emulsions are a mixture of two or more immiscible liquids in which one or more liquids are dispersed in another liquid (Jenjob *et al.*, 2019). Emulsions such as water-in-oil, oil-in-water, and oil-in-water-in-oil (O/W/O) or water-in-oil-in-water (W/O/W) have been used for pharmacological applications. Generally, proteins are encapsulated within polymeric nanoparticles by W/O/W emulsion systems (Iqbal *et al.*, 2015; Scherließ and Janke, 2021). PLGA can be used as a carrier

for protein antigen vaccines to increase the elicited adaptive response by means of increased antigen uptake, processing and presentation (Morales-Cruz *et al.*, 2012; Silva *et al.*, 2016; Scherließ and Janke, 2021). To be inhaled into the lungs, or nebulized, as pulmonary vaccines, nanoparticle formulations must be transformed into dry powder form. However, dry powders are considered to be too small in diameter to ensure maximum deposition in the lower airways due to exhalation (Uskokovic and Stevanovic, 2009) and so dry powder nanoparticles are formulated into nanocomposite microparticles ranging from 1-5µm, capable of reaching the respirable airways, by various techniques including spray drying (Figure 42) (Bailey and Berkland, 2009; Kunda *et al.*, 2014; Kunda, Alfagih, Dennison, *et al.*, 2015; Kunda, Alfagih, Miyaji, *et al.*, 2015). Upon encounter with the respiratory environment, nanocomposite microparticles dissolve, releasing the nanoparticles (Soppimath *et al.*, 2001). Spray drying prevents nanoparticle aggregation and agglomeration while maintaining nanoparticle and protein integrity and stability. Spray drying also eliminates cold chain requirements (Kunda *et al.*, 2012). The use of amino acids as microcarriers to increase aerosolization properties of spray dried powders, has been investigated and leucine has been shown to enhance the dispersibility and aerosolization properties of spray-dried powders for pulmonary delivery (Seville *et al.*, 2007; Kunda, Alfagih, Dennison, *et al.*, 2015). Moreover, PLGA nanoparticles with an acid terminated group, and cationic chitosan hydrochloride salt have demonstrated improved nanoparticle characteristics for pulmonary vaccine delivery (Pawar *et al.*, 2013; Kaneko *et al.*, 2021). As a salt, chitosan hydrochloride is also soluble in water and has further been shown to improve delivery of encapsulated material to mucosal sites (Pawar *et al.*, 2013). Notably, it has been shown that encapsulation of protein antigen within lipid-PLGA hybrid nanoparticles elicits significantly higher T-cell mediated antigen-specific immune response than adsorption of protein antigen onto nanoparticle surface or indeed free antigen due to long-term antigen persistence and presentation (Liu *et al.*, 2016).

Figure 42: Preparation of nanocomposite microparticles for nebulization. Image source: Kunda, Alfagih, Miyaji, *et al.*, 2015.

The aim of the work in this chapter was first to establish a platform to test novel nanoparticle-based vaccine formations *in vitro.* Then to determine whether novel nanoparticle-based pneumococcal

vaccines can support DC maturation and antigen specific T-cell activation *in vitro* (with minimal toxicity) to determine whether they might help to generate adaptive immunity against pneumococcal infection *in vivo* and provide a viable means of novel pneumococcal vaccine delivery.

5.3 Materials and Methods

5.3.1 Isolation of human peripheral blood mononuclear cells and monocytes

Buffy coats were obtained from NHS Blood and Transplant, Speke, Liverpool. Leucosep tubes were filled with 15ml of Ficoll using a serological pipette then centrifuged at 1011 x g (2200 rpm) for 1 minute at room temperature to bring Ficoll level below the barrier. Between 15ml and 30ml undiluted buffy coat was poured directly onto the barrier. Tubes were then centrifuged at 836 x g (2000 rpm) for 18 minutes at room temperature, with the centrifuge brake off. A sterile Pasteur pipette was used to collect the buffy layer from each tube at the interface above the frit and the buffer layer transferred to a new 50ml Falcon tube. $Ca/Mg^{-/-}$ HBSS was poured into each Falcon tube containing mononuclear cells to bring the volume up to 50ml and cells were washed by centrifugation at 252 x g for 10 minutes at room temperature. The supernatant from each tube was removed and cell pellets from the same donor were combined into a single 50ml Falcon tube. $HBSS^{-/-}$ was added to pooled cells to a total volume of 50ml. Cells were again washed by centrifugation at 252 x g for 10 minutes at room temperature. A further washing step was carried out and supernatant was then poured off and cells resuspended. Combined cells were resuspended in 50ml $HBSS^{-/-}$ and 10µl of cell suspension was diluted 1:10 Trypan Blue solution for cell counting.

5.3.1.1 Magnetic labelling using CD14 MicroBeads

All reagents and solutions were kept cold during the monocyte labelling and isolation. Peripheral blood mononuclear cells (PBMCs) were passed through a 30µm nylon mesh (pre-separation filter) and collected in a 15ml Falcon tube to obtain a single cell suspension. The filter was moistened with buffer prior to use. PBMC pellets were resuspended in 80µl of cold $PBS^{-/-}$ per 10^7 total cells. 20µl of CD14 MicroBeads (Mo-DC Generation Toolbox, human (Miltenyi Biotec)) were added per 10^7 total cells. The suspension was mixed well and then incubated for 20-30 minutes at 4°C on a rotator. Cells were washed by adding 1-2 mL of $PBS^{-/-}$ per 10^7 cells and centrifuging at 300 x g for 10 minutes. Supernatant was then aspirated completely. Up to 10^8 cells were resuspended in 500µL of $PBS^{-/-}$.

5.3.1.2 Magnetic separation of monocytes using CD14 MicroBeads

MACS columns (pre-cooled at 4°C) were placed in a MACS separator and columns were rinsed with the appropriate amount of buffer as specified by the manufacturer (500µL for MS columns or 3ml for LS columns). Following extremely slow flow-through of cell suspension and poor recovery of cells using the MS columns, the LS columns were used for separation thereafter. The cell suspension was then applied to the columns (one column per donor) and the flow-through of unlabeled cells collected in a Falcon tube. Columns were washed a further three times with buffer and the unlabeled cells that passed through were collected and combined with the effluent from previously. The columns were removed from the separator and placed on a suitable collection tube (for example, a 15ml Falcon tube). The appropriate amount of buffer (1ml for MS columns or 5ml for LS columns) was pipetted onto each column and the magnetically labelled cells were immediately flushed out by firmly pushing the plunger into the column. The columns were eluted a second time using the appropriate amount of buffer. The monocyte fraction was placed on ice to keep cold.

5.3.1.3 Freezing of unlabelled cells

The unlabelled cells (non-monocyte fraction) were centrifuged at 300 x g for 10 minutes at room temperature. An adequate quantity of CTL-CRYOABC medium was prepared according to manufacturer's instructions and cells were resuspended in the required volume to aliquot 1ml of $1x10^7$ cells into cryovials. Cells were stored in a Mr. Frosty at -80°C until long-term storage in liquid nitrogen.

5.3.2 Nanoparticle-based vaccine development

Nanoparticles capable of targeting lung dendritic cells were previously developed by the Formulation and Drug Delivery Research Group, School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University. The group provided five novel nanoparticle formulations to be used in this study. The nanoparticles will be referred to as A, B, C, D, E due to pending patent approval. Although the nanoparticles are the subject of a patent application and thus confidential, previously published work outlines the components used to develop the nanoparticles, although manufacturing processes are slightly different (Kunda *et al.*, 2014; Kunda, Alfagih, Miyaji, *et al.*, 2015; Kaneko *et al.*, 2021). All nanoparticles contain a core of PLGA (Mw 7-17kDa), comprising 50% lactic acid:50% glycolic acid. Spray drying nanoparticles produces dry powders, eliminating cold chain requirements whilst maintaining nanoparticle integrity and stability and also enabling the option of dry powder inhalation. Excipients ensure dispersion of nanoparticles in an aqueous environment, such as the humid lungs. Three dry powder formulations (A-C), testing different excipients, and two emulsion formulations (D and E), which had not

been spray dried, were tested in the current study. Nanoparticles A and B were produced by spray drying using excipients comprising leucine and oligosaccharides of the same family but with different structures, 'A' and 'B'. The oligosaccharides are water soluble to aid in the dispersion of nanoparticles in an aqueous environment and may aid in generating an immune response. Nanoparticle C was produced by spray drying using 100% leucine as an excipient. Nanoparticle D was coated with a water-soluble chitosan and nanoparticle E was coated with a fungus derived, water-insoluble chitosan. Nanoparticle D was subsequently loaded with PspA4 via the double emulsion method (Iqbal *et al.*, 2015; Scherließ and Janke, 2021) at a concentration of 16µg per 1mg of nanoparticle. PspA4 was encapsulated within the PLGA, and water-soluble chitosan coated the PLGA surface. Protein was quantified using a micro BCA protein assay kit. The PspA4 used is the same as that used throughout the entirety of this work, provided by the Miyaji laboratory (Instituto Butantan, São Paulo, Brazil) (Chapter 2.3.1).

5.3.3 Nanoparticle-induced Mo-DC maturation assay

5.3.3.1 Cell culture and Mo-DC differentiation

Following magnetic separation of monocytes from peripheral blood mononuclear cells (as outlined in 5.3.1.2), 10^7 monocytes were resuspended per 10ml of Mo-DC Differentiation Medium (Mo-DC Generation Toolbox, human (Miltenyi Biotec)) containing 1% penicillin–neomycin-streptomycin (PNS) (ThermoFisher Scientific) and mixed well. Cells were plated in a 6-well plate ($1x10^6$ cells in 1ml per well) and incubated for 3 days at 37°C, 5% CO_2 in air, and >95% humidity. Following 3 days of incubation, 1ml of Mo-DC Differentiation Medium (containing 1% PNS) was added to each well to feed cells. Cells were incubated for a further 4 days at 37°C, 5% CO_2 in air, and >95% humidity. On day 7, cells were harvested in a 50ml Falcon tube on ice; adhered cells were released using cold PBS + 2mM EDTA (10-minute incubation). Cells were resuspended in Differentiation Medium and were centrifuged at 300 x g for 10 minutes at 4°C. Supernatant was removed, and cells were washed in 20ml Differentiation Medium. After a second centrifugation step, supernatant was removed, and cells were resuspended in Differentiation Medium for counting. 200,000 Mo-DCs in 200µL Differentiation Medium were seeded per well in a round bottom 96 well plate.

5.3.3.2 Co-culture of Mo-DCs and nanoparticles

To test for nanoparticle-induced maturation of DCs, immature DCs from three donors were incubated at 37°C, 5% CO_2 in air, and >95% humidity for 48 hours in the presence of a maturation stimulus (positive control) (6000 IU/ml TNFα or 100ng/ml lipopolysaccharide, LPS), nanoparticle of desired concentration (15µg/ml, 30µg/ml, or 60µg/ml) or medium alone (negative control). Spray dried

nanoparticles were kept in a desiccator at room temperature until use. Emulsion nanoparticles were prepared fresh on the day by the Formulation and Drug Delivery Research Group and suspended in Differentiation Medium for immediate use.

5.3.3.3 Cell staining for flow cytometry analysis

Cell culture supernatant was discarded following centrifugation of the 96-well plate at 1500rpm for 5 minutes at 4°C. Cell pellets were resuspended in 25µl LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit (1µl in 500µl PBS) (ThermoFisher Scientific, reference: L34964), protected from light and incubated for 15 minutes at 4°C. 5µl of the antibody cocktail detailed in Table 18 was added to each well followed by 30 minutes incubation in the dark at 4°C. 125µl cold PBS was added to each well to wash cells and the plate was centrifuged at 1500rpm for 5 minutes at 4°C. Supernatant was expelled and cells were washed again with 200µl cold PBS. Supernatant was expelled and cell pellets resuspended in 200µl cold PBS for analysis on a Cytek® Aurora cytometer. Cells were kept on ice until acquisition. Data were analysed using FlowJo™ software version 10.7.1 (BD Biosciences).

Table 18: Markers of dendritic cell maturation antibody panel for flow cytometry analysis.

Antibody	Provider	Reference	Relevance
HLADR-FITC	Biolegend	307604	Marker of major histocompatibility complex (MHC) class II molecules expressed on antigen presenting cells and highly expressed on DCs; antigen expression to T-cells
CD86-PE	Biolegend	305406	Low expression on immature DCs, high expression on mature DCs; induces T–cell activation
CD83-APC	Biolegend	305312	Highly and stably expressed by mature dendritic cells
CD209-PerCPCy5.5	Biolegend	330110	Marker of differentiation from monocytes to DCs (increased expression on DCs)
CD40-PECy7	Biolegend	334322	Low expression on immature DCs, high expression on mature DCs; induces T-cell activation
CD14-AF700	Biolegend	301822	Highly expressed on monocytes, not expressed on mature DCs

5.3.4 T-cell activation assay

5.3.4.1 Co-culture of Mo-DCs, PBMCs and nanoparticles

To test for support of T-cell activation by Mo-DCs incubated with nanoparticle-based vaccines, DCs from three donors were co-cultured with PBMCs in the presence of PspA4-loaded nanoparticle, empty nanoparticle or PspA4 antigen (2µg). All conditions were duplicated, and maturation stimulus (100ng/ml lipopolysaccharide) added to duplicate wells for comparison with response from immature DCs in the presence of each stimuli. DCs were harvested on ice following 7 days in culture as detailed in 5.3.3.1 and $1x10^6$ cells in 1ml were seeded per well of a 12-well plate. Nanoparticle-based vaccine or protein antigen alone was added to the test wells and cells were rested for 6 hours at 37°C, 5% CO_2 in air, and >95% humidity. A maturation stimulus (100ng/ml lipopolysaccharide) was added to selected wells following the 6-hour resting period and all cells were incubated for a further 24 hours.

Previously frozen autologous PBMCs (5.3.1.3) were thawed as follows. Each cryovial containing 1 million donor PBMCs in 1ml CTL-CRYOABC medium was placed at 37°C for 10 minutes and inverted twice. Cells from each vial were transferred to a 15ml Falcon tube. Cells remaining in the cryovial were washed dropwise with 1ml pre-warmed (37°C) complete RPMI 1640 medium (RPMI 1640, REF: 52440-025; LOT: 2416388, + 10% foetal bovine serum (ThermoFisher Scientific) + 1% PNS) containing 1% deoxyribonuclease (DNase) (Sigma Aldrich) and added dropwise to the rest of the cells. 8ml of RPMI

containing 1% DNase was added dropwise to the cells, which were then centrifuged at 400 x g for 10 minutes at room temperature. Cell supernatant was removed. A further wash with 10ml RPMI-DNase and centrifugation step was carried out and supernatant removed. Cells were then resuspended in complete RPMI 1640 medium to achieve a final concentration of 1 million cells per 100µl medium. 1 million cells were seeded per well of a 96 well round bottom plate and left to rest for 2 hours at 37°C, 5% CO_2 in air, and >95% humidity.

Following the 24-hour incubation, DCs were harvested in a 50ml Falcon tube on ice; adhered cells were released using cold PBS + 2mM EDTA (10-minute incubation). Cells were resuspended in complete RPMI medium and were centrifuged at 300 x g for 10 minutes at 4°C. Supernatant was removed, and cells were washed in 20ml complete RPMI medium. After a second centrifugation step, supernatant was removed, and cells were resuspended to achieve 100,000 Mo-DCs in 100µL complete RMPI. 100,000 Mo-DCs in 100µL complete RMPI were seeded to each of the wells containing PBMCs. Cells were incubated for 24 hours at 37°C, 5% CO_2 in air, and >95% humidity.

5.3.4.2 Cell staining for flow cytometry analysis

Cells were stained for activation induced markers (AIM) (Table 19) in the same way as detailed in 5.3.3.3.

Table 19: Activation induced markers antibody panel for flow cytometry analysis.

Antibody	Provider	Reference	Relevance
CD3-APCCy7	Biolegend	344818	Protein complex and T-cell co-receptor involved in activating cytotoxic and helper T-cells
CD4-BV605	Biolegend	300556	Marker for $CD4^+$ T-cell subsets
CD8-BV785	Biolegend	344740	Marker for $CD8^+$ T-cell subsets
CD69-BV650	Biolegend	310934	Early marker of T-cell activation
CD154-PECF594	Biolegend	310840	Marker of antigen specific $CD4^+$T-cells
CD137-PECy5	Biolegend	309808	Marker of antigen-activated $CD8^+$ T-cells
CD134-APC	Biolegend	350008	Marker of activated $CD4^+$ T-cells

5.4 Results

5.4.1 Nanoparticles D and E induce non-specific DC maturation

The gating strategy used for selection of live monocyte-derived dendritic cells for flow cytometry analysis is shown in Figure 43. Geometric mean of $HLADR^+$, $CD86^+$, $CD83^+$, $CD40^+$, $CD209^+$ and $CD14^+$ cells was calculated as a proportion of total monocyte-derived viable DCs.

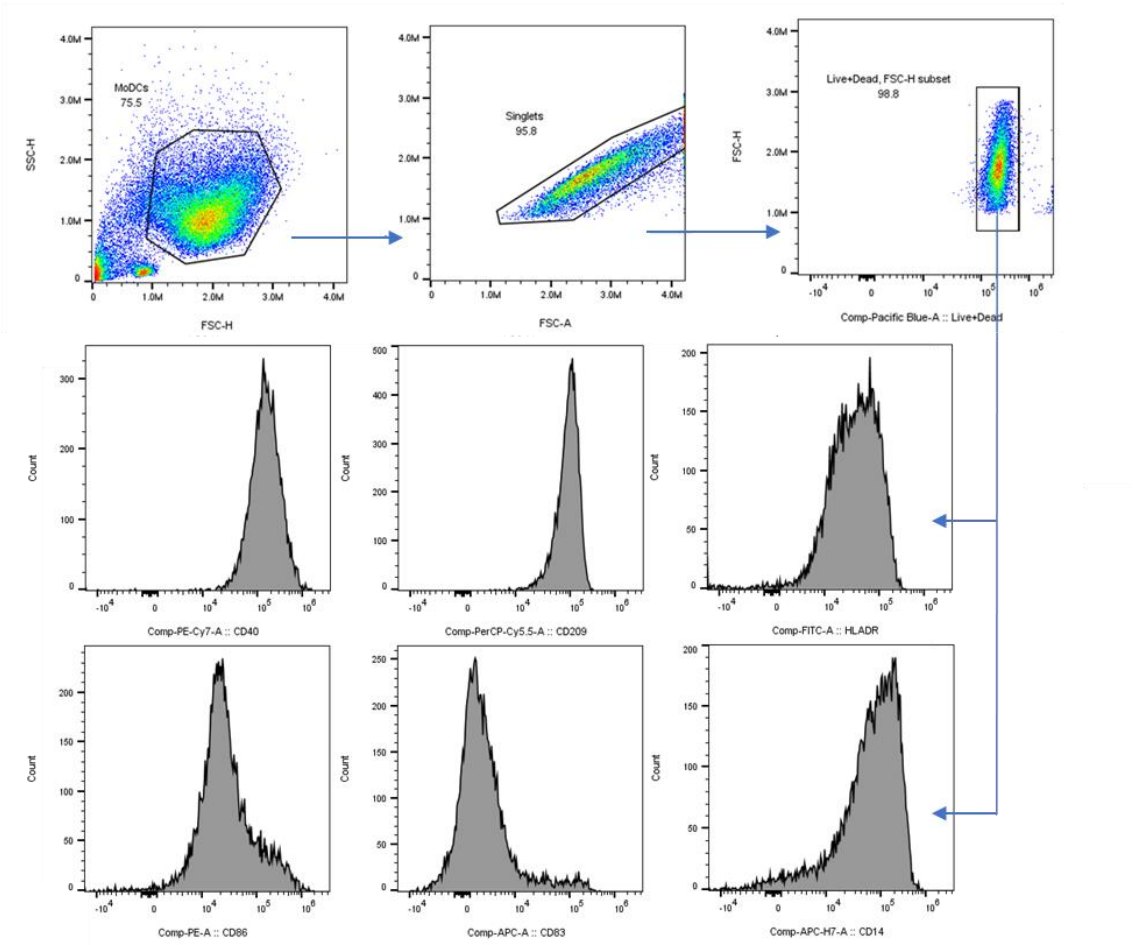

Figure 43: Gating strategy for monocyte-derived dendritic cells from debris, doublets, and dead cells. Cells were acquired using a Cytek® Aurora cytometer. A gate on total monocyte-derived dendritic cells (MoDCs) was set. Doublet cells were excluded from the analysis. A subgate on the live dendritic cells was then set. Counts of total live cells expressing markers of maturation (CD40, CD209, HLADR, CD86, CD83 and CD14) were then taken, as shown in the histograms.

Preliminary experiments showed that 48 hours was optimal to observe induction of DC maturation with positive control (as compared to 24hrs). Cell viability in the presence of each nanoparticle was comparable with that of the negative control, although cell viability decreased at 60µg of nanoparticle (Figure 44).

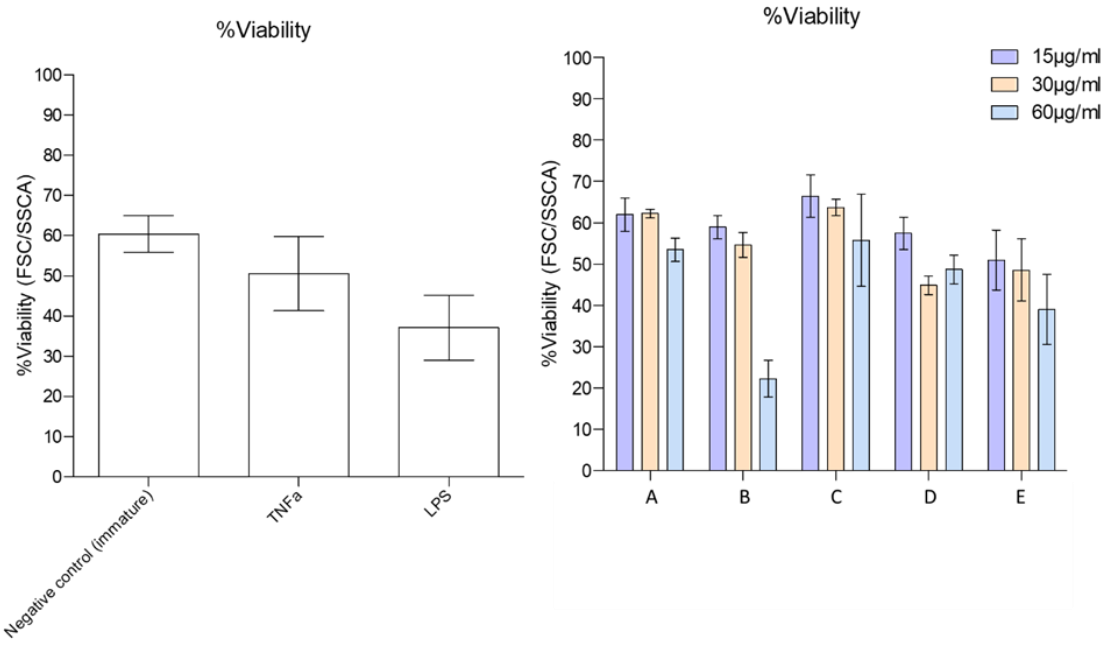

Figure 44: Viability of monocyte-derived dendritic cells in the presence of 15, 30 and 60µg/ml of five nanoparticles (A-E) as compared to media alone (negative control), TNFα or lipopolysaccharide. Immature DCs were incubated at 37°C, 5% CO2 in air, and >95% humidity for 48 hours in the presence of medium alone (negative control), a maturation stimulus (positive control) (6000 IU/ml TNFα or 100ng/ml lipopolysaccharide, LPS) or nanoparticle (A-E) of desired concentration. Cells were stained with LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit and analysed on a Cytek® Aurora cytometer. Data were analysed using FlowJo™ software version 10.7.1 (BD Biosciences) and GraphPad Prism (Version 5.04, GraphPad Software, La Jolla, CA). For 15 and 30µg/ml, n = 3; for 60µg/ml, n = 2 due to limited cell numbers. Bars indicate standard error of the mean across donors.

Dendritic cell maturation, as determined by increased expression in cellular markers CD83, CD86 and CD40, was induced most prominently by nanoparticles D and E (Figure 45). Levels of maturation marker expression were similar to those observed in response to treatment with positive controls. Minimal maturation was induced by nanoparticles A and C. Nanoparticle B showed some markers of maturation, although this was less marked at the lower nanoparticle concentrations with better cell viability and much less prominent than that of D and E. Nanoparticle E was subsequently discontinued by the manufacturer due to increased cytotoxicity and decreased stability during the nebulization process and thus was not taken any further in this present work.

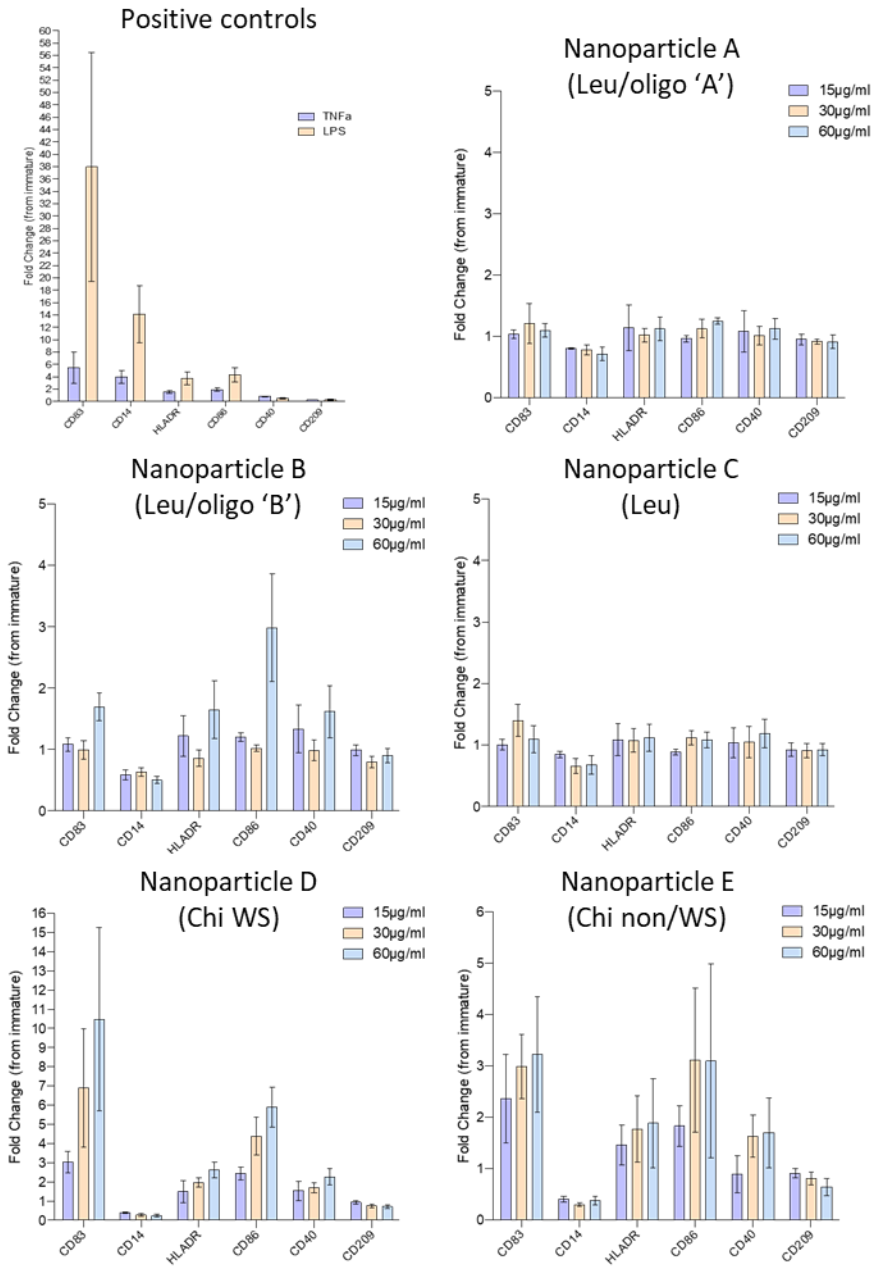

Figure 45: Induction of markers of dendritic cell differentiation and maturation by TNFα and LPS (positive controls) and nanoparticles A-E at 15, 30 and 60µg/ml as compared to medium alone (immature dendritic cells). Immature DCs were incubated at 37°C, 5% CO2 in air, and >95% humidity for 48 hours in the presence of a maturation stimulus (positive control) (6000 IU/ml TNFα or 100ng/ml lipopolysaccharide, LSP), nanoparticle of desired concentration or medium alone (negative control). Cells were stained for each marker and analysed using a Cytek® Aurora cytometer. HLADR$^+$, CD86$^+$, CD83$^+$, CD40$^+$, CD209$^+$ and CD14$^+$ cells were gated as a proportion of total live monocyte-derived DCs. Data were analysed using FlowJo™ software version 10.7.1 (BD Biosciences) and GraphPad Prism (Version 5.04, GraphPad Software, La Jolla, CA). For 15 and 30µg/ml, n = 3; for 60µg/ml, n = 2 due to limited cell numbers. Bars indicate standard error of the mean across donors. Leu = leucine; oligo = oligosaccharide; Chi = chitosan; WS = water soluble.

5.4.2 Nanoparticle D supports T-cell activation, but antigen-specificity is inconclusive

Nanoparticle D was subsequently taken forward into co-culture experiments to test for support of T-cell activation. As 30µg/ml nanoparticle induced good levels of maturation without compromising on cell viability, this concentration of nanoparticle was used for T-cell activation experiments. Two variations of nanoparticle D were tested, one loaded with PspA4 to test for enhancement of antigen-specific adaptive response and one lacking antigen for comparison. The gating strategy to test for markers of T-cell activation is shown in Figure 46.

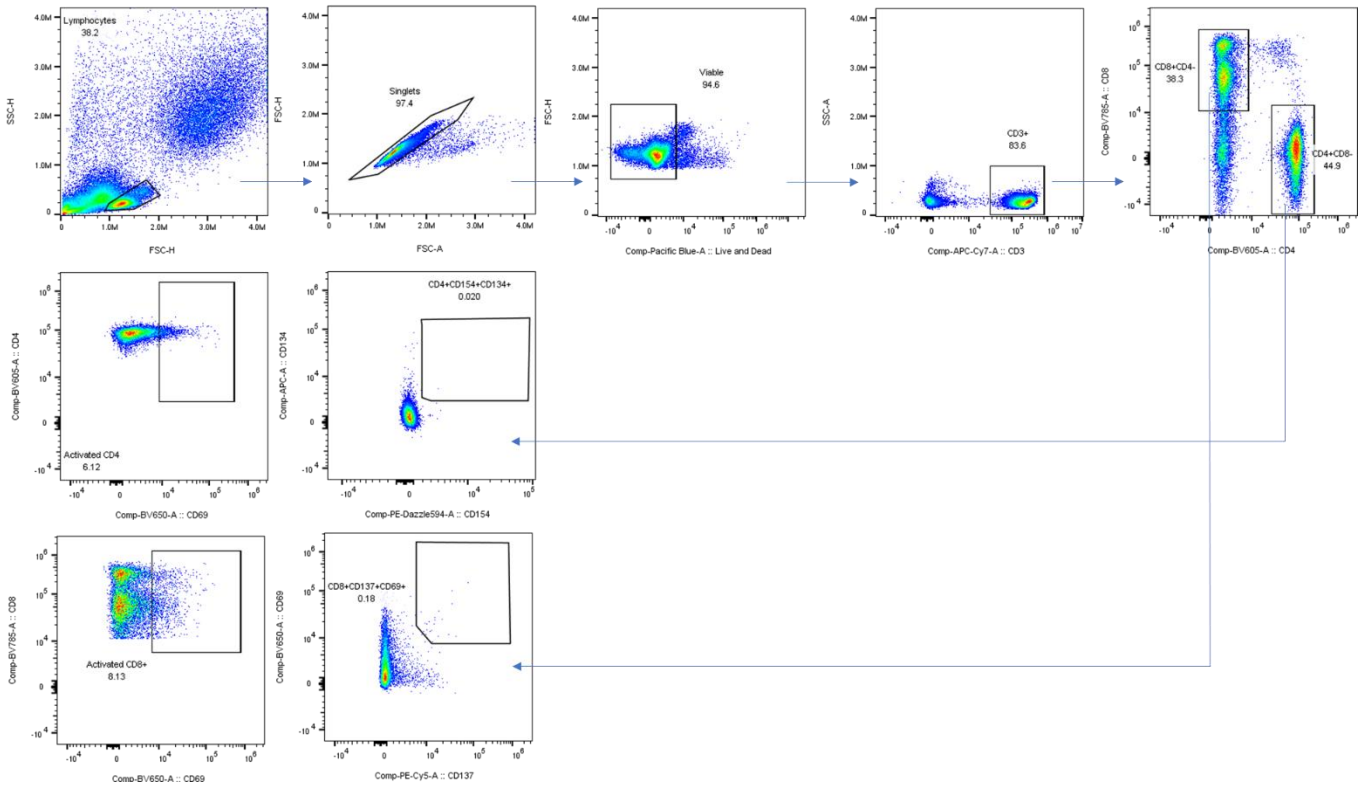

Figure 46: Gating strategy for activated lymphocytes. Cells were acquired using a Cytek® Aurora cytometer. A gate on total lymphocytes was set. Doublet cells were excluded from the analysis. A subgate on the live lymphocytes was then set. Live lymphocytes were then gated for $CD3^+$ T-cells and subsequently gated to separate the $CD4^+$ and $CD8^+$ cells. The $CD4^+$ cells were then plotted as CD4 vs CD69 and CD134 vs CD154 to assess the activation of the T-cells. The $CD8^+$ cells were plotted as CD8 vs CD69 and CD69 vs CD137 to assess activation.

Cell viability was consistent across controls and nanoparticle treated cells (Figure 47). As is depicted in Figure 48, results indicated that both mature and immature DCs (DCs treated with LPS for 24 hours and untreated DCs, respectively) exposed to unloaded nanoparticles stimulated non-specific $CD4^+$ T-cell activation. DCs exposed to PspA4 antigen alone induced some level of T-cell activation that was

greater than the level of activation seen by negative controls (immature DCs without any maturation or nanoparticle stimulus), indicating that the assay is successfully able to detect T-cell activation in response to protein antigen. The ability of protein-unloaded nanoparticle D to induce DC maturation supports the hypothesis that in the absence of maturation stimulus, nanoparticle D induced DC maturation, which subsequently promoted non-specific T-cell activation. However, the addition of PspA4 antigen to nanoparticles appeared to impair the ability of immature DCs to support $CD4^+$ activation. Indeed, the level of T-cell activation observed was reduced compared to that stimulated by DCs exposed to PspA4 antigen alone. $CD8^+$ activation was also stimulated in the presence of unloaded nanoparticle-exposed immature DCs, again supporting the notion that nanoparticle D promotes DC maturation and subsequent support of T-cell activation. However, the level of $CD8^+$ T-cell activation in the presence of immature DCs exposed to unloaded nanoparticle D was less marked than that induced by mature (LPS treated) DCs alone. Again, it appeared that exposure of immature DCs to PspA4-loaded nanoparticles impaired their ability to support $CD8^+$ T-cell activation; T-cell activation was reduced in comparison to that generated by DCs exposed to PspA4 antigen alone. These results suggest that the conjugation of protein to the nanoparticles

could alter nanoparticle properties, preventing DC maturation and thus subsequent non-specific T-cell activation.

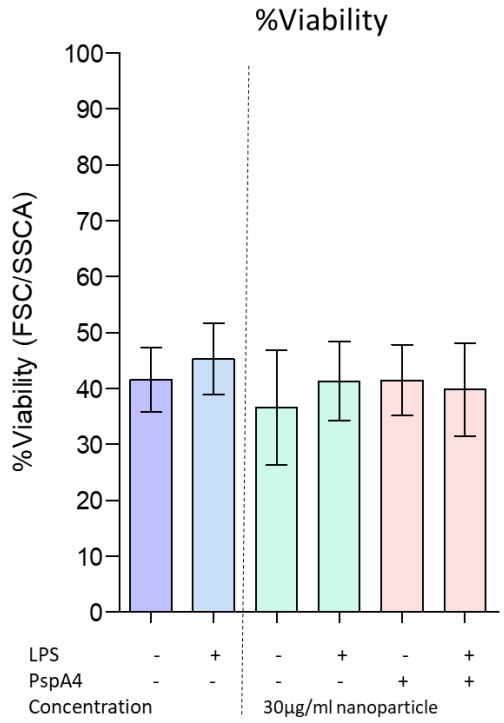

Figure 47: Viability of monocyte-derived dendritic cells when exposed to media alone, maturation stimulus or 30µg/ml nanoparticle D (unloaded and loaded with PspA4). Immature DCs were incubated at 37°C, 5% CO2 in air, and >95% humidity for 48 hours in the presence of a medium alone (negative control), maturation stimulus (positive control) (100ng/ml lipopolysaccharide, LPS) or 30µg/ml nanoparticle. Cells were stained with LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit and analysed on a Cytek® Aurora cytometer. Data were analysed using FlowJo™ software version 10.7.1 (BD Biosciences) and GraphPad Prism (Version 5.04, GraphPad Software, La Jolla, CA). Bars indicate standard error of the mean across 3 cell donors.

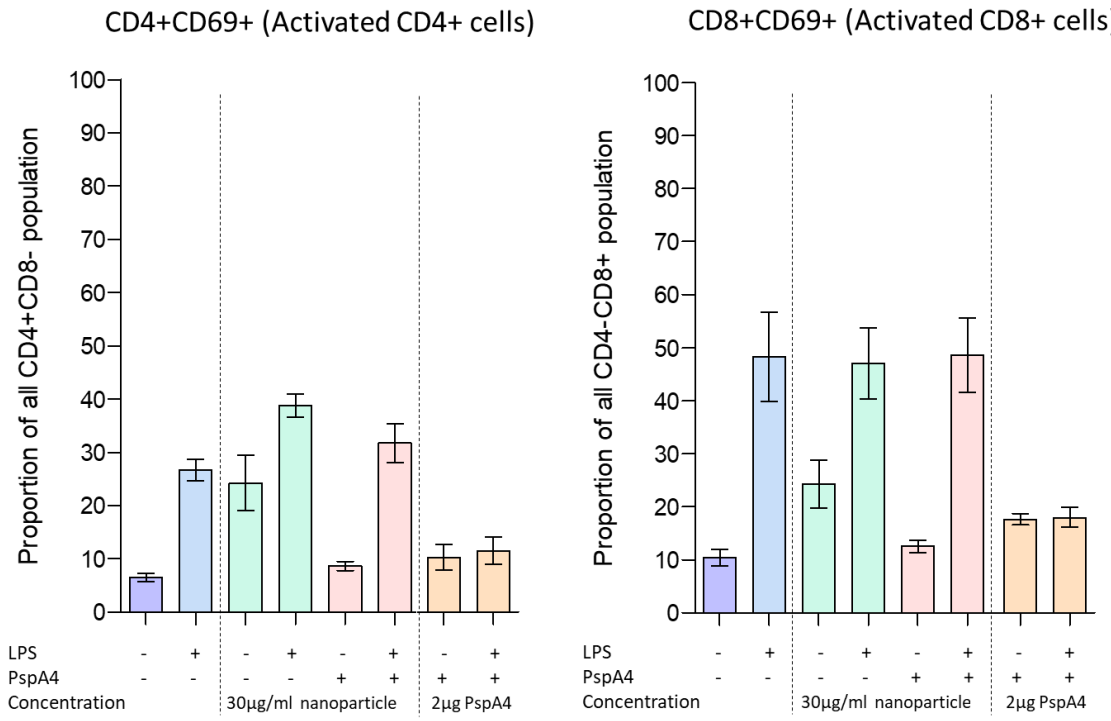

Figure 48: Induction of T-cell CD69 expression (T-cell activation marker) by monocyte-derived dendritic cells incubated with 30µg/ml nanoparticle D. Monocyte-derived dendritic cells from 3 donors were incubated for 24 hours at 37°C, 5% CO2 in air, and >95% humidity in the presence of media alone (negative control), 100ng/ml lipopolysaccharide (LPS) (positive control), unloaded nanoparticle D, nanoparticle D loaded with PspA4 antigen or PspA4 antigen alone. A maturation stimulus (100ng/ml lipopolysaccharide) was added to selected wells (positive control and duplicate wells for all test conditions) following a 6-hour resting period and all cells were incubated for a further 24 hours. 1 million PBMCs per donor (previous frozen) were thawed and seeded per well of a 96 well round bottom plate and left to rest for 2 hours at 37°C, 5% CO_2 in air, and >95% humidity. Dendritic cells were harvested after 24-hour incubation and 100,000 cells in 100µL complete RMPI were seeded to each of the wells containing PBMCs. Dendritic cells and T-cells were incubated for 24 hours at 37°C, 5% CO_2 in air, and >95% humidity. T-cells were stained for activation markers and analysed using a Cytek® Aurora cytometer. Bars indicate standard error of the mean across donors. No significant difference was observed between groups by Friedman test. Data were analysed using FlowJo™ software version 10.7.1 (BD Biosciences) and GraphPad Prism (Version 5.04, GraphPad Software, La Jolla, CA).

The stimulation of antigen-specific T-cell activation by mature DCs exposed to PspA4 antigen, that was greater than the response generated by mature DCs alone, confirmed that the co-culture assay is successful in providing a platform for investigating induction of antigen-specific T-cell activation (Figure 29). As is shown in Figure 49, antigen specific $CD4^+$ T-cell activation was supported by mature and immature DCs exposed to loaded and unloaded nanoparticle D and $CD4^+$ T-cell activation was much greater than that induced by mature DCs alone. However, loading of PspA4 antigen to nanoparticles did not enhance antigen-specific $CD4^+$ activation as compared to PspA4 alone as was expected. Nor was there any enhancement of response in the presence of mature DCs exposed to PspA4-loaded nanoparticle D as

compared to DCs exposed to unloaded nanoparticle D. The response stimulated by mature DCs exposed to loaded nanoparticles was no greater than that induced by mature DCs exposed to PspA4 alone and in the presence of immature DCs, antigen-specific response was less pronounced in the presence of DCs exposed to loaded nanoparticles than unloaded, although still greater than response stimulated by DCs exposed to PspA4 alone. It is thus unclear whether the T-cell response to PspA4 in the case of loaded nanoparticles is masked by the nanoparticles themselves or whether the nanoparticles offer no enhancement of response. It is also unclear whether in the presence of immature DCs, conjugation of PspA4 to nanoparticles enhances response to PspA4 or actually impairs the response to the nanoparticles themselves. Hence, no conclusion can be drawn as to whether nanoparticles are successful in generating a pneumococcal antigen-specific response or simply inducing non-specific immune activation. Antigen-specific CD8+ T-cell activation was somewhat greater in the presence of DCs exposed to loaded nanoparticles, particularly in the presence of mature DCs, but results were generally inconclusive.

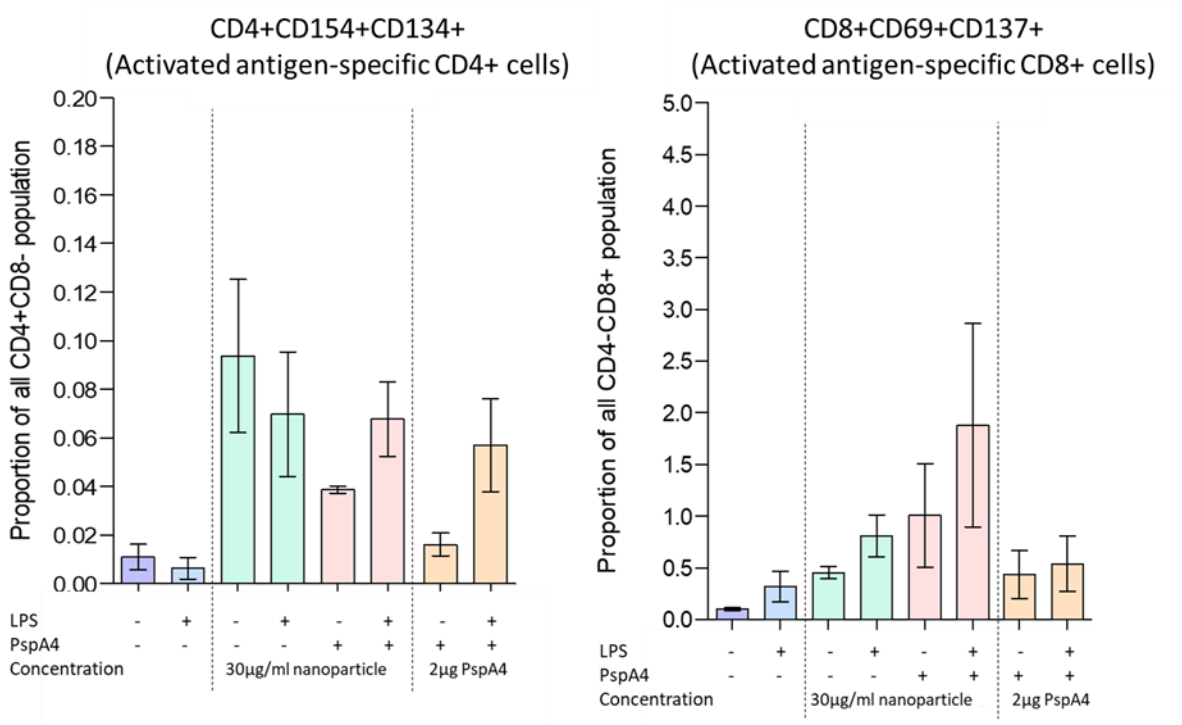

Figure 49: Induction of T-cell activation induced markers by monocyte-derived dendritic cells incubated with 30µg/ml nanoparticle D. Monocyte-derived dendritic cells from 3 donors were incubated for 24 hours at 37°C, 5% CO2 in air, and >95% humidity in the presence of media alone (negative control), 100ng/ml lipopolysaccharide (LPS) (positive control), unloaded nanoparticle D, nanoparticle D loaded with PspA4 antigen or PspA4 antigen alone. A maturation stimulus (100ng/ml lipopolysaccharide) was added to selected wells (positive control and duplicate wells for all test conditions) following a 6-hour resting period and all cells were incubated for a further 24 hours. 1 million PBMCs per donor (previous frozen) were thawed and seeded per well of a 96 well round bottom plate and left to rest for 2 hours at 37°C, 5% CO_2 in air, and >95% humidity. Dendritic cells were harvested after 24-hour incubation and 100,000 cells in 100µL complete RMPI were seeded to each of the wells containing PBMCs. Dendritic cells and T-cells were incubated for 24 hours at 37°C, 5% CO_2 in air, and >95% humidity. T-cells were stained for activation induced markers and analysed using a Cytek® Aurora cytometer. Bars indicate standard error of the mean across donors. No significant difference was observed between groups by Friedman test. Data were analysed using FlowJo™ software version 10.7.1 (BD Biosciences) and GraphPad Prism (Version 5.04, GraphPad Software, La Jolla, CA).

5.5 Discussion

Nanoparticles A-C did not appear to induce a mature DC phenotype. Failure to induce DC maturation could be associated with PLGA nanoparticles needing further optimisation to enhance immunogenicity (Kaneko *et al.*, 2021). Previous findings have shown that that L-leucine microparticles containing polymeric nanoparticles with adsorbed PspA4 were successfully internalized by DCs in cell culture medium, although no analysis of subsequent DC activation was conducted (Kunda, Alfagih, Miyaji, *et al.*, 2015). However, it was shown in the current study that nanoparticle D induced markers of dendritic cell maturation and supported non-specific T-cell activation. Nanoparticle D comprises water-soluble chitosan. Chitosan is widely used in experimental vaccine formulations and of a range of chitosan variants,

water-soluble chitosan in particular has been found to harbor properties that make it well-suited as a candidate for vaccine formulations (Kaneko *et al.*, 2021). Chitosan itself is immunogenic and has been shown to stimulate the murine immune response through activation of the STING-cGAS pathway to stimulate type-1 interferon dependent dendritic cell maturation and enhancement of antigen-specific Th1 responses (Carroll *et al.*, 2016). Chitosan has also been shown to enhance antigen-specific splenic $CD4^+$ proliferation in mice as well as protein-mediated humoral response (Zaharoff *et al.*, 2007). Moreover, chitosan-PsaA nanoparticles developed and used to intranasally immunise mice induced a more pronounced humoral response as compared to intranasal immunisation with PsaA alone and significantly increased protection against lethal challenge as compared to PsaA or chitosan alone (Xu *et al.*, 2015). In the current study, in the presence of mature DCs, the level of antigen-specific $CD4^+$ T-cell activation was similar in response to DCs exposed to unloaded nanoparticles, loaded nanoparticles and PspA4 antigen alone, suggesting that the nanoparticle offers no enhancement of PspA4 response. Whilst the antigen-specific $CD4^+$ response was greater in the presence of immature DCs exposed to loaded nanoparticles as compared to PspA4 antigen alone, the response was reduced in comparison to that supported by immature DCs exposed to unloaded nanoparticle. It is thus unclear whether in the presence of immature DCs, conjugation of PspA4 to nanoparticles enhances response to PspA4 or actually impairs the response to the nanoparticles themselves. Although a greater level of $CD8^+$ activation was observed in response to DCs exposed to PspA4-loaded nanoparticles as compared to PspA4 alone, more work is needed to determine whether this is due to the non-specific activation by nanoparticle D or an enhancement of PspA4-specific response.

Furthermore, loading of PspA4 to nanoparticles appeared to inhibit support of T-cell activation by exposed DCs. Chitosan is cationic and its coating of PLGA nanoparticles elicits a positive surface charge on nanoparticles to enhance interaction with the cell membrane and subsequent internalization (Salatin, Maleki Dizaj and Yari Khosroushahi, 2015). However, the protein loading process was found to reduce the positive surface charge of nanoparticles, although not completely. The effect of this reduction in positive charge could reduce uptake by dendritic cells (Foged *et al.*, 2005; Harush-Frenkel *et al.*, 2007; C. He *et al.*, 2010; Yu *et al.*, 2011; Foroozandeh and Aziz, 2018). Contrastingly, a greater positive charge has also been associated with increased agglomeration of nanoparticles and subsequently reduced cellular uptake (Rancan *et al.*, 2012; Guarnieri *et al.*, 2014). Nanoparticle size is also increased by the loading of protein antigen, which could again perturb efficiency of internalization by DCs (C. He *et al.*, 2010; Walkey *et al.*, 2012), although it has been shown that any nanoparticle size <500μm in diameter is optimal for DC uptake (Foged *et al.*, 2005).

Perhaps most crucially, the interlinked effects of protein corona formation on biological response to nanoparticles must be considered. Upon dispersion in cell culture media, as with any biological environment, nanoparticles will encounter a complex mixture of proteins. These proteins spontaneously adsorb onto the nanoparticle surface forming what is termed the "protein corona", which alters the physiochemical properties, such as surface charge, size and formation of agglomerates, of the nanoparticles (Gebauer *et al.*, 2012; Guarnieri *et al.*, 2014; Kurtz-Chalot *et al.*, 2014; Marichal *et al.*, 2019; Yu *et al.*, 2020). The protein corona is dynamic and changes over time (Mortensen *et al.*, 2013). It has been suggested that it is too simplistic to state for example, that more positively charged particles exhibit greater interaction with the cell membrane or indeed that any interaction between nanoparticle and cell surface is "direct" (Forest and Pourchez, 2017). Rather, interactions occur via the protein corona and the effects of the initial nanoparticle characteristics are important due to their effect on protein corona formation and its subsequent role in biological interactions and cellular uptake (Meiner, Potthoff and Richter, 2009; Saptarshi, Duschl and Lopata, 2013; Kurtz-Chalot *et al.*, 2014; Forest and Pourchez, 2017; Yu *et al.*, 2020). For example, smaller particles generally adsorb a higher density of proteins due to higher surface curvature and the composition of the protein corona also varies based on size, shape, hydrophobicity, surface functionality and charge of nanoparticles (Shannahan *et al.*, 2013; Walkey *et al.*, 2014; Saha *et al.*, 2016). A difference in properties between loaded and unloaded nanoparticles (such as shape, size and surface charge) may have induced differing temporal protein corona formations upon dispersion in media, altering the physiochemical and biological identities of the nanoparticles and thus biological response, including cellular uptake (Nel *et al.*, 2009; Walkey and Chan, 2012; Lesniak *et al.*, 2013; Mortensen *et al.*, 2013; Moore *et al.*, 2015; Forest and Pourchez, 2017).

It has been shown that the level of internalization of nanoparticles by cells is not directly dependent on the initial surface charge of nanoparticles as acquired charge in cell culture media is homogenized by the protein corona formation but rather by the level of agglomeration, which varies based on cell culture medium (Guarnieri *et al.*, 2014; Kurtz-Chalot *et al.*, 2014). There is conflict in the literature surrounding the effects of the protein corona on cellular uptake (Forest and Pourchez, 2017). For example, serum free media has been shown to limit protein corona formation around the nanoparticles and enhance internalization by cells (Lesniak *et al.*, 2013). However, authors have reported that medium containing serum diminishes aggregate formation as compared to serum-free medium as corona formation advantageously prevents nanoparticle/nanoparticle interactions and thus aggregation, enhancing cellular uptake (Maiorano *et al.*, 2010; Izak-Nau *et al.*, 2013; Tenzer *et al.*, 2013). The level of serum protein adsorption onto nanoparticles has also been positively correlated with uptake into cells

(Qiu *et al.*, 2010). Serum free medium was used in our experiments at the time of nanoparticle exposure to DCs as incubation of nanoparticles in the presence of serum has been shown to induce a change in nanoparticle surface charge that turns initially positively charged nanoparticles negative (Lundqvist *et al.*, 2008; Qiu *et al.*, 2010; Forest and Pourchez, 2017); although this greatly depends upon the type of surface coating/surfactant used, the concentration of that surfactant and the core nanoparticle materials. Thus, it is important to consider how results may differ in the presence of medium containing serum. The biological milieu greatly influences protein corona formation (Kurtz-Chalot *et al.*, 2014; Forest and Pourchez, 2017) and so it is vitally important to consider how interaction with the host environment *in vivo* may significantly alter the uptake of nanoparticle-based vaccines or their effect on the immune system as compared to observations made *in vitro* (Nel *et al.*, 2009; Forest and Pourchez, 2017). Biologically, proteins within the corona may act as opsonins, enhancing uptake by the mononuclear phagocyte system (Walkey *et al.*, 2012; Saha *et al.*, 2016).

Possible hypotheses that should further be explored for why PspA4 loading to nanoparticles might impair adaptive response include (i) diminished positive charge of nanoparticles impairs cellular uptake (ii) increased size, which is further enlarged upon dispersion in cell culture medium due to adsorption of proteins onto nanoparticle surface, impairs internalization (iii) altered physical properties of nanoparticles, such as size and shape, reduce the level of protein adsorption onto the nanoparticle surface, thus less prevention of nanoparticle/nanoparticle interactions takes place and a greater level of agglomeration ensues, limiting uptake.

It is important to note that samples used in this work were not obtained from volunteers in our human challenge model. Therefore, age, smoking status, recent pneumococcal exposure or indeed any health information is unknown. Future work might involve using the co-culture assay to evaluate responses to PspA4 antigen in volunteers known to be recently colonised with pneumococcus to establish whether the lack of antigen-specific response observed is due to a lack of induction by the nanoparticles or a lack of responsiveness in cells. Beyond this, responses to loaded nanoparticles can be evaluated prior to and post-controlled pneumococcal exposure to determine whether recent exposure boosts adaptive response to the vaccines. T-cell proliferation assays should also be conducted to assess whether cells are proliferating in response to stimulus. The structure of PspA4 released from nanoparticles should also be investigated. In previous work, it was found that the alpha helical content of PspA4 was reduced upon release, most likely due to the adsorption and desorption process or spray-drying (Kunda, Alfagih, Miyaji, *et al.*, 2015). Although antibody recognition and lactoferrin binding experiments provided an indication

that PspA delivered via nanoparticles or nanocomposite microparticles was stable and retained antigenicity for generating an immune response, PspA4 was previously adsorbed onto nanoparticle surfaces rather than encapsulated. Thus, further work should confirm the effect of the manufacturing process on protein structure. In addition, alternative proteins should be investigated to establish whether response differs with protein antigen.

Whilst steps were taken to ensure purity of nanoparticles during the manufacturing process including using pyrogen free water and consumables and autoclaving/filtering where required, no purification steps were taken beyond this. Previously, LPS checks have been conducted prior to *in vivo* studies (Rodrigues *et al.*, 2018), which found no LPS contamination and the same processes were followed for the current work. However, the purity of nanoparticles should be confirmed, particularly given the ambiguous results of the current study, as LPS is known to have a profound effect on T-cell populations (McAleer and Vella, 2008) and any contamination within nanoparticles could have affected the results of this study.

Work on this project was somewhat inhibited due to the ongoing Covid-19 pandemic. The experiments carried out here would ideally have been performed using lung cells obtained via bronchoalveolar lavage to determine the effects of nanoparticles on target cells, which could not go ahead due to the pandemic. Furthermore, plans to evaluate the effects of excipients alone (from nanoparticles A-C) were halted due to technical faults with the spray drier. Finally, work was limited due to a lack of supply of PspA4 antigen, obtained from the Miyaji laboratory (Instituto Butantan, São Paulo, Brazil).

5.6 Conclusion

In conclusion, a platform for *in vitro* testing of novel nanoparticle-based vaccine formulations on human cells has been developed. Whilst the results of the effect of nanoparticles on DC maturation and T-cell activation were inconclusive, they point to further work that can be conducted in the testing of nanoparticles as a delivery platform of immunogenic antigens to antigen-presenting cells (APCs) at mucosal sites. The assay developed in this work is functional and can be used to further investigate nanoparticle effects on cells *in vitro.*

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Chapter 6

General discussion

6.1 Introduction

The identification of serotype-independent cellular and humoral correlates of protection against pneumococcal infection is needed to accelerate development of serotype-independent vaccines. Protein-based vaccines are a promising method for serotype-independent immunisation, which could be effective in infants (Holmlund *et al.*, 2006; Zhang, Bernatoniene, Bagrade, Pollard, *et al.*, 2006; Pollard, Perrett and Beverley, 2009; Lebon *et al.*, 2011), the elderly (German *et al.*, 2018; Adler *et al.*, 2021) and potentially in high-risk groups, such as HIV positive individuals (Collins *et al.*, 2013). The study of host-pathogen interactions using a controlled human model of pneumococcal challenge elucidates immunological mechanisms by which colonisation with pneumococcus, itself an immunising event, is prevented or controlled.

This thesis provides new evidence on the adaptive immune response to pneumococcal protein antigens in young, healthy adults to inform protein-vaccine development. The main aims of this work were as follows:

- To develop a multiplex assay allowing rapid detection of protein-specific antibody responses to a library of highly conserved purified pneumococcal protein antigens.
- To determine correlates of natural protection against experimental pneumococcal colonisation of the nasal mucosa.
- To establish which pneumococcal protein antigens are immunogenic, generating a significant humoral immune response.
- To evaluate memory B-cell responses *in vitro* to selected pneumococcal proteins to determine whether the proteins induce adaptive immune response.
- To assess the potential efficacy of five novel nanoparticle-based vaccines in stimulating adaptive immunity to pneumococcal protein antigens *in vitro*.

6.2 Defining protection

Throughout most of this work, the key measurable for defining protein-mediated protection against pneumococcal infection was protection against experimental colonisation. Prevention of pneumococcal colonisation following intranasal inoculation with the bacteria is a measurable endpoint

that can be ethically tested for in human challenge models. However, when considering vaccine-mediated protection, prevention of carriage may not in fact be the desired outcome. As colonisation itself is an immunising event (Ferreira *et al.*, 2013; Mitsi *et al.*, 2020; Adler *et al.*, 2021), which may strengthen and reinforce immunity conferred by vaccination, vaccine-mediated protection may look like prevention against severe disease, in this instance pneumonia, or low colonising density and short carriage duration. Reducing colonising density may in turn prevent pneumonia episodes (Carr *et al.*, 2021) but maintaining a low level of carriage could provide some lung immunity against *S. pneumoniae* (Mitsi *et al.*, 2020). Moreover, given the serotype-independent nature of a protein-based vaccine, the issue of the nasopharynx providing a reservoir for serotype replacement would be ameliorated (Veenhoven *et al.*, 2003; Neill *et al.*, 2013). Additionally, replacement colonisation with other bacterial species might arise in the event of elimination of pneumococcal carriage. Veenhoven and colleagues found that whilst PCV reduced the prevalence of middle-ear fluid cultures with vaccine-serotype pneumococci in a population of paediatric patients aged 1-7 years with recurrent episodes of acute otitis media, there was three times increase in cultures positive for *S. aureus* (Veenhoven *et al.*, 2003). Competition between *S. pneumoniae* and *S. aureus* has been found in healthy children aged 4-9 years (Bogaert *et al.*, 2005). *H. influenzae* and *M. catarrhalis* are also common colonisers of the respiratory tract, particularly in children, and often co-colonise with *S. pneumoniae* (Sulikowska *et al.*, 2004; van Gils *et al.*, 2011; Cobey and Lipsitch, 2013). Thus, elimination of *S. pneumoniae* may have consequences on the prevalence of these pathogens and subsequent disease. A localised, robust and long-lasting immune response could provide protection against pneumonia and invasive disease whilst not disrupting the presence of pneumococci on the nasopharyngeal mucosa (McDaniel and Swiatlo, 2016).

Nevertheless, there is a strong argument to be made that elimination of carriage is ultimately necessary given that it is generally the pre-requisite to invasive disease and further, without complete elimination, mucosal disease would remain untreated. Whilst not life-threatening, otitis media is a cause of morbidity and can have major implications on a child's life such as affecting a child's education (Grindler *et al.*, 2014; Felipe, 2018). Therefore, a pneumococcal vaccine should strive to eliminate both non-life-threatening and life-threatening disease. It is plausible that higher levels of antibodies may be necessary to confer mucosal protection against *S. pneumoniae* compared to antibody levels needed for protection against systemic infection (Pelton *et al.*, 2003; Bogaert *et al.*, 2004; Dagan *et al.*, 2016). If we can define the colonising density and/or duration threshold below which *S. pneumoniae* will cease to become invasive or cause disease, particularly in the presence of viral coinfection, perhaps we could aim to reduce colonisation to that point. However, this might be unattainable in the case of wide-scale vaccination as

individual monitoring would likely be required (Bogaert, De Groot and Hermans, 2004). Suppression of *S. pneumoniae* invasion in the presence of influenza co-colonisation by a protein-based vaccine through reduction of bacterial density in the nasopharynx of mice has been reported (Khan, Xu and Pichichero, 2017).

Differential gene expression of various proteins involved in pathogenesis has been shown to vary across niches following intranasal challenge in murine models and in *in vitro* conditions mimicking biological environments (LeMessurier, Ogunniyi and Paton, 2006; Mahdi *et al.*, 2008; Ogunniyi *et al.*, 2012; Aprianto *et al.*, 2018). This variation in expression has also been shown across pneumococcal serotypes (Mahdi *et al.*, 2008). It is important to consider the different expression levels of proteins at different timepoints during infection and in various niches when designing vaccines to protect against pneumococcal disease, particularly if protection against carriage is difficult to achieve (Chapter 3.5). Proteins with lower levels of expression during colonisation but higher levels of expression during disease may not have induced a robust systemic IgG response in EHPC volunteers following colonisation in our model. Systemic anti-protein IgG profiles following a pneumonia episode for example may provide a different perspective on which proteins are immunogenic during disease and potentially protective against a subsequent disease episode.

6.3 Discussion of key findings

6.3.1 Utilising Luminex technology

Multiplex assays enable rapid analysis of antibody response to multiple analytes simultaneously using the minimum amount of sample, which is often very limited. The EHPC team is uniquely placed in its ability to utilise a biobank of samples taken from human challenge studies to screen for correlates of protection against experimental pneumococcal colonisation in a range of cohorts and evaluate immune response to pneumococcal carriage. The supply of a vast pneumococcal protein library by the Malley laboratory (Boston Children's Hospital, Harvard Medical School, MA, USA) has enabled the development of a Luminex-based platform for the evaluation of protein-mediated immune response to experimental pneumococcal colonisation. Multiplex Luminex assays have previously been demonstrated as a viable method of quantifying circulating IgG (Pickering *et al.*, 2002; Lal *et al.*, 2005; Pavliakova *et al.*, 2018) and previous studies have developed Luminex assays for the detection of anti-pneumococcal protein IgG (Shoma *et al.*, 2010; Lebon *et al.*, 2011; Jiménez-Munguía *et al.*, 2015). However, the platform described in the current work is unique in its ability to detect changes in levels of circulating IgG against conserved pneumococcal proteins in response to experimental exposure with pneumococcus under controlled

conditions using the EHPC model. In future work, this assay will enable the detection of response kinetics from baseline (prior to exposure) across multiple timepoints post-challenge with *S. pneumoniae.* Moreover, optimisation of the assay for detection of antibodies present in nasal wash samples will enable the measurement of mucosal IgG against the entire protein library, providing the opportunity to directly compare circulating and mucosal humoral response at the same timepoint.

Luminex technology does have its drawbacks. For example, Luminex assays have been deemed unreliable to evaluate antibody response to pneumococcal polysaccharide antigens for the assessment of humoral immunocompetence in clinical practice (Whaley *et al.*, 2010; Zhang *et al.*, 2013; Hajjar *et al.*, 2018; Sorensen and Edgar, 2018). Furthermore, a high level of variation between quantitative results from different laboratories using Luminex assays to quantify anti-pneumococcal antibody response has been observed, although qualitative agreement was good (Zhang *et al.*, 2013). High inter-assay variability of a commercial Luminex assay to determine cytokine concentrations in serum has also been shown (Chowdhury, Williams and Johnson, 2009). Indeed, at the beginning of the assay development process in the current work, a high level of inter-assay variation was observed, which likely arose from diluting small amounts of beads for each experiment. This variation was overcome by creating a concentrated 'master-mix' of panels, which was diluted for each experiment. Although the level of inter-assay variation observed was still relatively high, averaging 22.45%, the variation observed was comparable to similar studies conducted previously with *Staphylococcus aureus* (Verkaik *et al.*, 2008) and pneumococcal antigens (Lal *et al.*, 2005; Jiménez-Munguía *et al.*, 2015). Any proteins with an inter-assay CV >25% may be considered less reproducible (30.67% of all analytes in assay). Thus, it must be considered that as a platform, Luminex in general can have high inter-assay variability when it comes to absolute values, although general qualitative agreement is good. Therefore, extremely controlled conditions are necessary to generate directly comparable results across assays. Particularly, discrepancies between antibody quantification using this Luminex assay and previous results determined by ELISA or other multiplexing methods such as MSD technology may be likely. In addition, the need for a solid phase in bead-based assays might affect specific binding of IgG to proteins to a greater extent than is seen with MSD technology for example (Marchese *et al.*, 2009). Importantly, absolute MFI values obtained using the Luminex assay may not be entirely reproducible over time, although reproducibility to detect changes in immune response is likely to be more reliable (Cham *et al.*, 2008; Chowdhury, Williams and Johnson, 2009; Crompton *et al.*, 2010; Zhang *et al.*, 2013). We therefore emphasise the importance of always assessing inter-assay variation using an internal positive control consistently used across plates to ensure that results obtained across separate assays are comparable. In our work, pooled day 29 sera from immune EHPC volunteers was used

as an internal positive control across assays and variation in MFI values for positive controls between plates of which results were to be pooled or directly compared was always assessed to ensure comparability of results (<25% inter-assay CV for all proteins).

Nevertheless, there are many advantages to Luminex assays. Firstly, although variation is high, inter-assay CV for the detection of cytokine concentrations in human plasma is relatively comparable between MSD and Luminex techniques, ranging from 1.9-18.2% for Luminex and 2.4-13.9% for MSD (Günther *et al.*, 2020). Furthermore, whilst MSD has better sensitivity than Luminex, Luminex is more precise, with a higher limit of quantification for much fewer analytes in human plasma than other assays. Most importantly, Luminex assays enable the detection of a much higher number of analytes in multiplex, with the lowest amount of sample and are customizable, which has enabled this work to be conducted. Overall, Luminex technology is considered more robust in multiplexing than MSD technology as many more analytes can be run per specimen. The detection of the fold change in anti-PspA1 IgG from baseline to 29 days post-challenge by the Luminex assay was validated against an ELISA and results were highly comparable; Luminex appeared to detect a greater change in IgG response than did ELISA (Figures 23(B), 24).

6.3.2 Systemic humoral response prior to experimental pneumococcal challenge does not correlate with serotype-independent protection against colonisation

In agreement with previous studies (Ferreira *et al.*, 2013; Adler *et al.*, 2021; Araujo *et al.*, 2021), systemic anti-protein IgG at baseline against any of the pneumococcal proteins in our library did not correlate with protection against carriage following experimental exposure to serotype 6B or 15B pneumococcus. Where there was a trend in levels of anti-protein IgG and protection against experimental colonisation, this was not consistent between serotypes, i.e. different proteins associated with protection against experimental colonisation with Spn6B and Spn15B. In volunteers protected against colonisation with Spn6B, baseline IgG against SP0742 and SP0620 appeared to be higher than in those who were colonised, although both these proteins displayed generally low IgG titres. In volunteers protected against colonisation with Spn15B, baseline levels of IgG against PdB, SP2099, SP1833 and PspA1 tended to be higher in volunteers protected against experimental colonisation compared to those who were susceptible to colonisation. Differences in protein gene expression across the different serotypes at different stages in pathogenesis (LeMessurier, Ogunniyi and Paton, 2006; Mahdi *et al.*, 2008; Ogunniyi *et al.*, 2012; Aprianto *et al.*, 2018) may be a reason for this and the findings of this work further point to the

need for protein-based vaccines to incorporate various proteins in order to be truly serotype-independent.

As previously discussed (Chapter 3.5), although baseline levels of naturally-acquired circulating or mucosal anti-capsular IgG have also been shown not to correlate with protection against experimental pneumococcal acquisition (Pennington *et al.*, 2016), systemic anti-polysaccharide IgG, induced by PCV, plays a role in reducing colonisation density through antibody-mediated agglutination of bacteria and thereby leads to a decreased risk of disease to vaccinated individuals as well as reduced transmission (Collins *et al.*, 2015; Roche *et al.*, 2015; Mitsi *et al.*, 2016; German *et al.*, 2019). Higher levels of antibodies against pneumococcal proteins than those observed at baseline in our EHPC volunteers may be necessary to confer mucosal protection against *S. pneumoniae* carriage acquisition as compared to protection against systemic infection (Pelton *et al.*, 2003; Bogaert *et al.*, 2004; Xu, Casey and Pichichero, 2015; Dagan *et al.*, 2016). If a protein-based vaccine, or multiple carriage episodes induce increased titres of anti-protein IgG, protection against a subsequent acquisition episode may be conferred, with protection being defined as low carriage density and short carriage duration due to antibody-mediated agglutination of the bacteria and subsequent mucociliary clearance. It is likely that IgG against multiple proteins would be needed to confer serotype-independent protection against colonisation.

Based on previous findings, baseline levels of PspA1 or PspC6/9 isolated from the serotype 6B challenge strain might have been expected to correlate with protection against experimental colonisation with the homologous serotype (McCool *et al.*, 2002). IgG against PspA isolated from the same serotype as used for experimental challenge in a human model of infection in the US was previously shown to correlate with protection against experimental carriage but this was not seen in the case of challenge with a heterologous serotype (McCool *et al.*, 2002). Moreover, higher levels of anti-PspA4 IgG at baseline might have been expected to correlate with protection against colonisation as anti-PspA4 antibodies have been shown to be cross-reactive with PspA of different clades across families (Darrieux *et al.*, 2008; Moreno *et al.*, 2010), but this was not observed.

There is contrasting evidence surrounding the protective effect of anti-pneumolysin IgG (McCool *et al.*, 2003; Holmlund *et al.*, 2006; Zhang, Bernatoniene, Bagrade, Pollard, *et al.*, 2006; Mendy *et al.*, 2017), which did not significantly correlate with protection against experimental colonisation in the current work but did appear to be higher at baseline in volunteers protected against colonisation with Spn15B. As previously highlighted, pneumolysin is a component of multiple trial vaccine formulations (Kaur *et al.*, 2014; Entwisle *et al.*, 2017; Chan *et al.*, 2019), however, an immunogenic trivalent vaccine comprising

recombinant PcpA, PhtD, and pneumolysin conferred little protection against nasopharyngeal pneumococcal carriage in infants from the Gambia (Odutola *et al.*, 2017). Incremental efficacy in preventing acute otitis media over PCV13 in native American infants was also not demonstrated by the vaccine (Hammitt *et al.*, 2019). It is important to note that several factors could have contributed to the lack of protection conferred by the aforementioned novel trivalent protein vaccine, however. As outlined by Pichichero (2017), risk factors for high density pneumococcal nasopharyngeal colonisation prevail in trial populations, high density colonisation occurs in infants in low and middle-income settings (Usuf *et al.*, 2014) and nasopharyngeal colonisation in early life may adversely impact immune responses to vaccines (Madhi *et al.*, 2011; Licciardi *et al.*, 2014). Furthermore, otitis-prone children have been found to produce lower levels of serum and mucosal antibody to six pneumococcal protein antigens (PhtD, PhtE, LytB, PcpA, PspA and Ply) compared to non-otitis-prone children (Kaur, Casey and Pichichero, 2011; Xu *et al.*, 2016) and thus otitis-prone children may respond less prominently to vaccination with a protein-based vaccine than the general population. Differing immune responses based on age, prior pneumococcal exposure and socioeconomic conditions must also be considered (Lundgren *et al.*, 2012).

Computational biology analysis could be used to identify a group of proteins, rather than individual proteins, that confer protection against pneumococcal colonisation to inform vaccine development. This work is ongoing within our group.

6.3.3 Experimental pneumococcal colonisation induces systemic humoral response to pneumococcal proteins

Significantly increased systemic IgG against PspA1, PspC9, SP1032 and SP0346 was detected 29 days post-inoculation in the cohort colonised with Spn6B. The increase in anti-PspC6 post-colonisation with Spn6B showed borderline significance. In volunteers colonised with Spn6B, a ≥2-fold increase in median IgG was also elicited against SP1942 and SP0620. No significant difference was observed in anti-protein IgG response 14 days post-challenge with Spn15B, although a ≥2-fold increase from baseline in levels of IgG against SP0435, SP0604 and SP2207 was generated in response to Spn15B colonisation. In carriage negative participants, no significant change in anti-protein IgG was elicited in those challenged with Spn6B or Spn15B.

It is well-established that PspA and PspC are immunogenic proteins (McCool *et al.*, 2002, 2003; Ferreira *et al.*, 2013; Adler *et al.*, 2021; Araujo *et al.*, 2021). As significantly increased PspA1 and PspC9 IgG was detected post-colonisation in Spn6B-colonised volunteers but not in those colonised with Spn15B, it could be that IgG elicited in response to PspA and PspC is specific to the protein expressed by the

colonising strain and antibodies are not cross-reactive. It is well established that different serotypes express different clades of these proteins, which exhibit high variability in their N-terminal regions. Antibodies raised against different clades exhibit varying levels of cross-reactivity, for example, anti-PspA4 antibodies show high levels of cross-reactivity against other clades, whereas anti-PspA1 antibodies have limited cross-reactivity (Nabors *et al.*, 2000; Darrieux *et al.*, 2008; Moreno *et al.*, 2010), which is in line with the findings of our study. This may therefore explain why we only observe a significant increase in anti-PspA and anti-PspC IgG in response to colonisation with Spn6B but not Spn15B because the proteins coated to the Luminex microspheres are those expressed by Spn6B (PspA1, PspC6, PspC9). The fact that no increase in IgG against PspA4 (expressed by TIGR4 but not Spn6B) was observed in response to colonisation with Spn6B further substantiates this hypothesis. However, previous findings have shown that in serum taken from young, healthy adults (aged 18-30) experimentally challenged with Spn6B, anti-PspA4 IgG was reduced 27-29 days post-experimental challenge as compared to pre-challenge in individuals who did not develop subsequent colonisation (Araujo *et al.*, 2021). The decrease in anti-PspA4 IgG in serum is likely due to the migration of circulating IgG to the nasal mucosa to control colonisation by the challenge strain. Serum IgG against PspC3, 6, and 8 was also reduced 27-29 days post-challenge in carriage negative individuals, suggesting a role for these antibodies in protection against colonisation. We did not observe a significant decrease in anti-protein IgG post-challenge in the present study. A limitation of the present study is that the PspA and PspC clades expressed by the Spn15B were not included in the Luminex assay and therefore it cannot be determined whether the lack of induction in anti-PspA and anti-PspC IgG response by Spn15B colonisation is due to different protein expression to that of Spn6B or reduced immunogenicity of the strain.

SP0346, also known as Cps4A (Capsular polysaccharide biosynthesis protein), plays a vital role in capsule synthesis (Shainheit, Mulé and Camilli, 2014; Nakamya *et al.*, 2018) and the *Cps4A* gene has been found to be one of the most highly expressed pneumococcal genes in nasopharyngeal samples of healthy, colonised children in Peru (Sakai *et al.*, 2013). In a murine model of infection, SP0346 transcription was significantly upregulated during pneumonia and bacteraemia, suggesting that this protein plays a role in disease pathogenesis and thus may be particularly relevant in designing a novel vaccine against pneumonia (Gupta, Shah and Swiatlo, 2009). Using the PneumoExpress tool (https://veeninglab.com/pneumoexpress) developed by the Veening Lab (Aprianto *et al.*, 2018), SP0346 expression was shown to be increased in conditions mimicking the nose (colonisation) and lung (pneumonia) as well as migration from the nose to the lung and blood. Increased expression of SP0346 in the nose is interesting given that reduction in capsule expression during transparent morphology is

associated with colonisation (Hammerschmidt *et al.*, 2005; Kadioglu *et al.*, 2008; Li and Zhang, 2019). However, it would be expected that expression of SP0346 would increase during invasion, when increased capsule expression occurs (Trappetti *et al.*, 2011).

SP1032, also known as PiaA (pneumococcal iron acquisition ABC transporter), is a relatively established vaccine candidate (Brown *et al.*, 2001; Gor *et al.*, 2005; Entwisle *et al.*, 2017) and has been shown to confer protection against pneumococcal nasopharyngeal colonisation and systemic infection in mice and induce IL-17A production in adenoidal cells of children (Brown *et al.*, 2001; Lu *et al.*, 2018). Mucosal immunisation of mice with a vaccine of PiaA and PiuA elicited specific serum and mucosal antibody responses, and provided protection against fatal intranasal challenge with *S. pneumoniae* in a serotype-independent manner (Jomaa *et al.*, 2006). Furthermore, the protein is immunogenic from early infancy and has been shown to be clinically relevant, inducing IgG in acute and convalescent-phase patients with pneumococcal septicemia (Whalan *et al.*, 2005). Even more importantly, the induction of anti-SP1032 IgG in patients with pneumococcal septicemia is serotype-independent, as it was detected in patients with invasive disease caused by 13 different serotypes. SP1032 is thus a highly promising protein-vaccine candidate. Nevertheless, further work is needed to assess the level of protection conferred by the protein. PiaA is a component of a novel multi-protein-based vaccine, PnuBioVax, that has been shown to be safe and immunogenic in Phase I trials and will soon undergo Phase II testing (Entwisle *et al.*, 2017). A ≥2-fold increase in antibody titres against PiaA was observed for most participants following immunisation with the vaccine. The relevance of this protein as vaccine candidate further lies with its role as an ABC transporter responsible for iron-uptake; pneumococcal ABC transporters are promising protein-vaccine candidates due to their role in nutrient uptake and virulence (Brown *et al.*, 2001; Garmory and Titball, 2004; Basavanna *et al.*, 2009) and in this current work, although not significant, baseline IgG against the amino ABC transporter SP0620 was higher in volunteers protected against colonisation with Spn6B as compared to those susceptible to colonisation.

Anti-protein IgG at baseline and total colonisation density across the study or duration of carriage in volunteers colonised with Spn6B did not significantly correlate for any of the proteins. Higher levels of anti-SP0757 IgG at baseline showed a weak association with decreased carriage duration. Increased anti-protein IgG post-colonisation correlated with increased pneumococcal carriage duration and density, but this was not significant. Longer carriage duration and higher carriage density may have provided sustained immune stimulation, leading to elevated systemic anti-protein IgG. Increased anti-SP0742 IgG post-colonisation with Spn6B weakly associated with decreased colonisation density and duration, though

this was not significant. Future machine learning work within our group will assess whether hierarchical clustering of proteins shows a clearer correlation between anti-protein IgG at baseline and decreased duration and density of carriage.

It is undetermined why anti-protein humoral response differed so much between volunteers inoculated with Spn6B and those inoculated with Spn15B. One limitation of this study is that post-challenge serum samples from volunteers inoculated with Spn15B were taken 14 days post-challenge compared to 29 days-post challenge in the Spn6B cohort. A more robust anti-protein IgG response may have been detected at a later timepoint in volunteers colonised with Spn15B, although significant systemic anti-protein IgG response to colonisation has previously been detected at 14 days post initial challenge (Ferreira *et al.*, 2013). Nevertheless, these previous results were obtained in response to experimental inoculation with Spn6B and not Spn15B. Furthermore, the previous method of IgG detection was MSD technology, which is more sensitive than Luminex and thus might have detected more subtle changes in IgG levels; the fold-change in IgG levels from baseline found to be significant at 14 days post-challenge in the study conducted by Ferreira and colleagues was less pronounced than that found to be significant in the current study at 29 days post-challenge. Another possible explanation for differences between IgG response to Spn15B and Spn6B in this work is that the sample size of the cohort inoculated with Spn15B was smaller than the Spn6B cohort and perhaps a larger sample size may have found a more significant change in anti-protein IgG response to Spn15B. Moreover, differences in levels of protein expression across serotypes might have been a reason for such different results in each cohort (Mahdi *et al.*, 2008). However, it remains to be determined why Spn15B elicited a less marked protein-based humoral response than did Spn6B.

6.3.4 Nasopharyngeal carriage of *S. pneumoniae* induces systemic pneumococcal protein-specific memory B-cell response

As was observed with anti-protein IgG, no single protein antigen significantly correlated with higher numbers of protein-specific memory B-cells at baseline and protection against experimental colonisation, consistent with findings that circulating PspA- or PspC-specific memory B-cells conferred no protection against experimental colonisation with Spn6B (Pennington *et al.*, 2016). However, in contrast with the findings of Pennington and colleagues, where no increase in circulating PspA-specific IgG secreting memory B-cells was observed up to 35 days post-inoculation in the absence or presence of experimental colonisation with Spn6B, the current study found that at 29 days post-inoculation with Spn6B in colonised individuals, a significant increase in circulating PspA1-specific memory B-cells was

observed. As previously discussed, it is not clear from the published data whether the PspA used in the study conducted by Pennington and colleagues was homologous to Spn6B, which might explain differences between the findings of their work and that reported here, where the PspA protein used is homologous to the challenge strain. A significant increase in memory B-cells specific for the novel protein vaccine PnuBioVax was also observed 29 days post-inoculation in colonised individuals. PnuBioVax contains antigens PspA2, Ply, PsaA, PiaA (SP1032), PiuA, RrgB and RrgA (TIGR4 protein sequences) as well as phosphoglycerate kinase, translation elongation factor G, translation elongation factor Tu, enolase, pyruvate oxidase, pyruvate kinase, glyceraldehyde 3-phosphate dehydrogenase, endopeptidase O, aminopeptidase N, lipoprotein and others (Cecchini *et al.*, 2015; Hill *et al.*, 2018). A trend indicated that the vaccine may also confer memory B-cell mediated protection against experimental colonisation, evidenced by higher numbers of PBV-specific memory B-cells at baseline in protected volunteers. However, as previously mentioned, the memory B-cell ELISpot assay was optimised for the detection of B-cell responses against single antigens and further optimisation of the assay is necessary to determine response to multiple antigens simultaneously. Further optimisation is necessary to elucidate how response is distributed across all proteins included in the vaccine formulation. A larger sample size may have elicited significant results. Nevertheless, results are promising for the novel vaccine, which, subject to funding, will enter Phase II clinical trials in the EHPC model. Furthermore, these results suggest that a combination of proteins may be necessary to confer memory B-cell mediated protection against pneumococcal colonisation or infection.

Recent evidence suggests that human lungs contain class switched, predominantly of an IgG isotype, resident memory B-cells following exposure to pneumococcus (Barker *et al.*, 2021). In mouse models, *in vitro* stimulation of lung B-cells from naïve or pneumococcal-experienced mice followed by *ex vivo* culture and ELISpot analysis found that in the lungs of pneumococcal-experienced mice, reactivated lung-resident memory B-cells were more frequent as compared to naïve mice (Barker *et al.*, 2021). Cell culture supernatants of lung B-cells from experienced mice, but not supernatants of lung cells from naïve mice, contained IgG and IgM antibodies that bound to an acapsular strain of Spn3, indicating that the memory B-cells were specific for serotype-independent antigens. Furthermore, the BALF of experienced mice 96 hours post-intratracheal challenge with Spn3 contained significantly increased IgG, IgM, and IgA post-challenge able to bind acapsular Spn3. Thus, serotype-independent reactivation of lung resident memory B-cells in experienced lungs may occur and result in the rapid secretion of serotype cross-reactive antibodies. Importantly, protection from serotype-mismatched pneumococcal infection in mice which had recovered from past pneumococcal infections was found to be partially dependent on B-cell-

mediated immunity. Transfer of plasma from experienced mice to naïve mice conferred protection compared with plasma from naive mice, supporting the hypothesis that serotype-independent antibodies can contribute to lung defense against pneumococcus. However, it was found that lung B-cells did not substantially contribute to circulating antibody pools. These data suggest that lung resident memory B-cells might contribute to lung antibacterial immunity, likely via the local secretion of serotype-independent anti-pneumococcal IgG following reactivation. Thus, it is plausible that although circulating memory B-cells in human volunteers did not confer protection against colonisation in this study, lung-resident memory B-cells might confer protection against pneumococcal infection in an anamnestic manner mediated by antibody response.

As previously mentioned, upon experimental colonisation, B-cells in the nasal mucosa deplete, and depletion of nasal B-cell populations was associated with an expansion of total and pneumococcal polysaccharide-specific circulating plasmablasts, suggesting recirculation of activated B-cells from the nasal mucosa (Tangye *et al.*, 2003; Jochems *et al.*, 2019). A limitation of this current work is that B-cell response in the acute period following bacterial challenge was not investigated and so it is unknown whether there might have been a temporary depletion in circulating protein-specific memory B-cells in the short-term, suggesting a role in the control or prevention of carriage, as was observed by Pennington and colleagues regarding polysaccharide-specific B-cells (Pennington *et al.*, 2016). It must be considered that tissue-resident memory B-cells may protect against pneumococcal infection in a way that is not detected by our investigations into circulating memory B-cell response.

Importantly, colonisation has been shown to prime the memory B-cell response in mice and humans, which might enhance post-vaccine response (Rabquer *et al.*, 2007; Rohner *et al.*, 2008; Diks *et al.*, 2021). These data are in similarity with that showing colonisation augments immune response to immunisation with pneumococcal protein in mice (Neill *et al.*, 2013). Thus, further credence is given to the idea that a novel vaccine should not aim to completely eliminate nasopharyngeal carriage of *S. pneumoniae*, as this could enhance vaccine response, but to maintain nasopharyngeal colonisation at low density to prevent subsequent disease.

Ideally, the present study would have obtained nasal biopsies from volunteers to investigate local protein-specific memory B-cell response to challenge with pneumococcus at the nasal mucosa to compare with systemic response. However, the Covid-19 pandemic prevented this from being carried out.

To our knowledge, this is one of the first studies investigating potential serotype-independent B-cell response to pneumococcus. Further work is needed to determine what level of protection is conferred against homologous or heterologous challenge with pneumococcus following the induction of protein-specific memory B-cell response. It has been demonstrated that booster vaccination with PCV induces the appearance of memory B-cells at the nasal mucosa but this reflects the recirculation of systemic memory cells rather than the generation of tissue-resident cells (Clarke *et al.*, 2012). The establishment of lung resident memory B-cells against influenza has been shown to require local antigen encounter (Allie *et al.*, 2019). Mucosally administered vaccines may induce local B-cell memory that confers protection against pneumococcal colonisation or nfection in a way that is not observed by investigating systemic response.

6.3.5 Investigating induction of dendritic cell maturation and support of T-cell activation by novel nanoparticle-based vaccine formulations

Naturally acquired protection from pneumococcal disease following nasopharyngeal colonisation with *S. pneumoniae* requires both humoral and cellular immunity and T-cells have been shown to play a vital role in protection (McCool and Weiser, 2004; Malley *et al.*, 2005; Van Rossum, Lysenko and Weiser, 2005; Lu *et al.*, 2008; Richards *et al.*, 2010; Cohen *et al.*, 2011; Wilson *et al.*, 2015, 2017). Antigen-primed T-cells are essential for T-cell dependent B-cell activation (Kurosaki, Kometani and Ise, 2015; Akkaya, Kwak and Pierce, 2019). Production of mucosal antibodies (predominantly IgG1, along with lower levels of IgG3 and IgG4) in the nasopharynx of children against pneumococcal proteins PspC and Ply has been shown to be T-cell regulated (Zhang, Bernatoniene, Bagrade, Paton, *et al.*, 2006). Experimental human pneumococcal carriage in a cohort of healthy adults increased the proportion of IL-17A-secreting CD4+ memory T-cells in the lung, and was associated with enhanced killing capacity of opsonised pneumococci by alveolar macrophages (Wright *et al.*, 2013). In addition, Th17 response was shown to be the dominant naturally acquired T-cell response to pneumococcal protein antigens in young and elderly adults; this response was dependent upon the presence of antigen presenting cells (Schmid *et al.*, 2011). In adult mice, depletion of $CD4^+$ T-cells post vaccination with PhtD caused a loss of vaccine-induced protection against invasive pneumococcal pathogenesis (Khan, Xu and Pichichero, 2017). Depletion of $CD4^+$ T-cells post immunisation with PCV13 however did not result in a loss of vaccine-induced protection. In infant mice, passive transfer of both antisera and $CD4^+$ T cells from PhtD-vaccinated adult mice was required to cause a significant reduction in nasopharyngeal colonisation density. Thus, it is likely that both humoral and cellular mediated immunity must be conferred by a protein vaccine in order to confer significant protection against colonisation or disease.

Tissue resident memory T-cells (T_{RM}) have been identified in the airway epithelium and lungs, as well as the skin, bone marrow, spleen, intestine and lymph nodes (Farber, Yudanin and Restifo, 2014; Turner and Farber, 2014; Mueller and Mackay, 2015; Kumar *et al.*, 2017). Tissue-resident memory T-cells confer rapid protection against reinfection with a pathogen (Masopust and Soerens, 2019). $CD8^+$ and $CD4^+$ T_{RM} cells have been shown to confer protection against infection with respiratory viruses in mice (Teijaro *et al.*, 2011; Anderson *et al.*, 2012). Moreover, intranasal administration of live or killed pneumococcus in mice generated *S. pneumoniae*-responsive IL-17A-producing $CD4^+$ mucosal T_{RM} cells, which mediated long-lived, neutrophil-dependent protection against subsequent pneumococcal nasal challenge (O'Hara *et al.*, 2019). Most notably, parenteral administration of killed pneumococcus also generated IL-$17A^+$ $CD4^+$ T_{RM} cells at the nasal mucosa, in contrast to the paradigm that generation of mucosal T_{RM} requires mucosal vaccination (Belyakov and Ahlers, 2009; Holmgren and Svennerholm, 2012; Aguilo *et al.*, 2016). Work in mice has shown that regionally localised $CD4^+$ T_{RM} cells provide superior local tissue protection against *S. pneumoniae* infection to that mediated by systemic or central memory immune responses in a heterotypic way to prevent pneumonia (Smith *et al.*, 2017). Tissue-resident memory $CD8^+$ cells in the nose correlated with protection against experimental colonisation in a human challenge model (Jochems *et al.*, 2019). Novel vaccines should be designed to induce both this tissue-resident and circulating T-cell response for optimal protection against infections. The generation of tissue-resident memory cells is one of the principal advantages of mucosally administered vaccines. Mucosal administration of vaccines also offers the advantage over intramuscular vaccines of being needle-free and relatively painless, making it a more desirable method of immunisation for recipients.

There is evidence that regional activation of tissue-resident memory T-cells by antigen presenting DCs occurs in the airway submucosa, and is not restricted to activation in the lymph nodes (Constant *et al.*, 2002; Blank, Stumbles and Von Garnier, 2011; Thornton *et al.*, 2012). It has been suggested that inducible bronchus-associated lymphoid tissue (iBALT), which promotes the recruitment, priming and expansion of antigen-specific lymphocytes *in situ* and the sustained presence of which is dependent upon DCs, may be the location of such respiratory tract DC/T-cell interactions (Moyron-Quiroz *et al.*, 2004; Geurtsvankessel *et al.*, 2009; Halle *et al.*, 2009). However, it has been shown that T_{RM} cells in the lung can be maintained in niches independent of iBALT (Turner *et al.*, 2014).

All of this points to the development of a mucosally administered vaccine to confer localised protection against pneumococcal disease. Nanoparticle-based vaccines offer a promising new method of mucosal vaccination where antigens are delivered directly to mucosal sites, inducing local immunity.

Nanoparticles themselves harbor immunostimulatory properties as well as enhancing specific anti-pneumococcal response (Zaharoff *et al.*, 2007). Furthermore, nanoparticles in dry powder form eliminate the need for cold chain and improve stability of protein antigens as well as the nanoparticles themselves (Kunda *et al.*, 2012). Dry powder formulations offer the potential for inhalation for pulmonary delivery with controllable antigen release (Scherließ and Janke, 2021).

In the current study, the ability of five novel nanoparticle formulations to induce dendritic cell maturation was investigated. Two of the nanoparticle-based vaccines successfully induced DC maturation but as one was discontinued by the manufacturer due to increased cytotoxicity and decreased stability during the nebulization process, only one nanoparticle-based vaccine formulation, nanoparticle D, was taken forward to test for support of T-cell activation by nanoparticle-exposed DCs. Nanoparticle D comprises a PLGA core and a chitosan coating. Two formulations of the vaccine were investigated: unloaded and PspA4-loaded. In the presence of mature DCs, the level of antigen-specific $CD4^+$ T-cell activation was similar in response to DCs exposed to unloaded nanoparticles, loaded nanoparticles and PspA4 antigen alone, suggesting that the nanoparticle offers no enhancement of PspA4 response. Whilst the antigen-specific $CD4^+$ response was greater in the presence of immature DCs exposed to loaded nanoparticles as compared to PspA4 antigen alone, the response was reduced in comparison to that supported by immature DCs exposed to unloaded nanoparticle. It is thus unclear whether in the presence of immature DCs, conjugation of PspA4 to nanoparticles enhances response to PspA4 or impairs the response to the nanoparticles themselves. Although a greater level of $CD8^+$ activation was observed in response to DCs exposed to PspA4-loaded nanoparticles as compared to PspA4 alone, further work is needed to determine whether this is due to the non-specific activation by nanoparticle D or an enhancement of PspA4-specific response. Furthermore, loading of PspA4 to nanoparticles appeared to inhibit support of T-cell activation by exposed DCs.

Further work is needed to ascertain the effect of protein loading on the biological response to the nanoparticle-based vaccine. Altered surface charge, size and/or particle agglutination may have impaired uptake by dendritic cells. Perhaps most crucially, the interlinked effects of protein corona formation on biological response to nanoparticles must be explored and investigated. The biological milieu greatly influences protein corona formation (Kurtz-Chalot *et al.*, 2014; Forest and Pourchez, 2017) and so it is vitally important to consider how interaction with the host environment *in vivo* may significantly alter the uptake of nanoparticle-based vaccines or their effect on the immune system as compared to observations made *in vitro* (Nel *et al.*, 2009; Forest and Pourchez, 2017).

In addition, numerous factors need further elucidation. Optimal conditions for testing nanoparticle-based vaccines *in vitro* should be confirmed, such as the presence or omittance of serum in cell culture medium. The purity of nanoparticles needs to be more scrupulously assessed and LPS contamination in particular should be investigated given the profound effect on it has on DC populations, which in turn will have an impact on T-cell activation. The structure of PspA post-loading into nanoparticles should also be examined to ensure structure integrity. The effect of excipients alone on dendritic cells requires further investigation. Moreover, using samples from the EHPC model biobank, factors such as health status, age and recent pneumococcal exposure/colonisation status can be controlled for when testing response to nanoparticle-based vaccines. Responses to loaded nanoparticles can be evaluated prior to and post controlled pneumococcal exposure to determine whether recent exposure boosts adaptive response to the vaccines. T-cell proliferation assays should be conducted to assess whether cells are proliferating in response to stimulus. Cytokine profiling in response to nanoparticles should also been investigated to assess level of inflammation induced as well as specific T-cell response elicited. The induction of airway hyperresponsiveness upon administration of nanoparticle-based vaccines must be avoided (Stumbles, Upham and Holt, 2003). Finally, nanoparticle-based vaccines will ideally be tested on cells taken from mucosal sites to determine localised responses, which may differ from responses observed in experiments using peripheral cells.

Alternative protein vaccine candidates should be investigated for nanoparticle-based vaccine formulations as well as combinations of proteins. Notably, a nanoparticle formulation comprising adsorbed PspA4 was used for the immunisation of mice and induced both systemic and lung anti-PspA4 IgG (Rodrigues *et al.*, 2018). Whilst subcutaneous injection with purified protein and mucosal immunisation with the nanoparticle-based vaccine induced comparable levels of protection against lethal intranasal challenge with Spn3 (expressing clade 5, family 2 PspA), vaccination induced earlier control of infection. Importantly, mucosal immunisation with the vaccine induced much higher anti-PspA4 IgG titres in the lungs than did mucosal immunisation with the purified protein, which induced a negligible IgG response. Moreover, no increase in IgG titres was observed following immunisation with the empty vaccine formulation (no PspA4 adsorbed). However, no reduction in bacterial burden in the lungs following challenge with Spn19F, expressing clade 1 PspA (family 1) was observed following either immunisation with the vaccine or subcutaneous administration of purified protein. These results again point to the fact that for PspA to confer heterotypic protection, hybrid proteins need to be included in vaccine formulations. As has previously been discussed, a combination of proteins may be more likely to confer serotype-independent protection.

6.4 Relevance of findings for vaccine development

There is a distinct need for improved pneumococcal vaccines with serotype-independent protection. Current polysaccharide-based vaccines have numerous drawbacks, particularly serotype replacement. A novel pneumococcal vaccine should aim to combat pneumococcal pneumonia in particular as the burden of disease is the highest of all pneumococcal disease and current vaccines, while successful in reducing cases of invasive pneumococcal disease, have limited efficacy against pneumonia (Madhi, Whitney and Nohynek, 2008; Suzuki *et al.*, 2017). It has been suggested that acquired immunity towards non-capsular antigens drives immunity against re-acquisition of pneumococcal carriage and invasive pneumococcal disease (Lipsitch *et al.*, 2005; Granat *et al.*, 2009; Wilson *et al.*, 2017). It is likely that for a protein-based vaccine to confer protection against colonisation or pneumonia, the vaccine will need to be delivered mucosally to induce robust local immunity.

Numerous protein antigen vaccines are in development and aim to induce antibody-mediated immune protection against *S. pneumoniae* to prove immunogenicity (Appendix A). Importantly, there is now also a focus on conjugating capsular polysaccharides with pneumococcal proteins to develop new PCVs that might stimulate protein-specific responses in addition to polysaccharide-specific responses, conferring additional serotype-independent protection (Kaplonek *et al.*, 2022). Affinivax, Inc. (Cambridge, MA, USA) recently developed AFX3772, a vaccine candidate incorporating 24 pneumococcal polysaccharides and two conserved pneumococcal proteins, using the proprietary Multiple Antigen-Presenting System (MAPS™). The vaccine is designed to stimulate both antibody- and T-cell-mediated immunity against *S. pneumoniae* and Phase I and II clinical trials have shown the vaccine to be well tolerated and highly immunogenic in adults, generating both anti-capsular and anti-protein antibody responses. The identification of conserved protein antigens that could be used in such PCV formulations is therefore highly relevant in the current pneumococcal vaccine development landscape.

Both humoral and cell-mediated immunity is likely needed to confer protection against pneumonia. It is not fully clear to what extent protein-mediated antibody defense plays a role in naturally acquired protection against pneumococcal colonisation. Antibody-mediated response may play a role in protection against colonisation in infants (Obaro *et al.*, 2000; Holmlund *et al.*, 2006; Zhang, Bernatoniene, Bagrade, Pollard, *et al.*, 2006; Francis *et al.*, 2009) and possibly adults (McCool *et al.*, 2002), though, there is conflicting data, in line with this work, surrounding the extent to which naturally acquired protein-directed humoral immunity protects against colonisation (Ferreira *et al.*, 2013; Araujo *et al.*, 2021). Antibody-mediated protection may require a/multiple prior immunising event(s) to boost antibody levels

to a point that confers protection against colonisation, should this be the aim. Alternatively, whilst antibodies may associate with protection against colonisation, cellular immunity (Th17 in particular) may have a greater role in defense (McCool and Weiser, 2004; Malley *et al.*, 2005; Van Rossum, Lysenko and Weiser, 2005; Lu *et al.*, 2008; Trzciński *et al.*, 2008; Moffitt *et al.*, 2011). Although, in an area of high incidence of pneumococcal carriage, $CD4^+$ memory T-cell response did not correlate absolutely with protection against carriage (Mureithi *et al.*, 2009); it has been suggested that recurrent exposure to pneumococcal antigens might induce immune hyporesponsiveness (Ha *et al.*, 2008; Lagousi *et al.*, 2019). Baseline levels of antibody or B-cell populations might be indicative of response to immunisation (Papadatou, Tzovara and Licciardi, 2019). It has also been shown that, in mice, both humoral and cellular immunity against protein antigen is required to confer protection against nasopharyngeal colonisation (Khan, Xu and Pichichero, 2017). Vaccine induced pneumococcal protein antibodies have been shown to induce increased complement C3b deposition on the surface of *S. pneumoniae* and subsequent bacterial killing (Odutola *et al.*, 2017; André *et al.*, 2021; Nakahashi-Ouchida *et al.*, 2021). The failure of novel protein vaccines in clinical trials to confer significant protection against colonisation despite inducing antibody responses further substantiates that a mucosal vaccine may indeed be needed to achieve such a goal by inducing localised immunity (Odutola *et al.*, 2017; Hammitt *et al.*, 2019). In mice, intranasal immunisation with PspA confers protection against nasopharyngeal colonisation, pulmonary infection and sepsis but protection against colonisation is largely mediated by mucosal rather than systemic response (Wu *et al.*, 1997). It has been shown that higher levels of mucosal antibodies against pneumococcal proteins confer protection against acute otitis media caused by *S. pneumoniae* in infants (Xu, Casey and Pichichero, 2015). Indeed, it is also worth mentioning that in terms of future vaccine development, induction of antibodies alone is not enough to conclude immune protection. Functional capacity of antibodies should also be investigated using opsonophagocytic assays (Hill *et al.*, 2018); this is particularly relevant when considering immune response in immunocompromised individuals, such as those with HIV (Collins *et al.*, 2013).

Concerning protection against pneumonia, antibody-mediated response plays an important role in protecting against infection and pathogenesis. Protection against pneumonia and invasive disease in mice has been shown to be dependent upon antibodies against protein antigens (Wilson *et al.*, 2015, 2017). Results have shown that mice immunised with PlyD1 were protected against lethal intranasal pneumococcal challenge and protection was associated with PlyD1-specific IgG titres and *in vitro* neutralization titres (Salha *et al.*, 2012). A multi-antigen vaccine (MAV) comprising PspA and Ply has been shown to induce a robust antibody response against multiple pneumococcal serotypes, and confer

protection against pneumococcal pneumonia in rodents (Chan *et al.*, 2019). Passive transfer of serum from MAV-immunised rabbits to naïve mice conferred protection against systemic disease caused by homologous and heterologous pneumococcus. Resident memory B-cells in the lung have been shown to protect against pneumococcal pneumonia in mice in an anamnestic manner (Barker *et al.*, 2021). Direct antigen exposure is likely required to establish resident memory B-cells (Allie *et al.*, 2019) and thus inhaled mucosally administered vaccines may be necessary to confer robust protection against pneumococcal pneumonia. In line with what is already established, this work shows that colonisation is an immunising event, inducing both systemic antibody and memory B-cell responses. Moreover, lung resident memory T-cells have been shown play a large role in acquired immunity against pneumococcal pneumonia in mice (Smith *et al.*, 2017; Wang *et al.*, 2017; Shenoy *et al.*, 2020). Thus, novel vaccines that can be administered directly to mucosal sites may be the best chance at conferring robust protection. Nevertheless, parenteral immunisation of mice with live or killed *S. pneumoniae* has been shown to elicit protective IL-17A^{+}CD4^{+} tissue resident memory cells in the nasal mucosa (O'Hara *et al.*, 2019). T-cell response to protein antigens has previously been explored by our group and results, together with the results of the current work, are in preparation as a manuscript.

It has been shown that experimental colonisation with pneumococcus induces a significantly increased IgG response against several proteins (including PspC, PspA-UAB055, RrgA-Tigr4, PiuA, and PcpA) in the elderly, whereas no increase in anti-capsular IgG was induced by colonisation (Adler *et al.*, 2021). Furthermore, IgM responses to pneumococcal proteins have been shown to be sustained with age and are not associated with an age-related decline as is seen with antibody response to pneumococcal polysaccharide (German *et al.*, 2018). Thus, protein-based vaccines provide a promising method of immunising against pneumococcus in the elderly population. Interestingly, it has been shown that there is an age-related decline in anti-polysaccharide antibodies in women compared to men but anti-protein antibody concentrations were similar between the sexes (Simell *et al.*, 2008). On the other hand, PBMCs taken from older adults have shown reduced functional CD4^{+} T-cell to stimulation with pneumococcal protein antigen AliB (He *et al.*, 2021); PBMCs from older adults also demonstrated a tend towards lower IL-17A and IL-22 production (He *et al.*, 2021). Immunosenescence in CD4^{+} T cell responses to pneumococcal proteins might contribute to susceptibility to pneumococcal infections in the elderly and should be considered as this may limit protein vaccine effectiveness. Nevertheless, more work is required to determine acquired immune response to pneumococcal proteins in the elderly and this should be further explored using the EHPC model. Memory B-cell response should be investigated in older adults as

we know that B-cell populations decline with age (Shi *et al.*, 2005; Colonna-Romano *et al.*, 2008; Ademokun, Wu and Dunn-Walters, 2011; Frasca *et al.*, 2011; Scholz *et al.*, 2013).

Stratification of immunological data by sex may provide insight into differences in immune responses to *S. pneumoniae* between male and females, allowing better prediction of vaccine response in these groups, particularly regarding older adults. We have previously reported that males are significantly less likely than females to be experimentally colonised with pneumococcus (Cheliotis *et al.*, 2022). Previous studies have shown that females are more susceptible than males to infections of the upper respiratory tract whereas, males are more susceptible to lower respiratory tract infections (Falagas, Mourtzoukou and Vardakas, 2007). Vaccine efficacy in older adults is generally better in females than males (Cook, 2008; Fink and Klein, 2015). Sex-specific immune responses can result in varying vaccine responses, including safety, immunogenicity or efficacy (Fathi, Addo and Dahlke, 2021). Vaccine regimens could therefore be altered for the different sexes. Differences in age-related pneumococcal polysaccharide antibody responses in adults have been reported in males and females, although anti-protein response was consistent between sexes (Simell *et al.*, 2008). In addition, evidence suggests healthy females elicit a more robust antibody response to PPV23 than healthy males and the difference in response between sexes is more pronounced in adults over 50 years of age (Parker *et al.*, 2019; Chiarella *et al.*, 2021). Immune differences between males and females may be due to genetic and epigenetic differences, the influences of sex hormones (oestrogen and androgens), differences in the microbiome, variation in concomitant infections as well as social and cultural differences (Klein and Flanagan, 2016). Notably, it is unlikely that sex hormones would affect immune response in infants, whereas adults and adolescents will exhibit hormone effects on the immune system.

The results of the work reported here further substantiate that a combination of protein antigens will be necessary to elicit broad protection. Indeed, different antigens may stimulate either humoral or cell-mediated immunity and thus a combination approach should be used when designing vaccine formulations. Protein antigens included in novel vaccines should induce both arms of the adaptive immune system. It seems likely that regarding pneumonia, vaccine-induced localised immunity will be particularly important for conferring protection.

It has proven notoriously difficult to identify single protein antigens that confer protection against a given disease such as malaria (Bull *et al.*, 1998; Cockburn and Seder, 2018; Duffy and Patrick Gorres, 2020), pertussis (Xu *et al.*, 2021), typhoid (Das *et al.*, 2019), hepatitis B (Borges *et al.*, 2008) and tuberculosis (Gong *et al.*, 2022). Combinations of antigens and vaccine delivery systems that enhance

antigen immunogenicity are needed to ensure the success of protein-based vaccines. Booster vaccines may also be required to confer long lasting protection with a protein-based vaccine (Seiberling *et al.*, 2012). Currently, there is no mucosally administered protein-based vaccine licensed for use (Lavelle and Ward, 2021). A recent study reported that three engineered RSV G proteins containing single-point mutations are able to induce higher levels of IgG compared to wild-type RSV G as well as having improved safety profiles (Bergeron *et al.*, 2021) and engineered RSV G protein retains conformational epitopes to high-affinity protective antibodies (Castrejon *et al.*, 2022). Thus, structure-guided antigen design might be one method of improving protein antigen immunogenicity. Generally, protein antigens will require the addition of potent adjuvants to enhance immunogenicity. Assessing the ability of mucosal adjuvants to activate antigen presenting cells in tissues or lymph nodes may be a useful indicator of efficacy (Lavelle and Ward, 2021). The addition of aluminium adjuvant to a trivalent protein vaccine has been shown to improve vaccine immunogenicity without compromising safety in infants (Brooks *et al.*, 2015). Moreover, the use of $AS02_V$ adjuvant has been shown to significantly enhance functional antibody response to a vaccine comprising a combination of PhtD and detoxified pneumolysin in elderly volunteers (Pauksens *et al.*, 2014) and a PhtD vaccine in young and older adults (Leroux-Roels *et al.*, 2015) as compared to the use of alum adjuvant or plain protein vaccination. Further, PhtD/$AS02_V$ was associated with an increased frequency of PhtD-specific $CD4^+$ T-cells in both young and older adults as compared to PhtD/alum and $AS02_V$ also induced a significantly higher specific memory B-cell response in older adults as compared to alum/PhtD, similar to responses obtained in young adults (Leroux-Roels *et al.*, 2015). Effective adjuvants may thereby enhance immune response to protein vaccines in the elderly to a level close to that observed in younger adults. Enhancing $CD4^+$ T-cell function in the elderly could lead to improved antibody production and increase vaccine efficacy (Pashine, Valiante and Ulmer, 2005; Haynes and Swain, 2006). Administration of antigens with nanoparticles coated with immunogenic polymers such as chitosan, as discussed in the current work, can induce adaptive immune responses and enhance antigen-specific immunity (Zaharoff *et al.*, 2007; Xu *et al.*, 2015; Carroll *et al.*, 2016; Silva *et al.*, 2016).

The safety of nanoparticle-based vaccines as a method of mucosal vaccine delivery requires further investigation, however. Firstly, it is necessary to establish safety of these vaccines regarding translocation to and accumulation within the central nervous system. There is varying evidence surrounding the likelihood of extrapulmonary translocation of nanoparticles to the circulation and extrapulmonary organs (Yang, Peters and Williams, 2008). The translocation of inhaled nanoparticulates to extrapulmonary organs, as well as the olfactory bulb in rats suggests that further investigations need to be carried out in order to determine whether the same is observed in humans (Oberdörster *et al.*, 2008;

Zhu *et al.*, 2009; X. He *et al.*, 2010). The occurrence of such is likely dependent on nanoparticle properties (Yang, Peters and Williams, 2008). In addition, it is essential that nanoparticle-based vaccines do not elicit hyperresponsiveness in the lungs, particularly in individuals who may suffer from allergic inflammation. Further work, such as cytokine analysis, is needed to ascertain the specific T-cell response induced by the nanoparticle-based vaccines explored in the current work.

Whole-cell vaccines are one of the most promising protein-based immunisation methods. An increase in pertussis incidence has occurred since the introduction of the protein-based acellular pertussis vaccine over the original whole-cell vaccine, which elicits more side-effects but has greater efficacy and induces longer lasting immunity than the acellular vaccine (Jefferson, Rudin and DiPietrantonj, 2003; Bolotin, Harvill and Crowcroft, 2015; Locht, 2016; Syed, 2017). Possible reasons for the increase in pertussis cases include waning vaccine-induced immunity, pathogen adaptation to vaccination through changes in antigenic structure, change in aetiology, and poor vaccine quality, though advancements in diagnostic capabilities may also contribute to increased incidence (Syed, 2017). Whole-cell vaccines include molecules such as LPS, which act as PAMPs and activate host dendritic cells and macrophages to promote the induction of T-cell response (Gabutti *et al.*, 2015; Pichichero, 2017). Antigens are also presented in their natural configuration (Pichichero, 2017). Whole-cell vaccines are more affordable than acellular vaccines, making them more accessible to low- and middle-income countries (Gabutti *et al.*, 2015). Furthermore, immune response to the acellular pertussis vaccine has been shown to be Th2-mediated rather than Th1, as induced by whole-cell vaccines (Higgs *et al.*, 2012). However, whole-cell vaccines are more reactogenic than acellular vaccines (Gabutti *et al.*, 2015). Regarding pneumococcal whole-cell vaccines, various methods have been employed to reduce pathogen virulence and for vaccine development, including attenuation, chemical treatment, or preparations of whole-cell crude extracts (Lagousi *et al.*, 2019). A phase I study in a cohort of US adults recently showed that intramuscularly administered *S. pneumoniae* whole-cell vaccine (wSp), containing killed cells from a nonencapsulated strain of *S. pneumoniae* with aluminum hydroxide adjuvant, was safe and well tolerated with acceptable reactogenicity (Keech *et al.*, 2020). Furthermore, wSp elicited potentially significant IgG responses to multiple pneumococcal antigens, including PspA and pneumolysin. The highest does of wSp generated functional antibody responses. Increases in T-cell cytokine responses, including IL-17A, were also observed in response to vaccination. In mice, immunisation with a pneumococcal whole-cell vaccine conferred $CD4^+$ T-cell-mediated protection against nasopharyngeal colonisation and antibody-mediated protection against fatal aspiration-sepsis (Lu *et al.*, 2010). A non-adjuvanted intranasal γ-irradiated pneumococcal whole-cell vaccine has been shown to generate effective serotype-independent protection

against pneumonia and sepsis in mouse models, is mediated both humoral and innate IL-17 responses (Babb *et al.*, 2016).

Viral vectors expressing immunogenic protein antigens may also be a promising means of mucosal protein vaccine delivery (Kim *et al.*, 2018; Lavelle and Ward, 2021). Vaxart have recently begun a phase I clinical trial of an orally administered SARS-CoV-2 vaccine expressing the full-length S protein of the virus (NCT04563702). In the post Covid-19 pandemic world, mRNA-based vaccines offer exciting new possibilities (Walsh *et al.*, 2020). Given that protective responses against SARS-CoV-2 mRNA vaccines were antibody and Th1 mediated (Jackson *et al.*, 2020; Sahin *et al.*, 2020), it is possible that mRNA vaccines directed towards *S. pneumoniae* may be a possibility. mRNA-based SARS-CoV-2 vaccines have been shown to be safe and immunogenic in adolescents (Ali *et al.*, 2021; Frenck *et al.*, 2021) and older adults (Anderson *et al.*, 2020). Self-Amplifying mRNA (SAM) vaccines expressing antigens from Group A and Group B Streptococci induced fully functional serum antibodies in mice (Maruggi *et al.*, 2017), demonstrating that this technology could be used for immunisation against bacterial pathogens. Combination of mRNA vaccines with techniques such as nanoparticle delivery systems with immunostimulatory properties could enhance efficiency of mRNA vaccine induced protection (Oliveira *et al.*, 2021).

Particularly given that the results of this work were unable to determine constancies in response to single antigens across two cohorts colonised with different pneumococcal serotypes, there is a distinct need for machine learning and systems biology work to better elucidate global correlates of successful vaccination integrating all aspects of the immune system (Six *et al.*, 2012). Using the "immune response network theory" we can better understand drivers of protection as well as non-protective responses to protein antigens in order to predict vaccine response and develop new vaccine strategies (Oberg *et al.*, 2011). Nowadays, in this era of *in silico* analysis, systems biology and artificial intelligence (AI) can be used to streamline the vaccine development process (Russo *et al.*, 2020; Thomas *et al.*, 2022), enhancing epitope discovery (Lundegaard, Lund and Nielsen, 2011; Liu, Shi and Li, 2020), design of vaccines targeting the immunopeptidome (Jabbari and Rezaei, 2019; Vizcaíno *et al.*, 2020) and enabling prediction of outcomes through modeling of immune system interactions and behavior (Hagan *et al.*, 2015; Pappalardo *et al.*, 2015; Han and Kim, 2017) to identify novel vaccine candidates. AI has been used for vaccine development against SARS-CoV-2 (Keshavarzi Arshadi *et al.*, 2020). In a landmark event, in 2019, a clinical trial (NCT03945825) began in the USA of a seasonal influenza vaccine, the adjuvant of which was identified by AI (Thomas *et al.*, 2022). A general-purpose machine learning framework has also been

developed to predict vaccine efficacy, which is particularly relevant for improving monitoring for poor responses in the elderly, infants, or individuals with weakened immune systems (Lee *et al.*, 2016).

Finally, the EHPC model provides a unique platform to investigate immune responses to *S. pneumoniae* and pneumococcal vaccines in a highly controlled way, where we can control for confounding variables and better ascertain immune responses in a variety of cohorts. The models offers the opportunity to investigate innate and adaptive humoral and cellular immune responses to pneumococcal colonisation of the human nasopharynx in the upper respiratory tract, lower respiratory tract and systemic circulation (Ferreira, Jambo and Gordon, 2011). Prevention of nasopharyngeal carriage in response to intranasal inoculation with *S. pneumoniae* is both an ethically justifiable measurable endpoint and highly clinically relevant as colonisation is a pre-requisite of disease. It is highly exciting that the EHPC model has now been established in Malawi (Morton *et al.*, 2021), which will enable us to investigate immune responses to *S. pneumoniae* in a population with high pneumococcal burden and in a low- and middle-income setting. Comparing immune responses observed in a UK-based cohort to a Malawi-based cohort will better enable us to identify vaccine candidates that are relevant for the global population, particularly those in the most vulnerable communities/populations.

6.5 Recommendations for future work

- The Luminex assay should be used to investigate protein-mediated humoral response in different age groups, namely the elderly and children
- Machine learning work should be used to identify whether clusters of proteins confer protection against pneumococcal colonisation
- For proteins which elicited significantly higher baseline levels of IgG in carriage negative volunteers prior to correction for multiple comparisons, single antigen ELISAs could be carried out to assess significance of IgG against these proteins at baseline in EHPC volunteer sera
- Investigate protein-mediated protection against re-challenge with homologous and heterologous strains, which is now possible with samples stored in the EHPC biobank
- The Luminex assay should be optimised to identify IgG response in nasal wash and BAL to investigate protein-mediated antibody response in different niches
- Opsonophagocytosis assays could be used to measure serum antibodies' capacity to kill bacteria
- B-cell ELISpot assay should be optimised for the detection of memory B-cell response to multiple protein antigens simultaneously

- Protein-specific memory B-cell response should be investigated using samples taken from the nasal mucosa to identify B-cell response in this niche
- B-cell response in the elderly should be investigated as it is well-established that B-cell mediated immunity diminishes with increasing age
- Work has already been conducted investigating T-cell response to the entire protein library described in the current work and the combination of that work with the findings of this thesis into a manuscript is currently in progress
- Investigation of immune response to nanoparticles should be conducted using samples taken from EHPC volunteers to control for recent pneumococcal exposure, age, and health status
- Response to nanoparticle-based vaccines should be investigated using cells taken from the lungs to establish the immune response in the target environment
- T-cell proliferation assays should be conducted in response to exposure to nanoparticle-based vaccines
- Cytokine profile response to nanoparticle-based vaccines should be investigated
- Protein-directed immune responses in volunteers from the EHPC model in Malawi should be investigated and compared to responses seen in our UK population to identify proteins that are most relevant to immune protection against *S. pneumoniae* in a population with high pneumococcal prevalence and disease burden and to identify similarities in responses across the different populations

6.6 Conclusion

In conclusion, this work has resulted in the development of platforms for identifying vaccine candidates, has demonstrated that nasopharyngeal pneumococcal colonisation is an immunising event and has better elucidated the systemic protein-mediated humoral and cellular response to pneumococcal colonisation. Whilst further work is needed to determine serotype-independent correlates of protection against pneumococcal infection, this work has identified potential protein vaccine candidates and has highlighted the need for integrated approaches when developing a protein vaccine.

6.7 Final words

Overall, the wider aim of this work, and the work of our team, is to develop a vaccine, which confers broad protection against pneumococcal pneumonia and is effective across populations, including the most high-risk groups. We aim for a vaccine that is effective, affordable, and accessible to all communities around the world, particularly those most affected by pneumococcal disease. It is a sad fact

that the Covid-19 pandemic has highlighted, along with many triumphs for vaccine development, the huge disparity in vaccine equity around the world, with poorest communities being further disadvantaged and neglected. There remains a need to push for vaccines to be both equitably distributed and produced. International initiatives and collaborations such as Gavi and CEPI and Advanced Market Commitment have massively improved vaccine rollouts and access in the poorest communities - in 2019, pneumococcal vaccine coverage in Gavi-supported countries was higher than the worldwide average for the first time (Gavi, 2021) - but there is still a way to go.

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Appendices

Appendix A

Table I: Current pneumococcal vaccines in clinical trials (Pichichero, 2017; Lagousi et al., 2019).

Vaccine	Assignee	Indication	Identifier	Location	Age	Ph1	Ph2	Ph3	Status
PCV									
PCV15 (V114)	Merck	Safety & Immunogenicity	NCT01215175	USA & Finland	12–15mos & 18–45	X			Completed
PCV15 (V114)	Merck	Safety & Immunogenicity	NCT03950622	Canada, Japan, Spain, Taiwan, United States	≥50yrs			X	Completed
PCV15 (V114)	Merck	Safety & Immunogenicity	NCT03885934	Finland, Malaysia, Poland, Russian Federation, Thailand	7mos – 18yrs			X	Completed
PCV15 (V114)	Merck	Safety & Immunogenicity	NCT04031846	Australia, Belgium, Czechia, Estonia, Germany, Greece, Poland, Russian Federation, Spain	42-90 days			X	Not yet recruiting
PCV15 (V114)	Merck	Safety & Immunogenicity	NCT01215188		6–12wks		X		Completed
PCV15 (V114)	Merck	Safety & Immunogenicity	NCT02547649 NCT01513551		50yrs+		X		Completed
PCV15 (V114)	Merck	Safety & Immunogenicity	NCT02531373 NCT02037984		2mos-49yrs	X	X		Completed
PCV15 (V114)	Merck	Safety & Immunogenicity	NCT02573181		≥65yrs		X		Completed
Polyvalent PCV	Merck	Safety & Immunogenicity	NCT04168190		18yrs+	X			Not yet recruiting
PCV10 (GSK1024850A, Synflorix)	GlaxoSmithKline	Safety & Immunogenicity	NCT03197376	The Gambia	6-8 wks			X	Completed

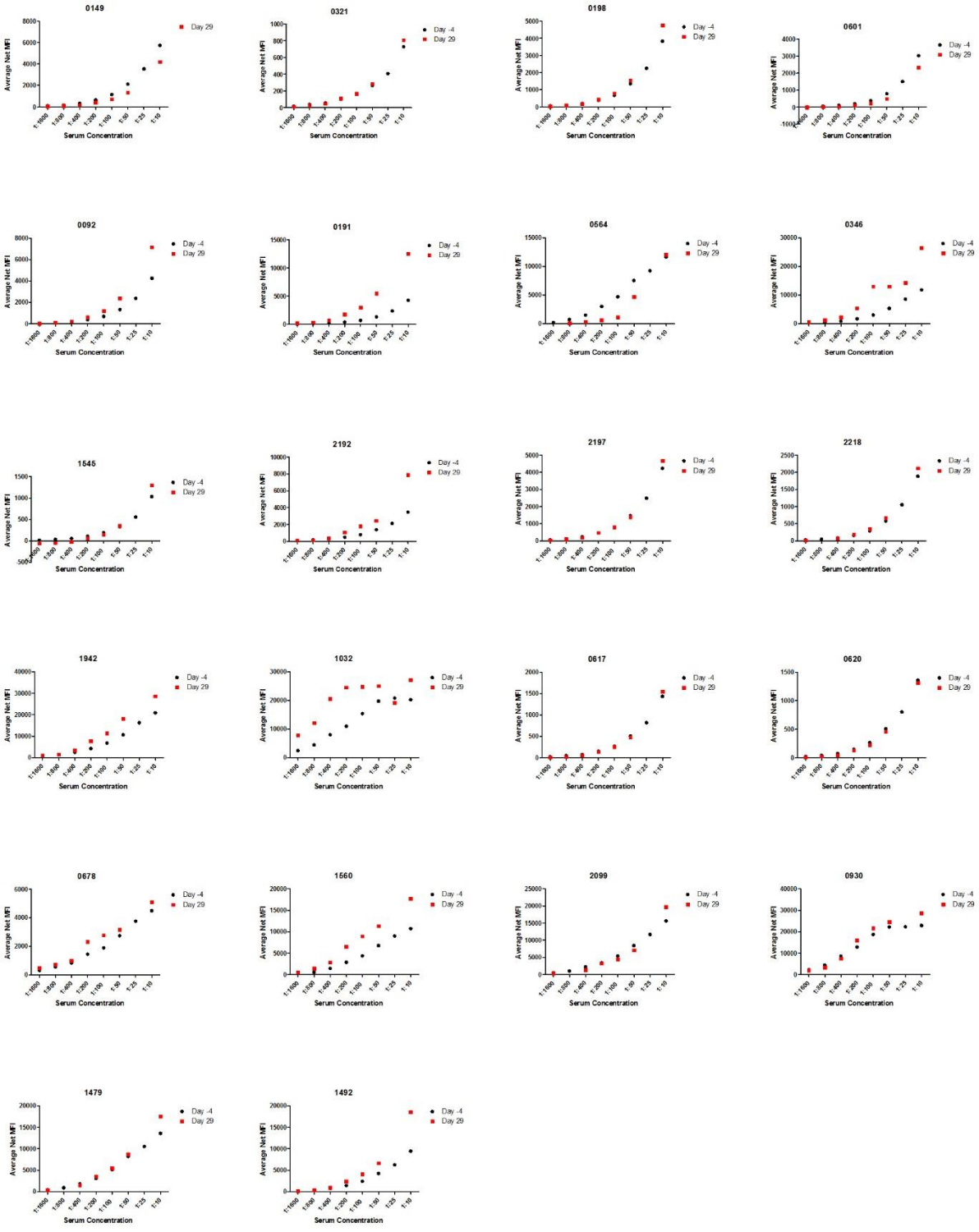
PCV10 (GSK1024850A, Synflorix)	GlaxoSmithKline	Safety & Immunogenicity	NCT00344318	Poland, Philippines	6-12wks			X	Completed
PCV10 (GSK1024850A, Synflorix)	GlaxoSmithKline	Impact on Carriage, Acute Otitis Media, Immunogenicity & Safety	NCT00839254	Finland	6-18mos			X	Completed
PCV10 (GSK1024850A, Synflorix)	GlaxoSmithKline	Immunogenicity	NCT00307541	Germany	8-16wks			X	Completed
PCV10 (GSK1024850A, Synflorix)	GlaxoSmithKline	Safety & Immunogenicity	NCT00390910	Greece	8-16wks			X	Completed
PCV12 (2830930A)	GlaxoSmithKline	Safety & Immunogenicity	NCT01485406	Germany	12–23mos	X			Completed
PCV11, PCV12 (2830929A, 2830930A)	GlaxoSmithKline	Immunogenicity	NCT01616459	Czech Republic, Germany, Poland, Spain	6–12wks		X		Completed
PCV12 (2830930A)	GlaxoSmithKline			The Gambia			X		Unknown
PCV10	Panacea Biotech	Safety & Immunogenicity					X		Unknown
PCV10	Serum Institute of India	Immunogenicity	CTRI/2015/12/006456	India	12–15mos		X		Unknown
Multivalent PCV	Pfizer	Safety & Immunogenicity	NCT02955160	USA	18-49yrs	X			Completed
PCV20	Pfizer	Safety & Immunogenicity	NCT04546425	Australia, Belgium, Czechia, Denmark, Estonia, Finland, Italy, Netherlands, Norway, Poland, Slovakia	42-112 days			X	Ongoing
PCV20	Pfizer	Safety	NCT02531373	Argentina, Canada, Chile, Czechia, Finland, Germany, Greece,	42-98 days			X	Not yet recruiting

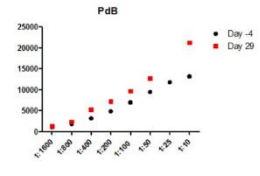
				Hungary, Puerto Rico, Spain, United States					
PCV20	Pfizer	Safety & Immunogenicity	NCT04642079	USA	15mos-17yrs			X	Ongoing
PCV10 (SIILPCV10)	PATH	Safety & Immunogenicity	NCT02308540	The Gambia	6-8wks, 12-15mos, 18-40yrs	X	X		Completed
PCV10 (PNEUMOSIL)	PATH	Safety & Immunogenicity	NCT03197376	The Gambia	6-8wks			X	Completed
LBVE (multivalent PCV)	LG Chem	Safety & Immunogenicity	NCT03467984	Republic of Korea	6-8wks		X		Completed
PCV24 (ASP3772)	Astellas Pharma Global Development, Inc.	Safety & Immunogenicity	NCT03803202	USA	18-64yrs	X			Completed
PCV13	CanSino Biologics Inc.	Safety & Immunogenicity	NCT04100772	China	6wks+	X			Not yet recruiting
EuPCV15	EuBiologics Co.,Ltd	Safety & Immunogenicity	NCT04830358	Republic of Korea	19-50yrs	X			Not yet recruiting
PCV15	Beijing Zhifei Lvzhu Biopharmaceutical Co., Ltd	Safety	NCT04108845	China	6wks+	X			Ongoing
PCV15	Beijing Zhifei Lvzhu Biopharmaceutical Co., Ltd	Safety & Immunogenicity	NCT04357522	China	2-3mos	X			Ongoing
Protein									
PhtD	GlaxoSmithKline	Safety & Immunogenicity	NCT01767402	Belgium	18–45yrs ≥65yrs		X		Completed
dPly,PhtD-dPly +/− PCV10	GlaxoSmithKline	Safety & Immunogenicity	NCT00707798[c]	Belgium	18–40yrs	X			Completed
PhtD-dPly (GSK2189242A)	GlaxoSmithKline	Safety & Immunogenicity	NCT00896064[c]	Belgium	18–41yrs		X		Completed
PhtD-dPly +/− PCV8	GlaxoSmithKline	Safety & Immunogenicity	NCT00756067	Sweden	65–85yrs	X			Completed

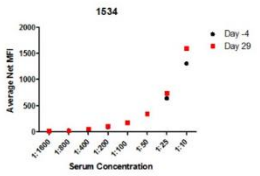
PhtD-dPly + PCV10	GlaxoSmithKline	Safety & Immunogenicity	NCT01262872	The Gambia	2–4yrs		X		Completed
PhtD-dPly + PCV10	GlaxoSmithKline	Efficacy NP colonisation	NCT01262872	The Gambia	8–10wks 2–4yrs		X		Completed
PhtD-dPly +/– PCV10	GlaxoSmithKline	Safety & Immunogenicity	NCT00985751	Czech Republic	12–23mos		X		Completed
PhtD-dPly + PCV10	GlaxoSmithKline	Safety & Immunogenicity	NCT01204658	Czech Republic, Germany, Poland	6–14wks		X		Completed
PhtD-dPly + PCV13	GlaxoSmithKline	Ear & Lung	NCT01545375	USA (Indian)	6–12wks		X		Completed
PlyD1	Sanofi Pasteur	Safety & Immunogenicity	NCT01444352	Switzerland	18–50yrs	X			Completed
PhtD	Sanofi Pasteur	Safety & Immunogenicity	NCT01444001	Switzerland	18–50yrs	X			Completed
PcpA, PcpA+PhtD	Sanofi Pasteur	Safety & Immunogenicity	NCT01444339	Switzerland	18–50yrs	X			Completed
PhtD+PcpA+PlyD1 (PPrV)	Sanofi Pasteur	Safety & Immunogenicity	NCT01764126, NCT01446926, U1111–1117-7316	Bangladesh	6–7wks 12–13mos 18–50yrs	X			Completed
PspA	Sanofi Pasteur	Safety & Immunogenicity	NCT01033409	USA	18-40yrs	X			Completed
PspA+PsaA	Sanofi-Pasteur/CDC	Safety & Immunogenicity	CN-00275765	USA	18-45yrs	X			Completed
PcpA +/- PhtD	Sanofi Pasteur	Safety & Immunogenicity	NCT01444339	USA	18-50yrs	X			Completed
SP2108+SP0148+SP1912 (GEN-004)	Genocea Biosciences	Safety & Immunogenicity	NCT01995617	USA	18–55yrs	X			Completed
SP2108+SP0148+SP1912 (GEN-004)	Genocea Biosciences	Efficacy NP colonisation	NCT02116998	UK	18–55yrs		X		Completed
PcsB, stkP, PsaA	Intercell AG	Safety & Immunogenicity	NCT00873431	Germany	18–65yrs	X			Completed
PnuBioVax	ImmunoBiology Limited	Safety & Immunogenicity	NCT02572635	UK	18-40yrs	X			Completed
Whole Cell									
WCV (Alum)	PATH	Safety & Immunogenicity	NCT01537185	USA	18–40yrs	X			Completed
WCV +/– PCV10 (Alum)	PATH	Safety & Immunogenicity	NCT02097472	Kenya	12–15mos 18–45yrs	X	X		Completed

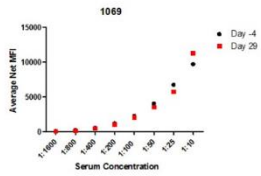
WCV (Aluminum hydroxide)	PATH	Safety & Immunogenicity	NCT02543892	Kenya	12–19mos 18–40yrs	X	X		Completed
S.typhi rPspA	Arizona State U.	Safety & Immunogenicity	NCT01033409	USA	18–40yrs	X			Completed

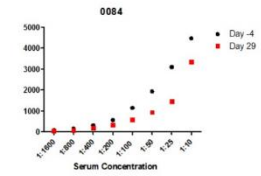
Appendix B

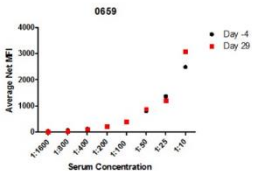

0149
0321
0198
0601
0092
0191
0564
0346
1545
2192
2197
2218
1942
1032
0617
0620
0678
1560
2099
0930
1479
1492
Day -4
Day 29
Average Net MFI
Serum Concentration
1:1600
1:800
1:400
1:200
1:100
1:50
1:25
1:10

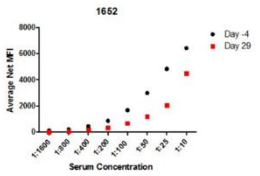

PdB
Day -4
Day 29

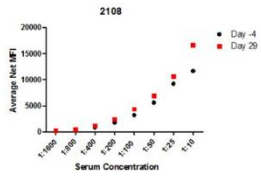

1534
Average Net MFI
Serum Concentration
Day -4
Day 29


1069
Average Net MFI
Serum Concentration
Day -4
Day 29


0084
Average Net MFI
Serum Concentration
Day -4
Day 29


0659
Average Net MFI
Serum Concentration
Day -4
Day 29


1652
Average Net MFI
Serum Concentration
Day -4
Day 29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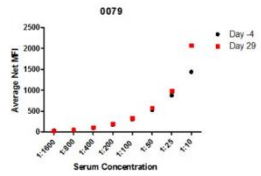
Average Net MFI
Serum Concentration
Day -4
Day 29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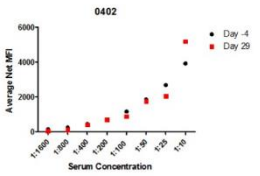
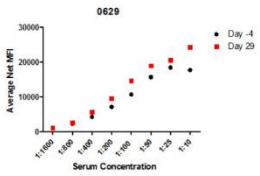
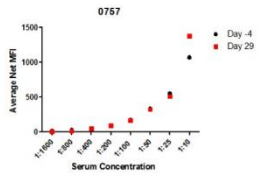
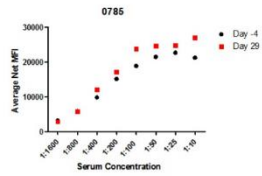
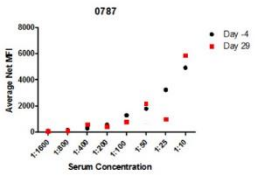
Average Net MFI
Serum Concentration
Day -4
Day 29


0402
Average Net MFI
Serum Concentration
Day -4
Day 29


0629
Average Net MFI
Serum Concentration
Day -4
Day 29


0757
Average Net MFI
Serum Concentration
Day -4
Day 29


0785
Average Net MFI
Serum Concentration
Day -4
Day 29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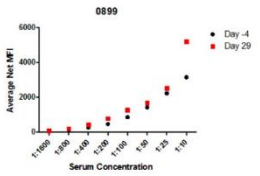
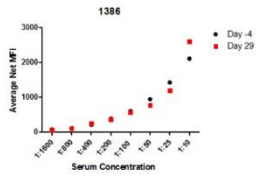
Average Net MFI
Serum Concentration
Day -4
Day 29


0899
Average Net MFI
Serum Concentration
Day -4
Day 29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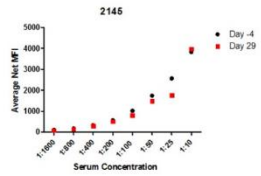
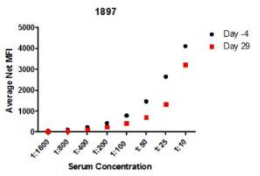
Average Net MFI
Serum Concentration
Day -4
Day 29


2145
Average Net MFI
Serum Concentration
Day -4
Day 29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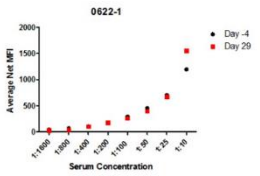
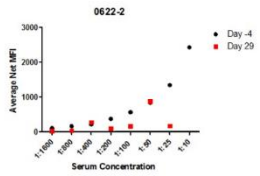
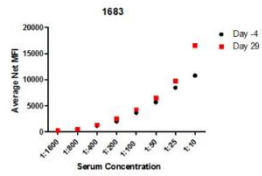
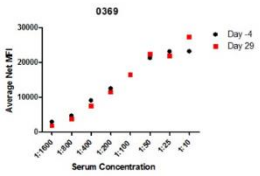
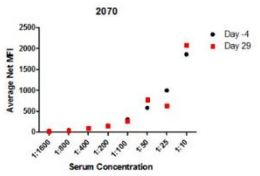
Average Net MFI
Serum Concentration
Day -4
Day 29

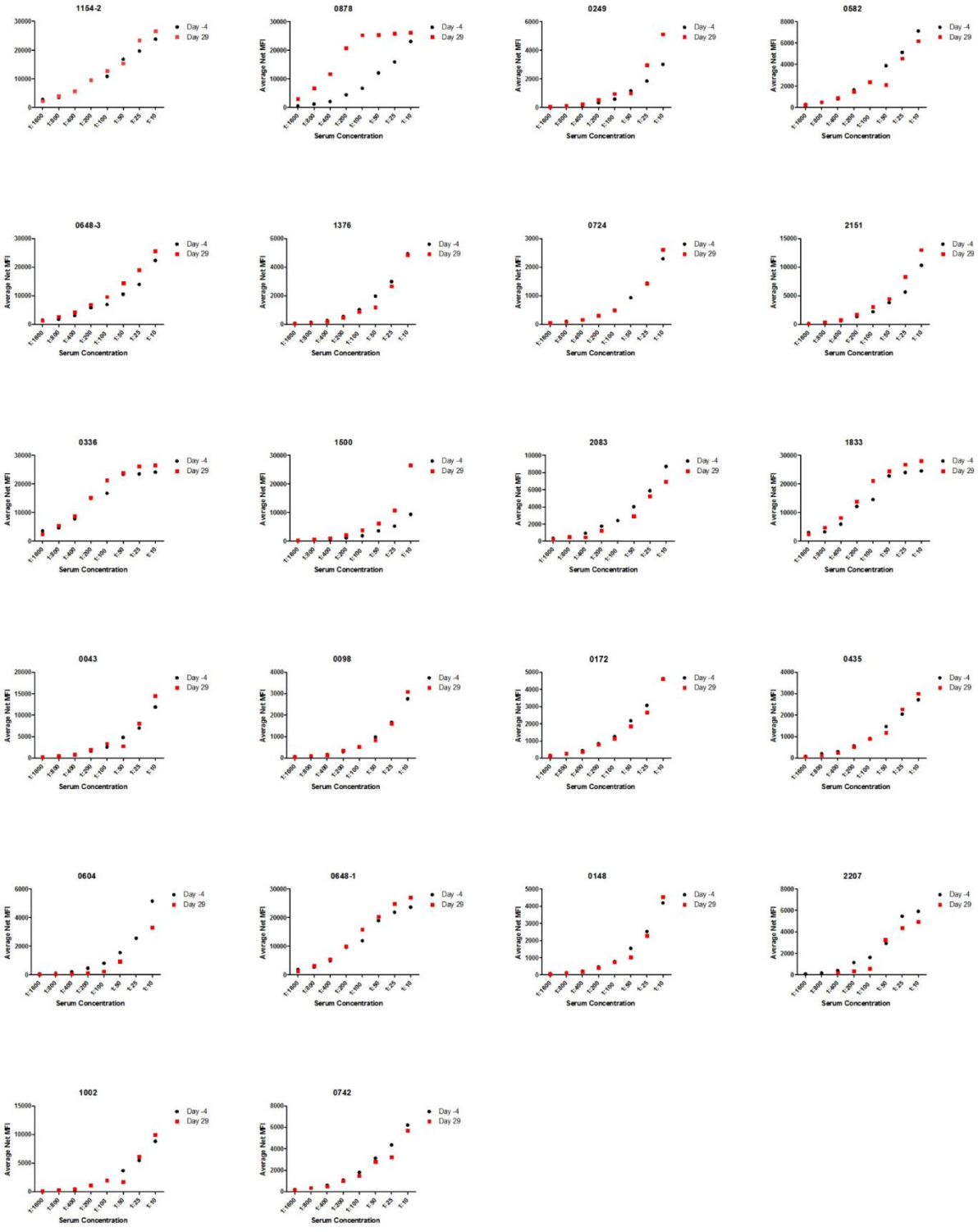

0622-1
Average Net MFI
Serum Concentration
Day -4
Day 29


0622-2
Average Net MFI
Serum Concentration
Day -4
Day 29


1683
Average Net MFI
Serum Concentration
Day -4
Day 29


0369
Average Net MFI
Serum Concentration
Day -4
Day 29


2070
Average Net MFI
Serum Concentration
Day -4
Day 29


1154-2
0878
0249
0582
0648-3
1376
0724
2151
0336
1500
2083
1833
0043
0098
0172
0435
0604
0648-1
0148
2207
1002
0742
Day -4
Day 29
Average Net MFI
Serum Concentration

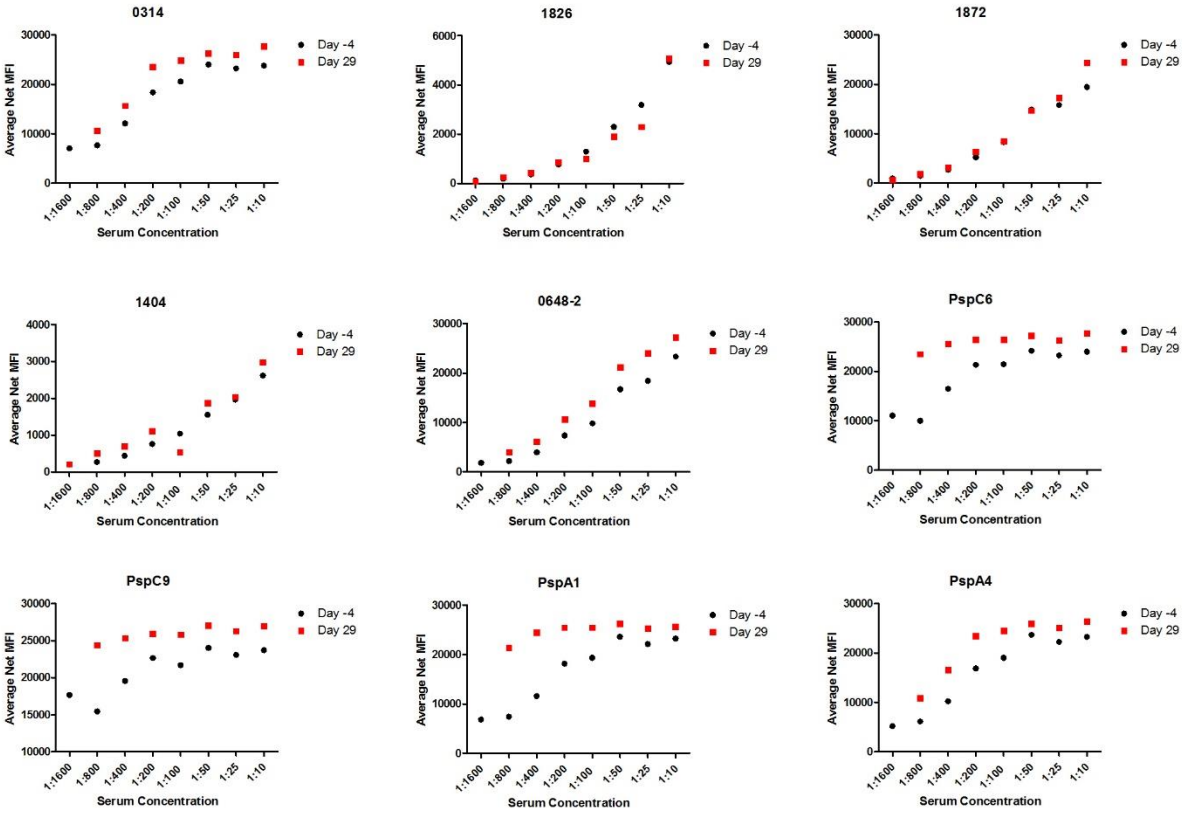


Figure I: Titration curves for anti-protein serum IgG against a library of 75 proteins. Sera was taken from participants 4 days prior to and 29 days post-challenge with *S. pneumoniae* serotype 6B (Spn6B). Baseline and day 29 sera from 10 participants who established pneumococcal carriage post intranasal inoculation with Spn6B in a human model of infection were pooled. IgG was measured as average net MFI in a Luminex assay.

Appendix C

Table II: Sample size for each protein against which antibody binding was quantified in a cohort of healthy adults.

	Carriage Negative			Carriage Positive		
Protein	**Pre-challenge**	**Post-challenge**	**Paired**	**Pre-challenge**	**Post-challenge**	**Paired**
SP0314	20	18	18	17	15	15
SP1826	11	11	11	10	10	10
SP1872	17	16	16	13	12	11
SP1404	11	11	11	10	10	10
SP0648-2	19	18	17	13	14	12
PspC6	20	19	19	15	17	15
PspC9	19	18	18	14	16	14
PspA1	19	17	14	15	15	14
PspA4	19	17	14	14	16	13

Appendix D

Full inclusion and exclusion criteria for EHPC studies detailed in Chapter 3.3 (EudraCT 2014-004634-26 and ISRCTN68323432) is as follows.

Inclusion criteria:

1. Adults aged 18-50 years ages (chosen to minimise the risk of pneumococcal infection, and to allow comparison with previously published experimental work done by our group)
2. Fluent spoken English (to ensure a comprehensive understanding of the research project and their proposed involvement)

Exclusion criteria:

1. Previous pneumococcal vaccination
2. History of pneumococcal illness
3. Close physical contact with at risk individuals (children under 5yrs, immunosuppressed adults, elderly, chronic ill health) - to minimise risk of pneumococcal transmission
4. Any current treatment for asthma – confounding effect of medications such as corticosteroids, and propensity to infection
5. Allergy to penicillin/amoxicillin
6. Taking daily medications that may affect the immune system e.g. steroids, steroid nasal spray, antibiotics. Also medication that may reduce immunity eg. Roacutanne
7. Current illness, acute illness within 3 days prior to inoculation or antibiotic treatment within 2 weeks of inoculation
8. Pregnancy - minimise risk of pneumococcal disease
9. Diagnosed as diabetic
10. Involved in another clinical trial unless observational or in follow-up (non-interventional) phase.
11. Have been involved in a clinical trial involving EHPC and bacterial inoculation
12. History of drug or alcohol abuse
13. Current regular smoker (smokes daily/ smokes > 5 cigarettes per week) - minimise risk of pneumococcal disease
14. Recent smoker i.e. within the last 6 months - minimise risk of pneumococcal disease

15. Ex-smoker with a significant smoking history (>10 pack years) – minimise risk of pneumococcal disease
16. Unable to give fully informed consent

Appendix E

Table III: Sample sizes for serum samples taken from volunteers inoculated with either *S. pneumoniae* serotype 6B or 15B in a human challenge model.

	Inoculated with *S. pneumoniae* serotype 6B						**Inoculated with *S. pneumoniae* serotype 15B**					
	Carriage positive			**Carriage negative**			**Carriage positive**			**Carriage negative**		
	Baseline	Day 29	Paired	Baseline	Day 29	Paired	Baseline	Day 14	Paired	Baseline	Day 14	Paired
SP0149	20	19	21	17	17	18	11	9	8	21	15	14
SP0321	20	19	21	17	17	18	11	9	7	21	15	14
SP0198	20	19	21	17	17	18	11	9	9	21	15	15
SP0601	20	19	21	17	17	18	11	9	9	21	15	15
SP0092	20	19	21	17	16	18	11	9	8	21	15	14
SP0191	20	19	21	17	17	18	11	9	8	21	15	12
SP0564	20	19	21	17	17	18	11	9	8	21	15	13
SP0346	20	19	21	17	17	18	11	9	9	21	15	15
SP1545	20	19	21	17	17	18	11	9	8	21	15	15
SP2192	20	19	19	17	17	17	11	9	9	21	15	14
SP2197	17	19	19	17	17	17	11	9	9	21	15	14
SP2218	20	19	19	17	17	17	11	9	9	21	15	14
SP1942	20	19	19	17	17	17	11	9	9	21	15	14
SP1032	20	19	19	17	16	16	11	9	9	21	15	14
SP0617	12	17	19	13	13	17	11	9	9	21	15	14
SP0620	19	19	19	17	16	17	11	9	9	21	15	14
SP0678	20	19	19	17	17	17	11	9	9	21	15	14
SP1560	19	19	19	17	17	17	11	9	9	21	15	14
SP2099	20	19	19	17	17	17	11	9	9	21	15	14
SP0930	20	19	16	17	17	17	11	9	9	21	15	14
SP1479	19	19	19	17	16	17	11	9	9	21	15	14
SP1492	20	19	19	17	17	17	11	9	9	21	15	14
PdB	20	20	19	18	17	16	11	9	9	22	15	14
SP1534	20	20	10	18	17	12	11	9	9	22	15	14
SP1069	18	19	18	17	16	16	10	9	9	21	15	14
SP0084	0	0	0	0	0	17	0	0	0	0	0	14
SP0659	20	20	19	18	17	17	11	9	9	22	15	14
SP1652	20	20	18	18	16	17	11	9	9	22	15	14
SP2108	20	20	19	18	17	17	11	9	9	22	15	14
SP0079	16	17	19	17	16	16	11	8	9	22	15	14
SP0402	20	20	18	18	17	17	11	9	9	22	15	14
SP0629	20	20	19	18	17	17	11	9	9	22	15	15
SP0757	20	20	19	18	17	17	11	9	9	22	15	15
SP0785	20	20	19	18	17	16	11	9	9	22	15	14
SP0787	19	20	17	18	16	0	11	9	9	22	15	15

SP0899	18	17	19	17	16	17	11	7	9	21	14	15
SP1386	18	19	19	17	16	16	11	9	9	22	15	15
SP2145	15	13	19	16	14	17	9	7	9	10	4	15
SP1897	20	20	14	18	17	16	11	9	8	21	15	15
SP0662-1	20	20	19	17	16	17	11	9	9	22	15	15
SP0662-2	19	20	19	17	15	17	11	8	9	20	15	15
SP1683	20	20	19	18	17	17	11	9	9	21	15	15
SP0369	20	20	19	18	17	17	11	9	9	22	15	15
SP1154-2	21	18	18	18	17	16	11	9	9	22	14	14
SP0878	21	19	16	18	17	16	11	9	7	22	14	15
SP0249	21	21	17	18	17	16	11	9	9	22	14	3
SP0582	21	20	12	18	17	14	11	9	6	22	14	15
SP0648-3	21	18	19	18	17	17	11	9	9	22	14	15
SP1376	21	20	19	18	17	16	11	9	9	22	14	14
SP0724	20	18	18	17	15	15	11	9	8	22	14	15
SP2151	13	13	19	9	11	17	11	9	9	22	14	15
SP0336	21	18	19	18	17	17	11	9	9	22	14	14
SP1500	21	21	18	18	17	17	11	9	9	22	14	14
SP2083	21	20	19	18	17	17	11	9	9	22	14	14
SP1833	21	21	21	18	17	17	11	9	9	22	14	14
SP0043	21	21	20	18	17	17	11	9	9	22	14	14
SP0098	21	21	18	18	17	17	11	9	9	22	14	14
SP0127	20	19	21	18	17	17	11	9	9	22	14	14
SP0435	21	21	17	18	17	14	11	9	9	22	14	14
SP0604	21	21	10	18	17	9	11	9	9	22	14	14
SP0648-1	21	20	18	18	17	17	11	9	9	22	14	14
SP0148	21	19	21	18	17	17	11	9	9	22	14	14
SP2207	21	21	19	18	17	17	11	9	9	22	14	14
SP1002	21	21	21	18	17	17	11	9	9	22	14	14
SP0742	15	15	21	11	13	17	11	9	9	22	14	14
SP0314	21	21	21	18	18	17	11	8	9	22	14	14
SP1826	21	21	18	18	18	17	11	7	9	22	14	14
SP1872	21	21	21	18	18	17	11	9	9	22	15	14
SP1404	21	21	21	18	18	17	11	9	9	22	15	14
SP0648-2	21	21	20	18	18	17	11	8	9	21	15	14
PspC6	21	21	19	18	18	17	11	8	9	20	14	14
PspC9	21	21	21	18	18	17	11	8	9	20	15	14
PspA1	21	21	21	18	18	17	11	9	9	22	15	14
PspA4	21	21	12	18	18	10	11	8	9	22	15	14

Appendix F

Table IV: Significance values before and after Benjamini Hochberg correction for the association between protein-specific IgG at baseline and protection against subsequent experimental colonisation with either *S. pneumoniae* serotype 6B or 15B.

	***S. pneumoniae* serotype 6B**		***S. pneumoniae* serotype 15B**	
Protein	*p*-value before correction	*p*-value after correction	*p*-value before correction	*p*-value after correction
SP0314	0.060	0.775	0.355	0.751
SP1826	0.118	0.775	0.222	0.69
SP1872	0.770	0.911	0.178	0.69
SP1404	0.693	0.911	0.585	0.801
SP0648-2	0.126	0.775	1	1
PspC6	0.426	0.910	0.079	0.69
PspC9	0.587	0.910	0.359	0.751
PspA1	0.094	0.775	0.063	0.69
PspA4	0.202	0.910	0.355	0.751
SP0149	0.727	0.911	0.439	0.78
SP0321	0.210	0.910	0.267	0.727
SP0198	0.949	0.949	0.275	0.727
SP0601	0.775	0.911	0.226	0.69
SP0092	0.506	0.910	0.796	0.894
SP0191	0.537	0.910	0.565	0.801
SP0564	0.366	0.910	0.874	0.925
SP0346	0.415	0.910	0.088	0.69
SP1545	0.075	0.775	0.088	0.69
SP2192	0.937	0.949	0.874	0.925
SP2197	0.074	0.775	0.648	0.816
SP2218	0.547	0.910	0.513	0.78
SP1942	0.573	0.910	0.506	0.78
SP1032	0.397	0.910	0.289	0.727
SP0617	0.213	0.910	0.578	0.801
SP0620	0.049	0.775	0.812	0.898
SP0678	0.285	0.910	0.634	0.812
SP1560	0.590	0.910	0.223	0.69
SP2099	0.510	0.910	0.042	0.69
SP0930	0.397	0.910	0.133	0.69
SP1479	0.779	0.911	0.223	0.69
SP1492	0.318	0.910	0.481	0.78
PdB	0.762	0.911	0.015	0.69
SP1534	0.455	0.910	0.632	0.812
SP1069	0.921	0.949	0.113	0.69

SP0659	0.549	0.910	0.925	0.951
SP1652	0.087	0.775	0.236	0.69
SP2108	0.838	0.939	0.089	0.69
SP0079	0.331	0.910	0.076	0.69
SP0402	0.501	0.910	0.51	0.78
SP0629	0.661	0.911	0.178	0.69
SP0757	0.279	0.910	0.169	0.69
SP0785	0.228	0.910	0.281	0.727
SP0787	0.346	0.910	0.633	0.812
SP0899	0.552	0.910	0.361	0.751
SP1386	0.668	0.911	0.864	0.925
SP2145	0.123	0.775	0.497	0.78
SP1897	0.629	0.911	0.551	0.801
SP0662-1	0.075	0.775	0.924	0.951
SP0662-2	0.800	0.911	0.403	0.751
SP1683	0.313	0.910	0.751	0.894
SP0369	0.553	0.910	0.114	0.69
SP2070	0.750	0.191	-	-
SP1154-2	0.183	0.910	0.778	0.894
SP0878	0.673	0.911	0.194	0.69
SP0249	0.526	0.910	0.411	0.751
SP0582	0.789	0.911	0.336	0.751
SP0648-3	0.856	0.946	0.778	0.894
SP1376	0.921	0.949	0.169	0.69
SP0724	0.464	0.910	0.789	0.894
SP2151	0.357	0.910	0.127	0.69
SP0336	0.126	0.775	0.396	0.751
SP1500	0.317	0.910	0.379	0.751
SP2083	0.921	0.949	0.233	0.69
SP1833	0.789	0.911	0.044	0.69
SP0043	0.254	0.910	0.299	0.727
SP0098	0.800	0.911	0.592	0.801
SP0127	0.573	0.910	0.789	0.894
SP0435	0.899	0.949	0.693	0.857
SP0604	0.382	0.910	0.468	0.78
SP0648-1	0.686	0.911	0.954	0.968
SP0148	0.735	0.911	0.113	0.69
SP2207	0.472	0.910	0.412	0.751
SP1002	0.944	0.949	0.456	0.78
SP0742	0.015	0.775	0.593	0.801

Appendix G

Table V: Correlation between colonising density of *S. pneumoniae* serotype 6B (Spn6B) in the nasopharynx of volunteers experimentally inoculated with the bacteria and fold change in protein-specific serum IgG between baseline and 29 days post-challenge. P-values are given prior to adjustment and after Bonferroni correction. CI denotes confidence interval

Protein	Lower CI	Upper CI	Estimate	*p*-value	Adjusted *p*-value
PdB	-0.15	0.68	0.33	0.17	1
PspA1	0.06	0.75	0.48	0.03	1
PspA4	0.00	0.73	0.43	0.05	1
PspC6	-0.04	0.71	0.40	0.07	1
PspC9	0.03	0.74	0.45	0.04	1
SP0043	-0.59	0.25	-0.21	0.37	1
SP0079	-0.15	0.77	0.41	0.14	1
SP0092	-0.60	0.28	-0.20	0.41	1
SP0098	-0.65	0.15	-0.30	0.18	1
SP0127	-0.72	0.11	-0.37	0.13	1
SP0148	-0.53	0.37	-0.10	0.69	1
SP0149	-0.59	0.30	-0.18	0.45	1
SP0191	-0.54	0.36	-0.11	0.65	1
SP0198	-0.58	0.30	-0.17	0.48	1
SP0249	-0.63	0.18	-0.27	0.24	1
SP0314	0.01	0.73	0.44	0.05	1
SP0321	-0.62	0.25	-0.23	0.35	1
SP0336	-0.61	0.29	-0.20	0.42	1
SP0346	-0.50	0.40	-0.06	0.81	1
SP0369	-0.06	0.73	0.41	0.09	1
SP0402	-0.02	0.75	0.44	0.06	1
SP0435	-0.68	0.10	-0.35	0.13	1
SP0564	-0.63	0.23	-0.25	0.30	1
SP0582	-0.56	0.31	-0.16	0.51	1
SP0601	-0.54	0.36	-0.11	0.66	1
SP0604	-0.68	0.10	-0.35	0.12	1
SP0617	-0.63	0.63	0.01	0.99	1
SP0620	-0.54	0.39	-0.09	0.72	1
SP0629	-0.18	0.66	0.30	0.21	1
SP0648-1	-0.59	0.26	-0.20	0.39	1
SP0648-2	-0.22	0.61	0.24	0.30	1
SP0648-3	-0.54	0.39	-0.09	0.72	1
SP0659	-0.14	0.69	0.34	0.16	1
SP0662-1	0.03	0.77	0.48	0.04	1
SP0662-2	-0.12	0.71	0.37	0.13	1

SP0678	-0.61	0.26	-0.22	0.37	1
SP0724	-0.72	0.14	-0.36	0.15	1
SP0742	-0.80	0.19	-0.43	0.16	1
SP0757	0.06	0.78	0.50	0.03	1
SP0785	-0.08	0.72	0.39	0.10	1
SP0787	-0.13	0.71	0.36	0.14	1
SP0878	-0.55	0.34	-0.13	0.59	1
SP0899	-0.08	0.76	0.43	0.10	1
SP0930	-0.61	0.27	-0.21	0.38	1
SP1002	-0.57	0.27	-0.18	0.42	1
SP1032	-0.55	0.35	-0.12	0.61	1
SP1069	-0.21	0.68	0.30	0.24	1
SP1154-2	-0.65	0.22	-0.27	0.28	1
SP1376	-0.61	0.20	-0.25	0.28	1
SP1386	-0.26	0.65	0.25	0.33	1
SP1404	0.08	0.76	0.49	0.02	1
SP1479	-0.59	0.32	-0.17	0.50	1
SP1492	-0.55	0.34	-0.13	0.59	1
SP1500	-0.53	0.32	-0.13	0.59	1
SP1534	0.21	0.83	0.60	0.01	0.46
SP1545	-0.55	0.34	-0.13	0.58	1
SP1560	-0.52	0.41	-0.07	0.78	1
SP1652	-0.09	0.71	0.38	0.11	1
SP1683	-0.04	0.73	0.42	0.07	1
SP1826	-0.05	0.70	0.39	0.08	1
SP1833	-0.56	0.29	-0.17	0.47	1
SP1872	-0.21	0.61	0.24	0.29	1
SP1897	-0.13	0.69	0.34	0.15	1
SP1942	-0.55	0.35	-0.13	0.60	1
SP2083	-0.66	0.16	-0.30	0.19	1
SP2099	-0.64	0.22	-0.26	0.28	1
SP2108	-0.23	0.63	0.25	0.31	1
SP2145	-0.05	0.85	0.54	0.07	1
SP2151	-0.10	0.88	0.56	0.09	1
SP2192	-0.59	0.29	-0.18	0.45	1
SP2197	-0.67	0.27	-0.26	0.34	1
SP2207	-0.57	0.27	-0.18	0.44	1
SP2218	-0.64	0.23	-0.25	0.29	1

Appendix H

Table VI: Correlation between duration of nasopharyngeal carriage of *S. pneumoniae* serotype 6B (Spn6B) in volunteers experimentally inoculated with the bacteria and fold change in protein-specific serum IgG between baseline and 29 days post-challenge. P-values are given prior to adjustment and after Bonferroni correction. CI denotes confidence interval

Antigen	Lower CI	Upper CI	Estimate	*p*-value	Adjusted *p*-value
PdB	-0.22	0.64	0.26	0.28	1
PspA1	0.07	0.76	0.49	0.03	1
PspA4	0.11	0.77	0.51	0.02	1
PspC6	-0.07	0.69	0.38	0.09	1
PspC9	-0.04	0.71	0.40	0.07	1
SP0043	-0.50	0.36	-0.08	0.72	1
SP0079	-0.52	0.54	0.02	0.95	1
SP0092	-0.65	0.20	-0.28	0.24	1
SP0098	-0.47	0.39	-0.05	0.82	1
SP0127	-0.62	0.28	-0.21	0.40	1
SP0148	-0.54	0.36	-0.11	0.64	1
SP0149	-0.39	0.51	0.08	0.76	1
SP0191	-0.46	0.45	-0.01	0.98	1
SP0198	-0.52	0.38	-0.09	0.72	1
SP0249	-0.56	0.28	-0.17	0.46	1
SP0314	0.26	0.83	0.62	0.008	0.19
SP0321	-0.54	0.36	-0.11	0.65	1
SP0336	-0.55	0.37	-0.11	0.66	1
SP0346	-0.34	0.56	0.14	0.58	1
SP0369	-0.18	0.66	0.30	0.21	1
SP0402	-0.09	0.71	0.38	0.11	1
SP0435	-0.63	0.18	-0.27	0.23	1
SP0564	-0.46	0.45	0.00	0.99	1
SP0582	-0.50	0.38	-0.07	0.75	1
SP0601	-0.36	0.54	0.12	0.64	1
SP0604	-0.47	0.39	-0.05	0.83	1
SP0617	-0.73	0.49	-0.20	0.59	1
SP0620	-0.66	0.21	-0.29	0.25	1
SP0629	-0.35	0.55	0.13	0.60	1
SP0648-1	-0.40	0.49	0.06	0.81	1
SP0648-2	0.20	0.81	0.58	0.01	0.38
SP0648-3	-0.56	0.36	-0.12	0.62	1
SP0659	0.12	0.80	0.54	0.02	1
SP0662-1	-0.12	0.70	0.36	0.13	1
SP0662-2	-0.21	0.66	0.28	0.26	1
SP0678	-0.49	0.41	-0.05	0.83	1

SP0724	-0.71	0.15	-0.35	0.16	1
SP0742	-0.84	0.07	-0.52	0.08	1
SP0757	0.05	0.77	0.49	0.03	1
SP0785	-0.15	0.68	0.33	0.17	1
SP0787	-0.29	0.61	0.20	0.43	1
SP0878	-0.54	0.36	-0.12	0.64	1
SP0899	0.01	0.80	0.50	0.05	1
SP0930	-0.50	0.41	-0.06	0.82	1
SP1002	-0.52	0.33	-0.12	0.61	1
SP1032	-0.39	0.51	0.08	0.76	1
SP1069	-0.30	0.63	0.21	0.42	1
SP1154-2	-0.53	0.40	-0.08	0.76	1
SP1376	-0.48	0.38	-0.06	0.80	1
SP1386	-0.34	0.60	0.17	0.52	1
SP1404	0.01	0.73	0.44	0.05	1
SP1479	-0.27	0.62	0.22	0.37	1
SP1492	-0.34	0.55	0.13	0.59	1
SP1500	-0.40	0.46	0.03	0.89	1
SP1534	0.15	0.81	0.57	0.01	0.80
SP1545	-0.41	0.49	0.05	0.84	1
SP1560	-0.64	0.24	-0.25	0.31	1
SP1652	-0.06	0.72	0.40	0.09	1
SP1683	0.04	0.77	0.49	0.03	1
SP1826	0.02	0.74	0.45	0.04	1
SP1833	-0.48	0.38	-0.06	0.79	1
SP1872	0.23	0.82	0.60	0.004	0.29
SP1897	0.01	0.76	0.46	0.05	1
SP1942	-0.51	0.40	-0.07	0.78	1
SP2083	-0.53	0.34	-0.12	0.63	1
SP2099	-0.49	0.42	-0.05	0.84	1
SP2108	-0.22	0.64	0.26	0.29	1
SP2145	-0.32	0.75	0.31	0.32	1
SP2151	-0.35	0.81	0.36	0.30	1
SP2192	-0.41	0.50	0.05	0.83	1
SP2197	-0.64	0.32	-0.21	0.44	1
SP2207	-0.43	0.43	0.00	1.00	1
SP2218	-0.52	0.39	-0.08	0.74	1