# Natural adaptive immunity to *Streptococcus pneumoniae* lung infection

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I, Robert Wilson, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm this has been indicated in the thesis.

# Acknowledgements

# Abstract

*Streptococcus pneumoniae* is an important respiratory pathogen and a leading cause of community-acquired pneumonia. As well as invasive disease *S. pneumoniae* also colonises the nasopharynx. Colonisation with *S. pneumoniae* is nearly universal in infants, dropping to 10% in adulthood. This frequent exposure has potential for developing and boosting natural adaptive immune responses. However naturally-acquired immune responses that protect against subsequent lung infection *with S. pneumoniae* are not fully understood. This thesis investigates the targets and function of naturally-acquired IgG to *S. pneumoniae* in humans and additionally the mechanisms of protection from lung infection following experimental colonisation in mice.

The target and function of naturally-acquired IgG in human sera and pooled intravenous immunoglobulin (IVIG) preparations was assessed. IVIG, pooled from >1000 adult donors provides a tool to investigate the natural antibody responses to *S. pneumoniae* within a population. Data indicate that naturally-acquired human IgG predominantly binds to non-capsular antigens on the surface of *S. pneumoniae* and can target surface exposed protein antigens. *In vitro* assays indicate that antibodies to non-capsular targets may be functional, enhancing phagocytosis and killing of *S. pneumoniae*. *In vivo* human IgG protected against lung infection. Cellular depletion demonstrated that protection within the lung required neutrophils and clearance of *S. pneumoniae* from the blood required macrophages.

A model of lung infection in the absence of bacteraemia using *S. pneumoniae* strain EF3030 was developed. This model allowed assessment of the immune responses to *S. pneumoniae* colonisation of the nasopharynx that protect against re-infection specifically within the lung. Prior nasal colonisation *with S. pneumoniae* EF3030 was protective against subsequent lung infection. Cellular depletion strategies and challenge in antibody-deficient mice demonstrated that protection against lung infection required the development of both humoral and cell-mediated immunity.

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# Abbreviations

ABC, ATP binding cassette

AM, Alveolar macrophage

ANOVA, analysis of variance

BALF, bronchoalveolar lavage fluid

BCA, bicinchoninic acid

BSA, bovine serum albumin

CBP, choline binding protein

CbpD, choline binding protein D

CbpE, choline binding protein E

CFU, colony forming unit

CPS, capsular polysaccharide

CRP, C-reactive protein

CSF, cerebral spinal fluid

CSP, competence stimulating peptide

CWPS, cell wall polysaccharide

DNA, deoxyribonucleic acid

ELISA, enzyme linked immunosorbance assay

Eno, enolase

FACS, fluorescence activated cell sorting

FAM-SE, 5-carboxyfluorescein, succinimidyl ester

FITC, fluorescein isothiocyanate

HIV, human immunodeficiency virus

HRP, horseradish peroxidase

hrs, hours

Hyal, hyaluronidase

Ig, immunoglobulin

IgA1ase, IgA1 protease

IN, intranasal

IP, intraperitoneal

IV, intravenous

KC, Keratinocyte chemoattractant

KDa, kilodaltons

KO, knockout

Lgt, diacylglyceral transferase

Lsp, lipoprotein signal peptidase

LytA, autolysin A

MFI, mean fluorescence intensity

mins, minutes

MLST, multi locus sequence type

MOI, multiplicity of infection

NanA, neuraminidase

ND, not detected

NLR, NOD-like receptor

NOD, nucleotide oligomerisation domain

ORF, open reading frame

PAMP, pathogen associated molecular pattern

PavA, pneumococcal adhesion and virulence A

PBMC. peripheral blood mononuclear cell

PBS, phosphate buffered saline

PCV, pneumococcal conjugate vaccine

PE, phycoerythrin

PerCP, peridinin-chlorophyll-protein complex

PFA, paraformaldehyde

PhtD, pneumococcal histidine triad protein D

PhtE, pneumococcal histidine triad protein E

PiaA, pneumococcal iron acquisition A

PilusA, pneumococcal pilus

PiuA, pneumococcal iron uptake A

PpmA, putative proteinase maturation protein A

PPS, purified polysaccharide

PRR, pattern recognition receptor

PsaA, pneumococcal surface adhesin A

PspA, pneumococcal surface adhesin A

PspC, pneumococcal surface protein C

RNA, ribonucleic acid

RT-PCR, reverse transcription polymerase chain reaction

SD, standard deviation

SlrA, streptococcal lipoprotein rotamase A

ST, serotype

TCR, T-cell receptor

TGF$\beta$, transforming growth factor $\beta$

THY, Todd-Hewitt yeast

TLR, toll-like receptor

TNF, tumour necrosis factor

WT, wild-type

# 1 Introduction

## 1.1 Introduction to *Streptococcus pneumoniae*

### 1.1.1 Basic microbiology of *S. pneumoniae*

*Streptococcus pneumoniae*, also known as the pneumococcus, is a Gram-positive encapsulated bacteria, first described in 1881 as a pathogenic diploccoci. It can be identified microbiologically on the basis of showing alpha-haemolysis on blood agar plates, optochin resistance and bile solubility (Garcia-Suarez Mdel et al., 2006). *S. pneumoniae* usually exists as a diplococcus, forming pairs of lancet-shaped cocci, but is also capable of forming small chains. It is a facultative anaerobe growing best in 5% $CO_2$, at 37° C, in broth or as colonies on blood agar.

As a Gram-positive bacteria *S. pneumoniae* has a single cytoplasmic membrane surrounded by a peptidoglycan cell wall, common to all serotypes. A number of Gram-positive bacteria including *S. pneumoniae* synthesise a large polysaccharide capsule that surrounds and is attached covalently to the cell wall. The pneumococcal capsular polysaccharide (CPS) was first described as a soluble carbohydrate in cultures of *S. pneumoniae* (Dochez and Avery, 1917, Heidelberger and Avery, 1923). It was subsequently determined that different S*. pneumoniae* isolates varied in the structure of polysaccharide they produced (Heidelberger et al., 1925). Distinct capsular polysaccharide structures form the basis of the 93 known serotypes of *S. pneumoniae* (Gladstone et al., 2011, Bentley et al., 2006).

### 1.1.2  *S. pneumoniae* infection

### 1.1.2.1  Colonisation

*S. pneumoniae,* along with a number of other potential bacterial pathogens including *Staphylococcus aureus* and *Haemophilus influenzae* asymptomatically colonises the nasopharynx in healthy individuals. The acquisition and clearance of colonisation is a dynamic process and *S. pneumoniae* is transmitted via aerosol droplet spread from other colonised individuals (Hartzell et al., 2003). Nasopharyngeal colonisation with *S. pneumoniae* is almost universal in infants; in a longitudinal study in the UK mean carriage (colonisation) rates were highest between 0-2 years of life (52% of individuals colonised at any one time), with carriage occurring more than once in 86% of children in this age group (Hussain et al., 2005). The average duration of the first episode of colonisation has been estimated at 63 days (Turner et al., 2012).  Carriage prevalence reduces with age, with mean carriage rates dropping to below 10% in those over 18 years old (Hussain et al., 2005). Although largely asymptomatic in itself, it has been suggested that nasopharyngeal carriage is a necessary pre-requisite for the development of invasive disease (Bogaert et al., 2004). Carriage of *S. pneumoniae* in the nasopharynx leads to a prolonged interaction with the immune system of the host. This interaction is likely to influence the adaptive immune response that assists prevention of future invasive pneumococcal disease (Cohen et al., 2011, McCool et al., 2002).

### 1.1.2.2    Symptomatic disease

Although most *S. pneumoniae* infection episodes involve colonisation of the nasopharynx, *S. pneumoniae* is also one of the most important human bacterial pathogens, responsible for up to 1.6 million deaths annually worldwide, including 1 million child deaths (Levine et al., 2006). *S. pneumoniae* infection can manifest as a number of different diseases (**Figure 1.1**). Aspiration of *S. pneumoniae* colonising the nasopharynx into the lungs may result in pneumonia. In the UK *S. pneumoniae* is the leading cause of community acquired pneumonia, with an annual incidence estimated at 36.5 per 100,000 people (Bewick et al., 2012). *S. pneumoniae* is also a leading cause of meningitis, an infection of the meninges surrounding the brain, and bacteraemia, the presence of bacteria in the blood. Both bacteraemia and meningitis have a case fatality rate of approximately 25% in adults (Ludwig et al., 2012). Bacteraemia is a relatively common complication of *S. pneumoniae* pneumonia, occurring in approximately 20% of cases admitted to hospital. Pneumonia has an overall mortality of around 5 to 10%, but is much commoner than septicaemia and meningitis so accounts for the highest burden of pneumococcal disease, and worldwide approximately 90% of deaths as a result of *S. pneumoniae* infection are due to pneumonia (O'Brien et al., 2009, Fitzwater et al., 2012). As well as severe invasive disease, *S. pneumoniae* is also a common cause of middle ear infection and is the most frequent bacteria isolated from middle ear fluid of individuals suffering acute otitis media (Jacobs et al., 1998, Sommerfleck et al., 2012). In addition to primary disease, *S. pneumoniae* is an important cause of secondary bacterial infection following influenza infection. It is estimated that secondary infection with *S. pneumoniae* may be present in up to 40% of patients with influenza (Wang et al., 2011). The presence of *S. pneumoniae* in influenza patients is associated with increased mortality (Palacios et al., 2009), and in murine models previous Influenza infection exacerbated the severity of subsequent infection with *S. pneumoniae* (Li et al., 2012).

The incidence of invasive pneumococcal disease is highest at the extremes of age, with significantly higher rates of disease in individuals under 2 or over 65 years of age (van der Poll and Opal, 2009).

Individuals with acquired immune deficiencies, including HIV infection, also have an increased risk of invasive pneumococcal disease, and *S. pneumoniae* is the leading cause of bacterial respiratory infection in adults and children with HIV (Janoff et al., 1992). There is also an increased incidence of invasive pneumococcal infection in individuals with chronic lung disease for example in patients diagnosed with Chronic obstructive pulmonary disease (COPD) (Chidiac, 2012).
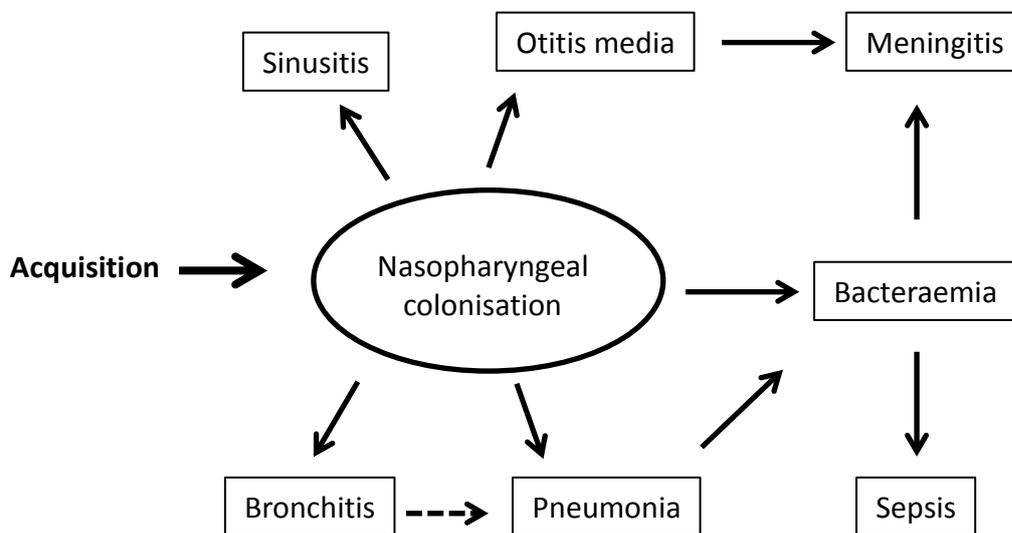
**Figure 1.1: Types of disease caused by *S. pneumoniae*.**

Some of the common clinical manifestations of *S. pneumoniae* disease following nasopharyngeal colonisation and the potential interactions between each disease type (Bogaert et al., 2004).

### 1.1.2.3   *S. pneumoniae* serotypes and disease

The prevalence of different pneumococcal serotypes varies widely, as does their ability to cause disease (Brueggemann et al., 2004). Between 2008 and 2010 the most common serotypes of *S. pneumoniae* detected in patients suffering community acquired pneumonia in a UK hospital were serotypes 14, 8 and 1 (Bewick et al., 2012).

For individual serotypes the incidence of invasive disease relative to the number of colonisation events has been estimated for the most prevalent serotypes. These ratios can be expressed as a serotype-specific 'attack rate', calculated from the incidence of invasive pneumococcal disease per 100,000 acquisitions for each serotype. This attack rate is a measure of a serotypes potential to cause invasive disease, often described as serotype 'invasiveness'. In a UK study, highly invasive serotypes (>20 cases of invasive disease per 100,000 acquisitions) included 4, 14, 7F, 9V, and 18C (Sleeman et al., 2006). There is an inverse relationship between both carriage duration or prevalence and invasiveness for each serotype (Sleeman et al., 2006, Brueggemann et al., 2004); serotypes with a low attack rate appear to stably colonise the nasopharynx for longer durations, in comparison to highly invasive serotypes which colonise for relatively short periods of time.  Recent epidemiological observations in the UK indicate that with increasing age an increase in serotype-specific 'attack rate' occurs, across all serotypes. This age-related increase seems to be more pronounced in less-invasive compared to highly-invasive serotypes (Bewick et al., 2012). As well as relative invasiveness, pneumococcal serotypes also vary in the type and severity of invasive disease they cause. Serotypes 1 and 5 are more commonly found in complicated pneumonias in children (Hausdorff, 2007). Serotypes 1 and 14 are more often isolated from the blood than serotypes 3, 19 and 23, which in turn are isolated more frequently from the cerebrospinal fluid (Hausdorff et al., 2000a).

### 1.1.2.4 Treatment of *S. pneumoniae* infection

Prior to the discovery of penicillin, serum therapy was the main treatment for invasive pneumococcal disease. Serum therapy involved the transfer to patients of type-specific serum, often from horses or rabbits vaccinated with pneumococcal polysaccharide (Goodner and Horsfall, 1937). Such therapy was reported to reduce death rates, and protect against bacteraemia in patients presenting with pneumococcal pneumonia (Bullowa and Wilcox, 1936), and also prevented bacteraemia in animal models of disease (Bull, 1915c). The toxicity of serum therapy and the emergence of effective antibiotics led to its abandonment. Currently β-lactam antibiotics are the first-line in the treatment of invasive pneumococcal disease (Feldman and Anderson, 2011). However *S. pneumoniae* has developed resistance to many major classes of antibiotics, including β-lactams, macrolides and fluoroquinolones (Feldman and Anderson, 2011). Although the incidence of resistance varies widely with geography, it is estimated that overall 15-30% of *S. pneumoniae* isolates worldwide are multi-drug resistant (resistant to 3 or more classes of antibiotics) (Lynch and Zhanel, 2009). Though currently antibiotic resistance may not be a major clinical problem, the potential lack of available treatment options due to increasing antibiotic resistance could have major consequences for the future case-fatality rate of *S. pneumoniae* infections. Increasing antibiotic resistance has led to the suggestion that antibody based therapies may have a role to play in future treatment of *S. pneumoniae* infections (Casadevall et al., 2004, Casadevall and Scharff, 1995).

### 1.1.3 Global serotype distribution

The global distribution of pneumococcal serotypes is not uniform. Serotypes responsible for both carriage and invasive disease vary with location. Serotypes 19F and 23F were the most common serotypes isolated from nasal washings in a longitudinal study of Thai children (Turner et al., 2012). In comparison the most common serotypes isolated form nasal washings of Italian children, over a similar time period, were 10A and 10F (Ansaldi et al., 2012).The *S. pneumoniae* serotypes responsible for invasive disease also vary depending on location. The most common serotypes responsible for invasive pneumococcal disease in Africa are serotypes 1, 5 and 6A whereas in Europe and North America serotypes 14 and 6B appear more commonly as causes of invasive disease (**Figure 1.2**) (Mehr and Wood, 2012, Donkor et al., 2013). In Latin America serotype 14 is dominant with 50% of isolates responsible for acute otitis media in Argentinian patients in 2009 being serotype 14, whereas in the USA serotype 14 was detected in approximately 10% of isolates, over the same time period (Rodgers et al., 2009). Differences in global serotype distribution are reflected in different coverage rates of the 13-valent pneumococcal vaccine, which is lower in Africa than Europe or North America (**Figure 1.2**). Vaccine introduction itself may reduce the circulation of certain serotypes within a population (Fitzwater et al., 2012); therefore uneven vaccine uptake may contribute to global variations in serotype prevalence
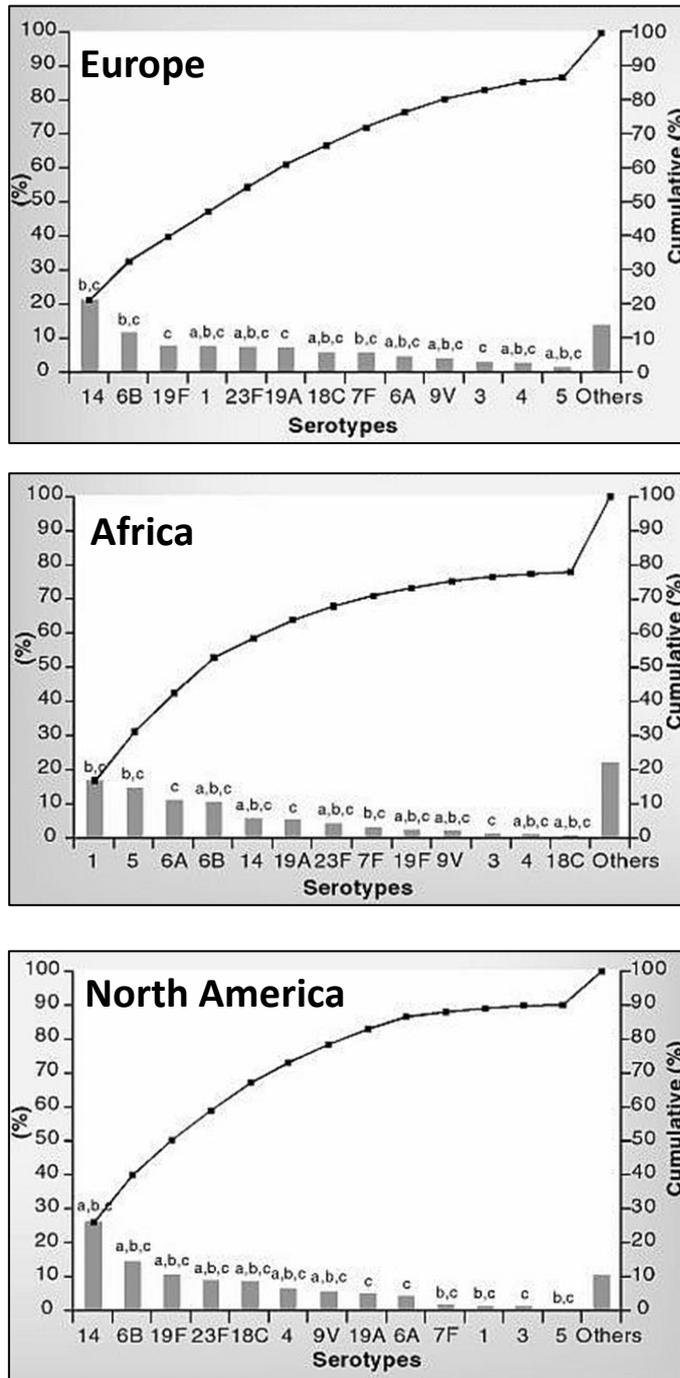
**Figure 1.2: Global distribution of *S. pneumoniae* serotypes.**

Estimated prevalence of invasive pneumococcal disease in different geographical areas, for the 13 serotypes included in the 13-valent conjugate vaccine, prior to the introduction of PCV-7 into national immunisation programmes. Bars indicate the mean percentage of different serotypes and line indicates the cumulative mean (McIntosh and Reinert, 2011).

## 1.2  *S. pneumoniae* surface envelope and genetics

### 1.2.1  *S. pneumoniae* capsule

Based on the relatedness of their capsular polysaccharide structure the 93 serotypes of *S. pneumoniae* can be categorised into 46 serogroups. The pneumococcal capsule is a virulence factor that aids pneumococcal survival in the host, and unencapsulated *S. pneumoniae* do not cause invasive infections in animal models of disease (Watson and Musher, 1990).  The capsule may aid virulence via a number of mechanisms. Firstly, the presence of a polysaccharide capsule can inhibit phagocytosis by neutrophils (Hyams et al., 2010a).The composition of different polysaccharide serotypes also affects their susceptibility to neutrophil mediated phagocytosis. Highly encapsulated serotypes such as 19F and 23F display increased resistance to neutrophil mediated phagocytosis, which has been linked to a higher carriage prevalence (Weinberger et al., 2009, Hyams et al., 2010c). The capsule may also help *S. pneumoniae* evade host defence mechanisms by masking immunogenic surface molecules (Alugupalli and Gerstein, 2005). For example, the ability of pneumococcal surface protein C (PspC) to bind factor H, a negative regulator of the complement pathway is affected by capsular serotype (Yuste et al., 2010) and there is a positive correlation between serotype-invasiveness and factor H binding (Hyams et al., 2013). The binding of antibodies to pneumococcal surface protein A (PspA) may also be affected by capsular serotype (Abeyta et al., 2003). Investigations using capsular-switched isogenic strains of *S. pneumoniae* have indicated that the capsule may alter the surface-exposure of the pneumococcal proteins CbpA and PsrP, which are adhesins,  thus affecting virulence (Sanchez et al., 2011). The metabolic demand of synthesising different pneumococcal polysaccharides varies between serotypes, and this may affect the ability of *S. pneumoniae* strains to colonise the nasopharynx (Hathaway et al., 2012).

As well as serotype variation the degree of capsular polysaccharide expression by a particular strain can vary, causing phase variation. Transparent phase variants have lower levels of capsular polysaccharide (Kim and Weiser, 1998) , exercise increased adherence to host cells and can more

easily colonise the nasopharynx (Weiser et al., 1994). Opaque variants on the other hand display higher levels of capsular polysaccharide and exhibit greater resistance to phagocytosis, but decreased adherence to host cells.
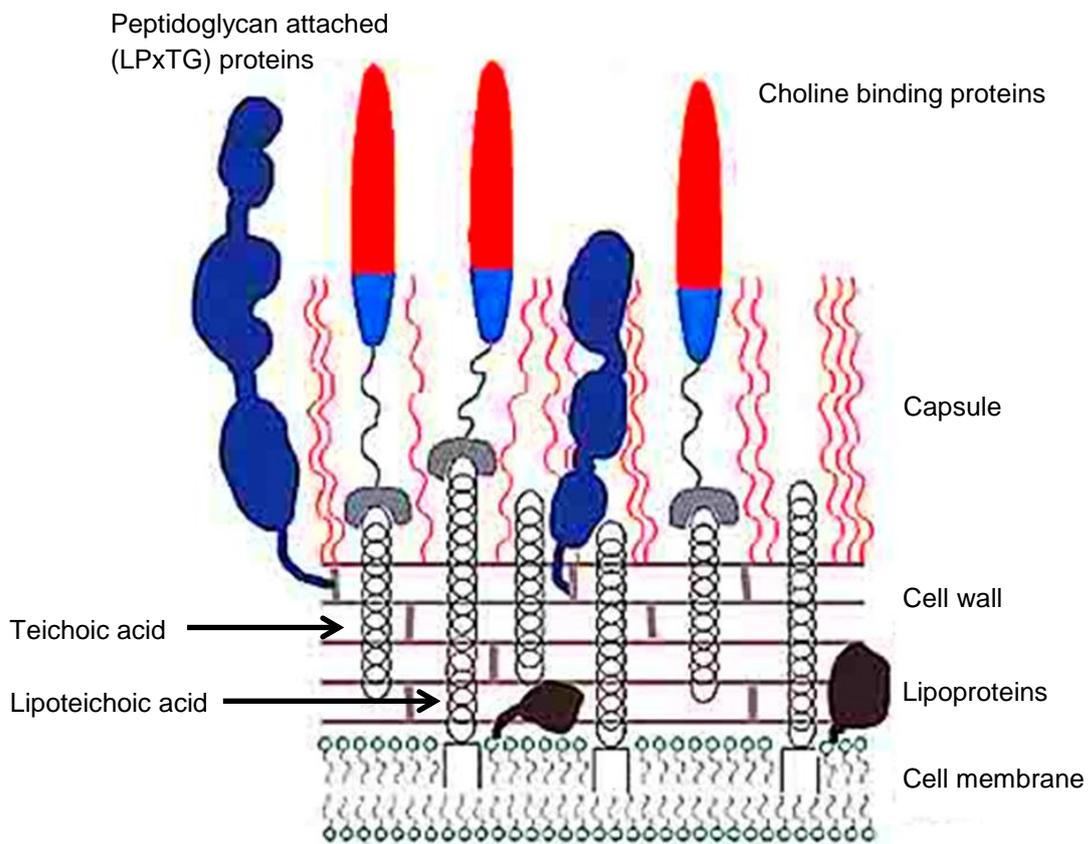
**Figure 1.3: Basic structure of *S. pneumoniae* cell surface.**

Basic schematic of *S. pneumoniae* surface structure including the lipid cell membrane, peptidoglycan cell wall, polysaccharide capsule and three classes of surface protein (Jedrzejas, 2004).

### 1.2.2 *S. pneumoniae* cell wall

In common with other Gram-positive bacteria *S. pneumoniae* has a peptidoglycan cell wall that is essential for maintaining the structural integrity of the cell (**Figure 1.3**). Peptidoglycan in the *S. pneumoniae* cell wall is composed of chains of the glycan N-acetylmuramic acid and N-acetyl-D-glucosamine (Bui et al., 2012), cross-linked by short peptides (Navarre and Schneewind, 1999). The cell wall of *S. pneumoniae* also contains accessory structures including teichoic acid and lipoteichoic acid (Navarre and Schneewind, 1999). Teichoic acids are covalently bound to cross-linked peptidoglycan via a phosphodiester bond. Lipoteichoic acids are anchored to the lipid cell membrane (**Figure 1.3**). Both teichoic acid lipoteichoic acids contain phosphorylcholine residues, to which one class of pneumococcal surface proteins (choline binding proteins) can bind. Peptidoglycan associated teichoic and lipoteichoic acids make up the pneumococcal cell wall polysaccharide (CWPS) antigen (Lu et al., 2009).

### 1.2.3 *S. pneumoniae* surface proteins

Surface proteins of *S. pneumoniae* are important for its interaction with its environment and therefore contribute to disease pathogenesis. Surface exposed proteins may also be targets for opsonizing antibody. Detection of genes with common motifs, suggestive of surface localisation, indicates that 4% of the pneumococcal genome may code for proteins that are surface located (Wizemann et al., 2001). A number of mechanisms of localisation and attachment of proteins to the bacterial surface have been described (Bergmann and Hammerschmidt, 2006). Table 1.1 shows an overview of some important surface proteins, grouped by their mechanism of attachment to the bacterial surface.

| Lipoproteins | | Roles |
| --- | --- | --- |
| PpmA | Putative proteinase maturation protein A | Adherence to epithelial cells |
| SlrA | Streptococcal lipoprotein rotamase A | Modulates virulence factors |
| PsaA | Pneumococcal surface adhesin A | Adhesion to host cells, Manganese transport |
| PiaA | Pneumococcal iron acquisition A | Iron transport |
| PiuA | Pneumococcal iron uptake A | Iron transport |
| **Choline binding proteins** | | |
| PspA | Pneumococcal surface adhesin A | Binds apolactoferrin, Inhibits complement deposition |
| PspC | Pneumococcal surface protein C | Binds complement factor H, Role in adhesion to host -tissue |
| 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 |
| **LPxTG proteins** | | |
| NanA | Neuraminidase | Cleavage of terminal sialic acid residues on host cell glycolipids and glycoproteins |
| IgA1ase | IgA1 protease | Cleaves IgA1 |
| PrtA | | Serine protease |
| HtrA | High-temperature requirement A | Heat-shock induced serine proteases, resistance to oxidative stress |
| Hyal | Hyaluronidase | Breakdown of host extracellular matrix |
| **Non-classical** | | |
| 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 |
| PavA | Pneumococcal adhesion and virulence A | Binds fibronectin |
| Eno | Enolase | Binds to plasminogen, promotes degradation of extracellular matrix |

Table 1.1: Summary of some of the well-characterised surface proteins of *S. pneumoniae* grouped according to their mechanism of attachment to the bacterial surface (Bergmann and Hammerschmidt, 2006, Kadioglu et al., 2008).

### 1.2.3.1 Choline binding proteins

The choline binding protein PspA was first identified as a protein that lacked the features of streptococcal LPxTG proteins, but instead bound to choline residues on cell wall lipoteichoic acid via its C-terminal region (Yother and Briles, 1992). The release of PspA from the bacterial surface can be induced by either incubation of bacteria in high concentrations of choline, or deletion of the C-terminal region (Yother and Briles, 1992).This mechanism of attachment is shared by a number of other pneumococcal surface proteins, all sharing a common C-terminal choline binding domain, consisting of a repeating 20 amino acid sequence (Wren, 1991). The common C-terminal domain of these proteins binds choline residues attached to pneumococcal cell wall teichoic and lipoteichoic acid, resulting in their non-covalent attachment to the cell surface (**Figure 1.3**). Other well characterised CBPs of *S. pneumoniae* include autolysin (LytA) (Garcia 1986) and pneumococcal surface protein C (PspC) (Rosenow et al., 1997).

### 1.2.3.2 LPxTG motif proteins

Surface proteins with an LPxTG amino acid signal sequence represent a class of proteins common to Gram-positive cocci (Fischetti et al., 1990). The presence of an LPxTG signal sequence at the C-terminus targets these proteins to the bacterial cell surface (Navarre and Schneewind, 1999). This signal sequence is cleaved by sortase, a transpeptidase, and the resultant cleaved proteins are then covalently attached via sortases to peptidoglycan of the pneumococcal cell wall (Mitchell and Mitchell, 2010). Genome analysis indicates the presence of 19 LPxTG anchored surface proteins in the TIGR4 strain of *S. pneumoniae* (Bergmann and Hammerschmidt, 2006). Some pneumococci express pili, long appendages involved in adhesion that extend beyond the capsule (Barocchi et al., 2006). The pneumococcal pilus is attached to the cell surface via a LPxTG signal sequence (Lofling et al., 2011).

### 1.2.3.3 Lipoproteins

Cell surface lipoproteins are covalently linked to phospholipids of the pneumococcal cell membrane (**Figure 1.3**). This mechanism of attachment to the cell surface is conserved among bacteria (Sutcliffe and Harrington, 2002). Lipoproteins are initially secreted as prolipoproteins, following secretion they are covalently attached to the membrane phospholipid diacylglyceryl via the enzyme diacylglyceryl transferase (Lgt) (Tokunaga et al., 1982). Following attachment to the phospholipid membrane, the N-terminal signal peptide is cleaved by the enzyme lipoprotein signal peptidase (Lsp), forming the mature lipoprotein. Some lipoproteins form important components of transmembrane ABC transporters, these include the iron-uptake transporters Piu, Pit and Pia (Brown et al., 2001) and the manganese uptake transporter PsaA (Dintilhac et al., 1997). Other lipoproteins are not components of ABC transporters, for example, PpmA is an adhesin involved in pneumococcal adherence to host tissues (Gor et al., 2005).

### 1.2.3.4 Non-classical surface proteins

There are a number of pneumococcal surface proteins that fall outside any of the three categories already described, and are attached to the cell surface by other novel and often poorly understood mechanisms that would not be detected by genome analysis. These proteins can be collectively grouped into 'non-classical' surface proteins and include, for example, the pneumococcal histadine triad (Pht) family of proteins (Adamou et al., 2001). As their name suggests the Pht proteins contain a repeated histadine triad motif and have been demonstrated as surface located by flow-cytometry. A pneumococcal serine rich repeat protein (PsrP) has also been described (Rose et al., 2008). This protein is a member of a family of serine rich repeat proteins (SRRP) found in numerous pathogenic bacteria and has a role in adhesion to host tissue and the development of invasive disease (Sanchez et al., 2010). Additional *S. pneumoniae* proteins have transmembrane insertions, which mean they are partially expressed on the cell surface. For example the pneumococcal serine/threonine kinase (Stk-P) has a transmembrane region anchoring it to the cell surface (Echenique et al., 2004).

## 1.3 Genetic diversity of *S. pneumoniae*

### 1.3.1 Genome structure

*S. pneumoniae* contains a single circular chromosome estimated at between 2,240 to 2,270 kilobase pairs (KBP) in length (Gasc et al., 1991). The first pneumococcal genome to be sequenced was the serotype 4 strain TIGR4, which contains approximately 2200 open reading frames (ORFs) (Tettelin et al., 2001). Subsequently the genomes of other pneumococcal strains have been sequenced, all containing approximately 2000 ORFs (Barocchi et al., 2007). Significant genomic diversity between strains of *S. pneumoniae* exists on a number of levels.

#### 1.3.1.1 Transformation

*S. pneumoniae* is naturally competent. Competence refers to the ability of an organism to undergo genetic transformation, incorporating DNA into its genome via homologous recombination. Induction of competence in *S. pneumoniae* is controlled by the concentration of a secreted peptide competence stimulating peptide (CSP) (Steinmoen et al., 2002). The induction of a competent state is dependent upon the concentration of CSP, this allows populations of *S. pneumoniae* to respond to their own density, a process known as quorum sensing.

Colonisation of the nasopharynx provides a site for the exchange of genetic information between bacteria (Leung et al., 2011). This exchange allows adaptation to environmental pressures, for example, the development of anti-microbial resistance (Zhu and Lau, 2011), or the acquisition of genes involved in virulence (Brown et al., 2001). Investigating the evolution over time of the PMEN1 serotype 23F strain of pneumococcus has allowed the identification of recombination 'hot spots', where horizontal gene transfer events were detected at increased frequency. Recombination 'hotspot' loci include genes encoding the pneumococcal surface proteins PspA, PspC PsrP and capsule biosynthesis. Selective pressures may have led to the removal or alteration of genes encoding the pneumococcal proteins PspA and PsrP in this isolate (Croucher et al., 2011). All the

genes required for type-specific polysaccharide production are located together in genetic 'cassette' (Dillard and Yother, 1994). This facilitates capsule switching during transformation.  Sequence typing indicates that *S. pneumoniae* of the same sequence type may have different capsular polysaccharide serotypes, probably as a result of capsule switching. Capsule switching has been demonstrated in response to the introduction of the pneumococcal conjugate vaccine, where serotype 19A isolates have emerged from a previously 23F expressing lineage (Croucher et al., 2011).

### 1.3.2    Sequence type

As well as by capsular serotype, *S. pneumoniae* may also be categorised based on its genetic sequence type. Multi-locus sequence typing (MLST) classifies *S. pneumoniae* based on the sequence of seven 'core' housekeeping gene fragments (Hanage et al., 2005), therefore allowing grouping into 'sequence type'. It has been demonstrated that a number of isolates responsible for invasive disease from different countries were of the same sequence type, but not necessarily the same serotype (Coffey et al., 1998). These isolates are probably from the same clone and have different capsular polysaccharides due to horizontal transfer of genes encoding the capsule. *S. pneumoniae* strains of different sequence type may therefore have distinct virulence phenotypes independent of differences in their capsular polysaccharide (Sjostrom et al., 2006). More recently it has been demonstrated that the isolates responsible for invasive disease in west Africa are more likely to be clonal (of the same sequence type) than the those identified as common in carriage (Donkor et al., 2013).

### 1.3.3 Regions of difference

As well as global differences in their genomes, genomic variation of *S. pneumoniae* can also occur at specific sites. Studies of the pneumococcal pathogenicity island (PPI-1) were amongst the first to indicate genetic variation at a particular site. PPI-1 is a collection of genes involved in virulence, the 3' region of which is highly variable between different strains. Some variations in PPI-1 have been associated with alterations in the virulence of *S. pneumoniae* (Brown et al., 2001, Harvey et al., 2011)*.* Later further *S. pneumoniae* genes were shown to vary substantially between strains and termed 'regions of diversity' (van der Poll and Opal, 2009). Some regions of diversity are involved in virulence and can distinguish between invasive and non-invasive strains. Comparison of the genome sequence of highly invasive, compared to a non-invasive isolates of *S. pneumoniae* has identified 8 regions of the genome specific only to highly invasive isolates, these include variable regions of the PPI-1, ABC transporters and metabolic enzymes (Harvey et al., 2011).

Along with regions of diversity, single genes are present or absent in different strains. Overall analysis of seventeen pneumococcal genomes indicated that less than 50% of genes were conserved between all 17 pneumococcal strains examined (Hiller et al., 2007). This diversity has led to the 'supra-genome' hypothesis being applied to *S. pneumoniae*, which states that all the genes available to a species exist in a hypothetical pool, with every strain deriving and contributing genes to this pool (Hiller et al., 2007).

Genetic diversity means that certain pneumococcal proteins that may be protective antigens are not present in all serotypes. For example significant differences in the expression of the pneumococcal adhesin PsrP have been demonstrated between serotypes (Munoz-Almagro et al., 2010), and the pneumococcal pili are also only present on certain pneumococcal serotypes (Basset et al., 2007b).

### 1.3.4    Allelic variation

Whilst many pneumococcal surface proteins are structurally conserved between serotypes and strains, some of these proteins display allelic variance, providing an additional level of genetic variation between strains. For example, the gene sequence for the choline binding protein PspC  is highly variable between strains, and based on sequence identity it has been classed into 11 different groups (Iannelli et al., 2002). Antibodies to one allele of PspC do not necessarily cross react with other alleles; mice immunised with recombinant PspC from a type 3 strain (HB565) were protected against homologous challenge, partially protected against infection with a serotype 2 *S. pneumoniae* (D39), but not protected against challenge with a serotype 4 strain (Ricci et al., 2011). These differences in protection were linked to the level of variation in the amino acid sequence of PspC between strains.  PspA is also structurally variable between serotypes, alleles of PspA are divided into two main families and further into different clades (Hollingshead et al., 2000). The development of antibodies to PspA may be family specific (Melin et al., 2008), and although antibodies raised against one PspA family can cross-react with PspA from both families (Nabors et al., 2000), the level of cross-reactivity varies between families (Nabors et al., 2000). Similar to PspC, comparisons have been made of the ability of polyclonal mouse sera raised against PspA fragments from different clades to bind to PspA, with the cross-reactivity of anti-PspA antibodies related to the level of similarity of PspA between strains (Darrieux et al., 2008). In contrast lipoproteins (for example, PsaA, PiuA and PiaA) are highly conserved between pneumococcal serotypes (Sampson et al., 1997, Jomaa et al., 2005), and antibodies raised against the lipoproteins Piu/Pia from *S. pneumoniae* D39 (serotype 2), mediated increased phagocytosis of a number of other *S. pneumoniae* serotypes (Jomaa et al., 2005).

### 1.3.4.1 Gene expression

Pneumococcal gene expression is a dynamic process. Analysis of gene expression by microarray indicated that expression of a number of genes can be altered when *S. pneumoniae* are exposed to altered environments, which may affect virulence (Orihuela et al., 2004). 17 out of 20 of the genes classified as 'virulence genes' were differentially expressed when exposed to different physiological niches (blood, cerebrospinal fluid, and cultured epithelial cells). Changes included, for example, enhanced expression of the pneumococcal surface protein PspC in the presence of cultured epithelial cells. An increased expression of PspA was observed in all three physiological environments and enhanced expression of the pneumococcal surface protein PhtD was demonstrated in CSF. Investigation of gene expression by RT-PCR confirmed alterations in gene expression in different organ compartments *in vivo*. In addition, PspC was more abundantly expressed in *S. pneumoniae* isolates from the nasopharynx than the lungs and blood of mice 72hrs post intranasal (IN) infection with the D39 strain (LeMessurier et al., 2006). Microarray studies have also shown pronounced differences in gene expression between *S. pneumoniae* strains when exposed to the same physiological conditions (Ogunniyi et al., 2012).

## 1.4 Immunity to *S. pneumoniae*

### 1.4.1 Immunodeficiency and *S. pneumoniae*

There are a number of primary immune deficiencies that may result in reduced or absent immunoglobulin production. These diseases include X-linked agammaglobunemia, caused by a defect in B-cell differentiation and common variable immune deficiency (CVID), a heterogeneous group of primary immune deficiencies all associated with deficient immunoglobulin production. Patients with primary immunoglobulin deficiencies display increased susceptibility to a number of infections, including an increased incidence of pneumonia (Oksenhendler et al., 2008). *S. pneumoniae* and *H. influenzae* are the most common causative agents of pneumonia in these patients (Rosen et al., 1995). The incidence of respiratory tract infection in immunoglobulin deficient patients can be partially reversed by intravenous immunoglobulin (IVIG) replacement therapy (Quinti et al., 2011). IVIG is pooled, purified immunoglobulin (IgG) from over a thousand donors, administered intravenously (IV) (Schwab and Nimmerjahn, 2013). The 'trough' concentration of IgG in patients' blood between infusions of IVIG has been used to assess treatment efficacy and some studies have indicated that higher trough levels of IVIG may be protective against pneumonia (Orange et al., 2010). It has also been noted that IVIG therapy may reduce the deterioration in pulmonary function in patients with CVID (measured by FEV1) (de Gracia et al., 2004), presumably due to prevention of recurrent lung infection. The increased incidence of pneumococcal lung infection in immunoglobulin deficient patients, and its partial reversal following replacement therapy with pooled IgG, suggests an important role for IgG in natural immunity to lung infection with *S. pneumoniae.*

As well as antibody deficiencies, other immune deficiencies are associated with increased susceptibility to infection with *S. pneumoniae*. Patients with deficiencies in the classical pathway of complement activation have increased incidence of pneumococcal septicaemia, meningitis and

bacterial pneumonia (Jonsson et al., 2005). Phagocytosis of *S. pneumoniae* was reduced when opsonised in the sera of patients with a classical complement defect, compared to healthy controls (Yuste et al., 2008). Patients with cellular immune deficiencies are also more susceptible to pneumococcal disease, and the incidence of invasive *S. pneumoniae* may be up to 30 times higher in HIV positive patients compared to healthy controls (Chidiac, 2012). Impaired CD4+ T-cell responses to *S. pneumoniae* have been demonstrated in Malawian adults with HIV (Glennie et al., 2011). Asplenic patients have compromised immunity associated with increased susceptibility to infection, in particular sepsis, with encapsulated bacteria such as *S. pneumoniae.* It is estimated that 50-90% overwhelming infections post-splenectomy are down to *S. pneumoniae* (Davidson and Wall, 2001, Waghorn, 2001). In animal models of bacteraemia, experimental splenectomy significantly reduced the clearance of *S. pneumoniae* from the blood (Shinefield et al., 1966, Okinaga et al., 1981).

### 1.4.2 Innate Immunity to *S. pneumoniae*

### 1.4.2.1 Host-recognition of *S. pneumoniae*

The host response to invading bacteria requires cells to identify the bacterial pathogen. This is mediated by pattern recognition receptors (PRRs) on host cells, which recognise conserved pathogen associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are one well-described family of intra and extra-cellular pattern recognition receptors and a number of TLR's may recognise different components of *S. pneumoniae*. TLR2 recognises lipoteichoic acid and lipoproteins of Gram-positive bacteria including *S. pneumoniae* (Mogensen et al., 2006). TLR2 KO mice displayed impaired clearance of pneumococcal colonisation (van Rossum et al., 2005). In addition TLR4 recognises the pneumococcal toxin pneumolysin, and TLR9 recognises unmethylated CpG motifs on bacterial DNA (Paterson and Orihuela, 2010). Another family of PRRs are the NOD-like receptors (NLRs) which identify PAMPs within the cell cytosol. NOD-2 recognises peptidoglycan, and detects internalised *S. pneumoniae* (Opitz et al., 2004). Certain NLRs can form intracellular signalling complexes called inflammasomes, which can be activated by the pneumococcal toxin pneumolysin (McNeela et al., 2010). Other PRRs that may detect *S. pneumoniae* include scavenger receptors and SIGN-R1 on macrophages. The scavenger receptor MARCO is required for maximal TLR2 and NOD2 signalling, in response to colonisation with *S. pneumoniae* in mice (Dorrington et al., 2013). SIGN-R1 recognises the pneumococcal polysaccharide capsule (Paterson and Orihuela, 2010).

### 1.4.2.2 Role of complement in immunity to *S. pneumoniae*

The complement system consists of a series of plasma and cell surface proteins involved in the opsonisation and killing of pathogens. Complement is an important component of innate immunity to *S. pneumoniae*, and individuals with an inherited deficiency in the central complement component C3, or the classical pathway component C2 are highly susceptible to pneumococcal infection (Paterson and Orihuela, 2010, Jonsson et al., 2005). There are three main pathways of complement activation, classical, lectin and alternative. The classical pathway is initiated by the binding of immunoglobulin, or acute phase proteins such as C reactive protein (CRP) and serum amyloid P (SAP) to phosphorylcholine of the pneumococcal cell wall (Casal and Tarrago, 2003). The lectin pathway is initiated by the binding of mannose binding lectin (MBL) to mannose on the pathogen surface (Eisen, 2010). The alternative pathway involves the deposition of complement component C3 directly on the bacterial surface. In murine models of disease the classical pathway has been shown to be the dominant complement pathway in innate immunity to *S. pneumoniae*. (Brown et al., 2002b). The importance of complement in immunity to *S. pneumoniae* is highlighted by the complement-evasion strategies it employs. These include the binding of the complement regulator factor H by PspC (Yuste et al., 2010) and inhibition of complement deposition by the capsular polysaccharide (Hyams et al., 2010a).The pneumococcal surface protein PspA also inhibits complement activity by competing with CRP for binding to phosphocoholine residues on bacterial surface (Mukerji et al., 2012). Opsonisation of *S. pneumoniae* by complement leads to phagocytosis and assists bacterial clearance. Complement is essential for effective clearance of *S. pneumoniae* from the blood (Yuste et al., 2005). Studies of type 3 pneumococcus have shown that a number of complement proteins are present in the lungs of animals challenged with *S. pneumoniae* (Coonrod and Yoneda, 1981), and complement depletion resulted in increased CFU in the lungs, in animal models of *S. pneumoniae* pneumonia (Yuste et al., 2005).

**1.4.2.3    Innate immunity to colonisation with *S. pneumoniae***

The primary clearance of *S. pneumoniae* from the nasopharynx depends upon neutrophils and macrophages (van Rossum et al., 2005, Zhang et al., 2009). In murine models of colonisation, neutrophils were recruited to the lumen of the nasopharynx and depletion of neutrophils resulted in normally asymptomatic colonisation becoming invasive (Matthias et al., 2008). Neutrophils may also facilitate the delivery of pneumococcal antigen to lymphoid tissue (Matthias et al., 2008). However alone neutrophils may not be sufficient to clear primary *S. pneumoniae* colonisation, as the decline in bacterial numbers in the nasopharynx of colonised mice failed to correlate with neutrophil influx (Matthias et al., 2008). Instead it appears clearance of primary colonisation depends upon the recruitment of monocytes and macrophages as depletion of these cells reduced pneumococcal clearance from the nasopharynx (Zhang et al., 2009). Neutrophils on the other hand appear to be important in the clearance of *S. pneumoniae* from the nasopharynx following secondary challenge, in previously colonised mice. Clearance of *S. pneumoniae* may be influenced by the presence of other bacterial species co-colonising the nasopharynx. In comparison to inoculation with *S. pneumoniae* alone, mixed inoculation of both *H. influenzae* and *S. pneumoniae* into the nasopharynx of mice resulted in a rapid clearance of *S. pneumoniae*, associated with a greater influx of neutrophils (Lysenko et al., 2005).

**1.4.2.4    Innate immunity to lung infection with *S. pneumoniae***

The first line of immunity to *S. pneumoniae* reaching the lungs is the respiratory epithelium. *S. pneumoniae* may be cleared from the airways by the mucociliary clearance system of respiratory epithelial cells (Pittet et al., 2010). Epithelial cells secrete surfactant proteins which are capable of binding to and agglutinating pneumococci and appear to contribute to host defence (Jounblat et al., 2005). Epithelial cells also secrete numerous anti-microbial peptides such as α and β-defensins and cathelecidin in response to bacterial infection (Schaller-Bals et al., 2002), which may also contribute to the clearance of *S. pneumoniae* from the lungs.

Alveolar macrophages are resident phagocytic cells of the lungs, and make up the majority (<95%) of cells in bronchoalveolar lavage fluid (BALF) under normal conditions (Gordon and Read, 2002). Bacterial inoculum into the lung are usually efficiently cleared by alveolar macrophages (Marriott and Dockrell, 2007). This clearance occurs without the recruitment of inflammatory cells and release of inflammatory mediators. However, if the resident alveolar macrophages are unable to initially clear this inoculum, inflammation associated with a recruitment of neutrophils occurs. Alveolar macrophages release inflammatory mediators including tumour necrosis factor alpha (TNF-$\alpha$), interleukin-6 (IL-6) and interleukin-1 (IL-1). These cytokines can stimulate an inflammatory response. For example, IL-1$\beta$ and TNF-$\alpha$ release by macrophages facilitates the release of the neutrophil attracting chemokine CXCL8 (IL-8) from epithelial cells (Standiford et al., 1990, Sun et al., 2007). Neutrophils are usually the first cells recruited following bacterial inoculum into the lungs (Yamada et al., 2011, Dallaire et al., 2001), where they control *S. pneumoniae* numbers by phagocytosis (Hyams et al., 2010a). In murine models of  pneumonia neutrophil influx appeared to peak by 12hrs post-infection with *S. pneumoniae* (Kadioglu et al., 2000).

Neutrophil recruitment is promoted by chemokines released in response to infection. Neutrophils express two CXCR receptors (CXCR1 and CXCR2) which respond to CXC chemokines, resulting in neutrophil migration. CXCR1 responds to CXCL6 (granulocyte chemotactic protein-2) and CXCL8 (IL-8) (Stillie et al., 2009). Additionally CXCR2 responds to a range of other chemokines including CXCL1 (GRO-$\alpha$), CXCL2 (GRO-$\beta$) and CXCL3 (GRO-$\gamma$).The CXC chemokine IL-8 appears to be particularly important in the migration of neutrophils into the lung following bacterial infection. Patients with bacterial pneumonia have been shown to have increased lung concentrations of IL-8 (Baggiolini et al., 1994), and antibody neutralisation of KC (Keratinocyte chemoattractant, CXCL1), considered the murine functional homologue of IL-8 (Singer and Sansonetti, 2004), reduces neutrophil influx in response to experimental bacterial pneumonia in mice (Craig et al., 2009). Bacterial products themselves may mediate neutrophil chemotaxis directly. N-formyl-peptides released by bacteria bind to formyl peptide receptors (FPRs) on neutrophils (Gauthier et al., 2007). Inhibition of FPRs by

cyclosporine H reduced neutrophil numbers in the alveolar space of mice 6hrs following high dose challenge with *S. pneumoniae* (Gauthier et al., 2007). Neutrophils are recruited into the alveoli of infected lungs via the integrins MAC-1 and $\alpha_4\beta_1$ (Kadioglu et al., 2011b).

Phagocytosis of *S. pneumoniae* by neutrophils is enhanced by opsonisation with complement and Immunoglobulins. IgG bound to the surface of *S. pneumoniae* may enhance phagocytosis directly, by interaction with FC$\gamma$ receptors, or indirectly by facilitating complement deposition (Mold et al., 2002). Complement bound to *S. pneumoniae* is recognised by complement receptors on the surface of neutrophils (Williams et al., 2003). Once internalised, there are a number of mechanisms by which neutrophils can kill bacteria. Neutrophils can produce reactive oxygen species (ROS), including hydrogen peroxide and hydroxyl radicals via the enzyme NADPH oxidase (Segal, 2005). Neutrophils also contain anti-microbial peptides within cytoplasmic granules. *In vitro* experiments have indicated that human neutrophils kill *S. pneumoniae* primarily via antimicrobial serine proteases, rather than the generation of ROS (Standish and Weiser, 2009) as inhibition of theses peptides reduced intracellular killing of *S. pneumoniae*, but inhibition of NADPH oxidase did not.

Neutrophil recruitment appears to be crucial to the resolution of pneumococcal pneumonia (Calbo and Garau, 2010), and a robust Influx of neutrophils into the lungs may clear *S. pneumoniae*; however excessive neutrophil influx may contribute to inflammation and lung damage characteristic of pneumonia (Dockrell et al., 2012). Antibody-depletion of neutrophils led to enhanced bacterial numbers in the alveolar space 24-hours post IN infection with *S. pneumoniae* (Sun and Metzger, 2008). However the physical migration of neutrophils into the alveolar space may enhance bacterial invasion into the blood (Marks et al., 2007), and antibody depletion of neutrophils protected mice from bacteraemia post-challenge with a serotype 8 *S. pneumoniae* (Marks et al., 2007).

### 1.4.2.5    Innate immunity to *S. pneumoniae* bacteraemia

As already discussed CRP binding to phosphorylcholine enhances the deposition of complement component C3 on the surface of *S. pneumoniae* (Horowitz et al., 1987), and is important for the clearance of *S. pneumoniae* from the blood, transfer of human CRP protected mice from experimental pneumococcal bacteraemia (Horowitz et al., 1987). The acute phase protein serum amyloid P (SAP) also improves complement deposition on the surface of *S. pneumoniae* and enhances its clearance from the blood (Yuste et al., 2007). Natural poly-reactive IgM is also important for the innate clearance of *S. pneumoniae* from the blood (Brown et al., 2002b, Baxendale et al., 2008). IgM produced in the absence of antigenic stimulation is considered natural IgM.  A particular subset of B-cells, B1a are responsible for the production of germ-line encoded poly-specific natural antibody, primarily IgM (Baumgarth, 2011). Natural IgM is capable of binding to a range of pneumococcal polysaccharide serotypes and passive transfer of natural IgM protected mice in a model of pneumococcal sepsis (Baxendale et al., 2008). IgM appears to target bacteria in the blood to the marginal zone of the spleen, for removal (Zandvoort and Timens, 2002). The spleen is important for the removal of *S. pneumoniae* from the blood, splenectomised mice have impaired clearance of pneumococcal bacteraemia (Shinefield et al., 1966), and in humans *S. pneumoniae* is responsible for more than 50% of overwhelming infections post-splenectomy (Davidson and Wall, 2001). Tracking radiolabelled bacteria following *in vivo* infection indicated that *S. pneumoniae* in the bloodstream is primarily cleared by phagocytic cells of the liver and spleen (Brown et al., 1983). Macrophages located in the marginal zone of the spleen express the C-type lectin SIGN-R1 which binds to pneumococcal capsular polysaccharide and therefore promotes phagocytosis and clearance of *S. pneumoniae* from the blood (Kang et al., 2004).

### 1.4.3    Natural adaptive immune response to *S. pneumoniae*

### 1.4.3.1    Development of humoral immunity

A decrease in the incidence of pneumococcal disease with increasing age in children, following a period of high colonisation incidence, indicates the development of adaptive immunity. As previously discussed, individuals with acquired defects in humoral immunity are at greater risk of *S. pneumoniae* infection (Oksenhendler et al., 2008), demonstrating an important role for antibody in naturally-acquired immunity to *S. pneumoniae*. Purified capsular polysaccharide is an effective serotype-specific vaccine against *S. pneumoniae* through induction of anti-capsular polysaccharide antibody, and anti-polysaccharide immune serum can passively protect against serotype-specific pneumococcal disease (Casadevall and Scharff, 1994). It has therefore been assumed that the main mechanism of naturally-acquired immunity to *S. pneumoniae* is via the generation of antibodies to the pneumococcal polysaccharide (Musher et al., 1993). Longitudinal observational studies have indicated that nasopharyngeal colonisation does result in the development of anti-capsular antibodies (Goldblatt et al., 2005), and there is some epidemiological evidence this causes serotype-specific immunity for at least some capsular serotypes (Weinberger et al., 2008). However, whilst anti-capsular antibodies may play a role in adaptive immunity there is also epidemiological evidence to suggest that other mechanisms are important in the development of naturally-acquired immunity to *S. pneumoniae*. A parallel reduction in the incidence of invasive disease occurs across all serotypes with increasing age in children (**Figure 1.4**) (Lipsitch et al., 2005) , which suggests the development of a common rather than serotype-specific mechanism of immunity. Furthermore, the age over which the reduction in invasive disease occurs does not necessarily coincide with the time at which an increase in anti-capsular antibodies is observed (Lipsitch et al., 2005). Longitudinal studies of *S. pneumoniae* colonisation in children have demonstrated that individuals acquire IgG to a number of *S. pneumoniae* protein antigens following periods of colonisation with *S. pneumoniae* (Prevaes et al., 2012). Data from murine models of experimental colonisation suggest that colonisation with *S.*

*pneumoniae* induces high titres of IgG to *S. pneumoniae*, including antibody to specific pneumococcal protein antigens (Richards et al., 2010, Cohen et al., 2011).
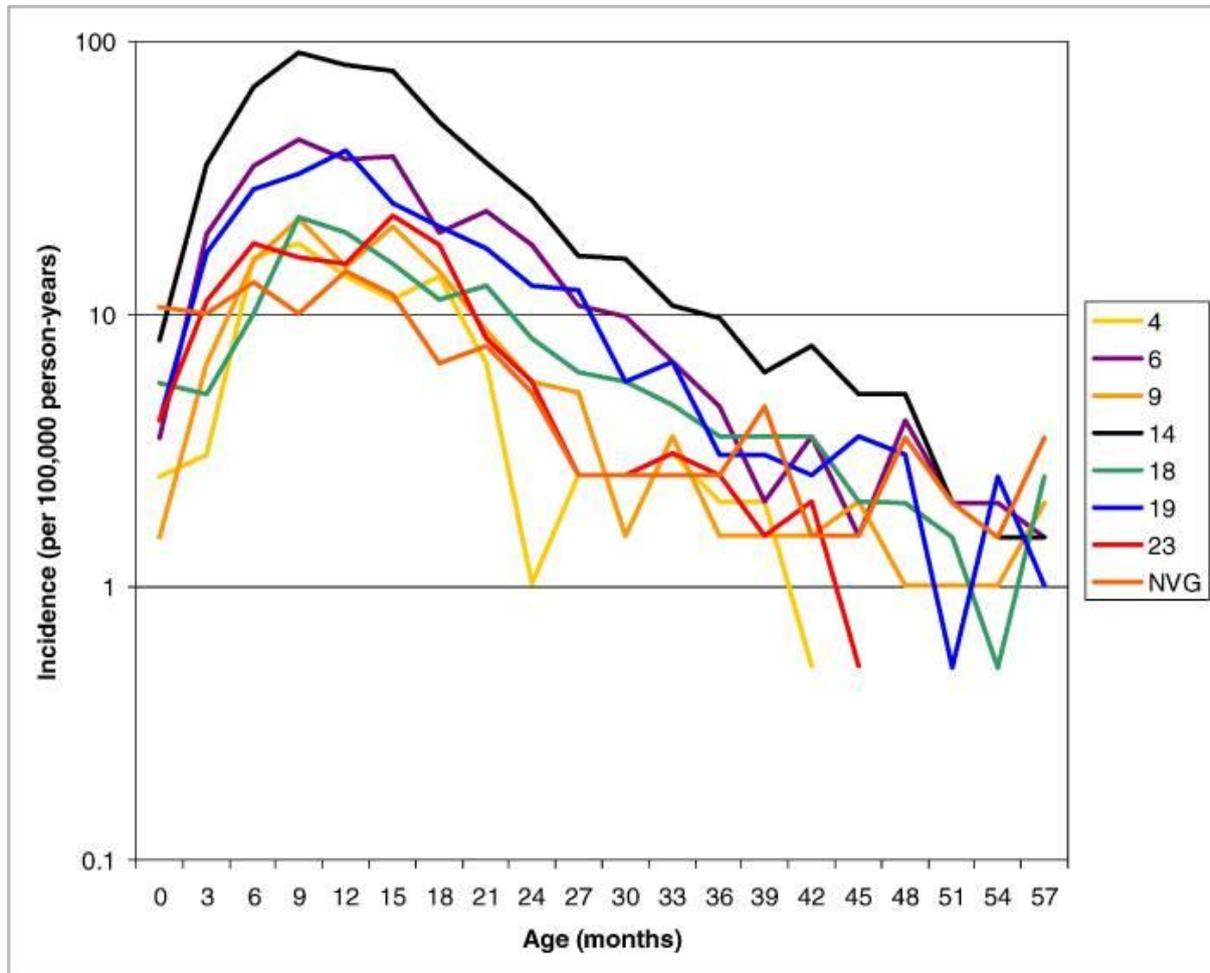


**Figure 1.4: Incidence of invasive *S. pneumoniae* by age.**

Specific incidence of invasive *S. pneumoniae* disease per 100,000 person years in the United States by serogroup (in children of increasing age). 'NVG' indicates those serotypes not included in the 7-valent vaccine combined (Lipsitch et al., 2005).

### 1.4.3.2  Role of IgM/IgA

In addition to IgG, IgA and IgM may have some role in adaptive immunity to *S. pneumoniae*. IgM is also produced by B-cells in response to pathogens, and experimental infection with *S. pneumoniae* serotype 3 induced the production of anti-phosphorylcholine IgM in mice (Koppel et al., 2005). Following experimental colonisation of mice small rises in *S. pneumoniae*-specific IgM have been demonstrated (Cohen et al., 2012), associated with the development of IgM to the capsular polysaccharide. IgA is found in abundance at mucosal surfaces. There are two subclasses of IgA, IgA1 and IgA2, the predominant subclass in the human airway is IgA1 (Kadioglu et al., 2008). Secretory IgA (sIgA) is formed of dimers of IgA1 or IgA2 connected by a J-chain. sIgA  is secreted across epithelial cells by the action of the polymeric Ig receptor (pIgR) (Pabst, 2012). IgA may have a role in protective immunity to *S. pneumoniae*, secretory IgA was produced following IN vaccination with the pneumococcal surface protein PspA. IgA was required for protection in this model as IgA deficient mice were not protected (Fukuyama et al., 2010) . *S. pneumoniae* expresses an enzyme, IgA1ase, capable of cleaving human IgA1, therefore preventing opsonisation by IgA1in the nasopharynx (Wani et al., 1996).  The vast majority of patients with specific IgA deficiencies do not appear to have any clinical manifestations (Pabst, 2012). However, recent a study has suggested that individuals with a selective IgA deficiency may have an increased incidence of respiratory tract infection (Jorgensen et al., 2013).

### 1.4.3.3 Antigen targets for naturally-acquired antibody

Experimental colonisation in mice can result in both the development of anti-CPS IgM and IgG against protein targets (Richards et al., 2010, Cohen et al., 2011), though the dominant antibody responses following murine colonisation appear to be against protein rather than polysaccharide antigens (Cohen et al., 2011). Reduced CFU of *S. pneumoniae* in the nasopharynx of previously colonised mice correlated with both anti-CPS IgM and IgG against the surface protein PspA (Richards et al., 2010). Importantly in mouse models of nasopharyngeal carriage experimental carriage with one serotype was protective against subsequent invasive disease caused by another serotype (Richards et al., 2010, Roche and Weiser, 2010), suggesting the development of serotype independent immunity. In experimental human colonisation models, protection from acquisition of *S. pneumoniae* was associated with pre-existing IgG to the pneumococcal surface protein PspA, but not with serotype specific anti-CPS antibody (McCool et al., 2002). Humans experimentally colonised with *S. pneumoniae* developed both serum IgG against the capsular polysaccharide and IgG against a number of pneumococcal surface proteins (Ferreira et al., 2013). Sera from these individuals, when passively transferred to mice was protective against challenge with a different serotype of *S. pneumoniae* (Ferreira et al., 2013). In unvaccinated human serum the concentration of IgG to the capsular polysaccharide does not necessarily correlate with binding of IgG to different *S. pneumoniae* serotypes (Hyams et al., 2011) (**Figure 1.5**), suggesting antigens other than the capsular polysaccharide may be important targets of naturally-acquired IgG. The *S. pneumoniae* capsule may also mask targets of IgG binding to sub-capsular antigens (Hyams et al., 2010a).
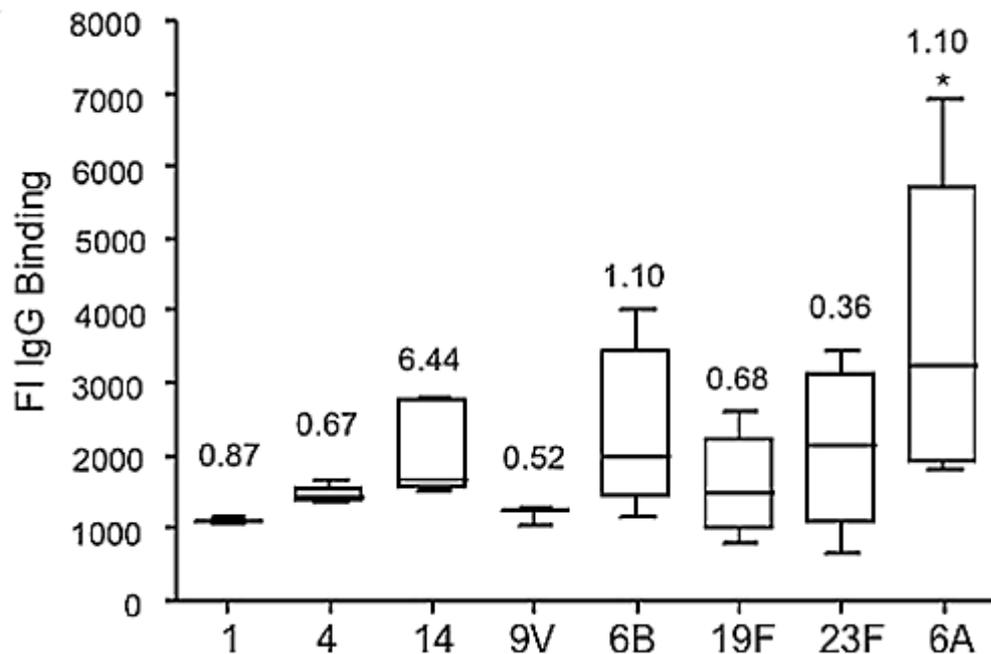
**Figure 1.5: IgG binding to different *S. pneumoniae* serotypes**.

IgG binding to different *S. pneumoniae* clinical isolates following incubation in pooled human sera, and the concentration of capsular specific IgG (μg/ml) in that sera (Hyams et al., 2011).

Low levels of IgG to the pneumococcal surface proteins PhtD, PhtE, and Ply have been demonstrated in children prone to pneumococcal otitis media (Sharma et al., 2012). This was associated with a lower percentage of memory B-cells in otitis-prone children recognising these pneumococcal surface antigens (Sharma et al., 2012). Higher antibody levels to a range of pneumococcal proteins (NanA, PpmA, PsaA, SlrA, SP0189, and SP1003) correlated with reduced risk of respiratory tract infection in 14 month old children (Lebon et al., 2011) and to a reduced risk of otitis media (Kaur et al., 2011). Higher antibody responses to the pneumococcal proteins CbpA and pneumolysin (salivary and serum IgG) have also been demonstrated in children who are culture negative compared to those colonised with *S. pneumoniae* (Zhang et al., 2006b). However, recent epidemiological studies observing serum IgG responses to a range of pneumococcal proteins suggested that although nasopharyngeal

colonisation may elicit antibodies to a range of pneumococcal proteins, levels of IgG to pneumococcal surface proteins were not associated with a reduced risk of subsequent carriage in a population of children (Prevaes et al., 2012). Murine models indicate that whilst antibodies to pneumococcal protein antigens correlated with protection from future colonisation, they are not required for this protection (Trzcinski et al., 2005), and in mice acquired immunity to colonisation depends upon cellular responses, in particular the development of Th17 responses, and antibody may be redundant for protection (Zhang et al., 2009).

**1.4.3.4    Mechanisms of antibody mediated protection**

1.4.3.4.1    Antibodies to *S. pneumoniae* CPS

There are a number of mechanisms by which antibodies may protect against *S. pneumoniae* infection. Binding of antibodies to the polysaccharide capsule may facilitate complement deposition on the bacterial surface, enhancing phagocytosis (Brown et al., 1982), and capsular polysaccharide-specific antibody can also facilitate phagocytosis by directly interacting with FC receptors on the surface of phagocytes, enhancing phagocytosis (Gordon et al., 2000). Antibody targeting the pneumococcal capsular polysaccharide may also protect by agglutination. For a number of years it has been known that factors in sera can lead to the agglutination of bacteria *in vivo* and the protective capacities of serotype specific anti-pneumococcal sera have been correlated with its capacity to cause pneumococcal agglutination (Bull, 1915c, Bull, 1915a). Serotype-specific anti-capsular IgG can induce pneumococcal agglutination *in vitro* (Dalia and Weiser, 2011). Agglutination results in pneumococci more sensitive to complement deposition and complement dependent phagocytic killing correlates with the degree of antibody-mediated pneumococcal agglutination. A novel mechanism of protection by antibodies to the *S. pneumoniae* capsular polysaccharide has been described; binding of polysaccharide specific antibodies increased the transformation frequency of cultures of *S. pneumoniae*, resulting in competence induced bacterial killing (Yano et al., 2011).

1.4.3.4.2   Antibodies to *S. pneumoniae* proteins

Antibodies to specific protein targets of *S. pneumoniae* can passively protect against invasive disease. Monoclonal antibodies to PspA mediate protection following IV or IP challenge with *S. pneumoniae* (Briles et al., 1989) and monoclonal antibodies raised against protease-sensitive antigens of *S. pneumoniae* protected mice from invasive disease (McDaniel et al., 1984). Immunisation of mice with recombinant pneumococcal proteins induces specific antibody responses (Jomaa et al., 2005, Green et al., 2005). Sera from mice immunised with a number of different *S. pneumoniae* surface proteins mediates enhanced opsonophagocytosis of *S. pneumoniae in vitro* (Jomaa et al., 2005, Harfouche et al., 2012).  Passive transfer of sera containing antibodies to a range of pneumococcal proteins induced by colonisation protected mice from invasive *S. pneumoniae* challenge (Cohen et al., 2011). Subcutaneous immunisation with purified *S. pneumoniae* PspA and PdB (a detoxified derivative of pneumolysin) protected against infection with an *S. pneumoniae* 19F strain (EF3030) that remained within lungs, and protection was associated with the induction of IgG against these protein antigens (Briles et al., 2003). Sera form older human subjects has reduced antibody  titres to both the *S. pneumoniae* capsular polysaccharide and the *S. pneumoniae* protein antigens CbpA, LytC, PhtD (Simell et al., 2008), and aged sera displays reduced opsonophagocytosis of *S. pneumoniae in vitro* (Simell et al., 2011). Plasma from diabetic patients may be deficient in anti-PspA antibodies, compared to controls, and  sera from these patients demonstrated impaired phagocytosis of a ST14 *S. pneumoniae* associated with reduced complement deposition (Mathews et al., 2012). Antibodies may also neutralise the function of *S. pneumoniae* virulence proteins. Antibodies to the pneumococcal cytotoxin pneumolysin may inhibit its function and are protective in models of pneumococcal lung infection (Salha et al., 2012, Briles et al., 2003). Antibodies to the pneumococcal proteins PhtD, PhtE PcpA and PsaA can block the adherence of *S. pneumoniae* to epithelial cells *in vitro* (Khan and Pichichero, 2012, Khan et al., 2012, Romero-Steiner et al., 2003).

## 1.4.4    Development of cellular immunity to *S. pneumoniae*

### 1.4.4.1    CD4+ T-cells

Antigen specific T-cells have a role to play in adaptive immunity to *S. pneumoniae*. CD4+ 'T-helper' cells can differentiate into a number of subsets (Th1, Th2, Th17, iTregs), defined by the range of cytokines they produce (Zhu et al., 2010). Differentiation of T-cells into distinct lineages involves the up-regulation of specific 'master regulators' of transcription, with the transcription factors T-bet, GATA3, RORγt and FOXP3 being expressed by Th1, Th2, Th17 and iTregs respectively (Zhu et al., 2010). Th1 cells are characterised by the expression of IFN-γ. Peripheral blood mononuclear cells (PBMCs) from isolated from humans are capable of producing IFN-γ following stimulation with pneumococcal antigen (Mureithi et al., 2009), and human monocytes promoted Th1 responses by CD4 cells (IFN-γ production) in response to stimulation with live pneumococci *in vitro* (Olliver et al., 2011). However, IFN-γ receptor KO mice or mice treated with an IFN-γ neutralising antibody were better able to control pneumococcal infection than WT mice (Rijneveld et al., 2002) , suggesting that IFN-γ is not necessarily protective during *S. pneumoniae* pneumonia. Furthermore, IFN-γ production is responsible for the inhibition of host defence during secondary bacterial pneumonia following influenza infection (Sun and Metzger, 2008) .

Th2 cells are important for directing B-cell responses to pathogens. This can occur through physical contact, for example, through CD40-CD40L interactions (Moens et al., 2008) and via the production of Th2 cytokines (IL-4, IL-5, IL-13) (Mosmann et al., 1986). T-cell help improves B-cell activation, increasing the affinity of immunoglobulin via the processes of somatic hyper-mutation and class switching. B-cell production of antibodies against the pneumococcal proteins PspC and Ply, is dependent upon the presence of CD4+ T-cells *in vitro* (Zhang et al., 2006a).

Th17 CD4+ T-cells are defined by the production of IL-17 and IL-22, IL-23 has a role in the differentiation of T-cells into a Th17 phenotype (Rudner et al., 2007). Th17 cells are important in

mucosal host defence against extracellular pathogens, including *S. pneumoniae*. In murine models of colonisation acquired immunity to colonisation depended upon the development of Th17 cells (Zhang et al., 2009). IL-17 is able to act on epithelial cells, activating the production of CXC cytokines involved in neutrophil recruitment. IL-17 can stimulate the production of IL-8 (CXCL8) in cultures of human bronchial epithelial cells (Laan et al., 1999), and treatment with recombinant IL-17 induced a neutrophil influx into the airways of rats (Laan et al., 1999). IL-17 may also act on epithelial cells directly to increase the production of antimicrobial peptides, for example IL-17 can augment the production of β-defensin in cultured human airway epithelial cells (Kao et al., 2004). IL-17 may also directly stimulate killing of *S. pneumoniae* by alveolar macrophages (Wright et al., 2013).

Regulatory T-cells (Tregs) are characterised by the production of the inhibitory cytokines IL-10 and TGF-β, and have an immunosuppressive function (Vignali et al., 2008). The increased susceptibility to *S. pneumoniae* infection in the CBA/Ca mouse strain compared to BALB/c is due to reduced numbers of TGF-β expressing Tregs (Neill et al., 2012). Inhibition of TGF-β impaired the ability of BALB/c mice to resist IN infection with *S. pneumoniae,* whereas adoptive transfer of Tregs prolonged the survival of previously susceptible CBA/Ca mice (Neill et al., 2012), indicating an important role for these cells in protection from *S. pneumoniae* infection. Conversely however, IL-10 may abrogate immune responses that can protect against *S. pneumoniae* infection, and IN administration of recombinant IL-10 resulted in increased lung bacterial counts 40hrs following *S. pneumoniae* challenge in C57/BL6 mice (van der Poll et al., 1996).

CD4+ T-cells may contribute to the pathogenesis of invasive pneumococcal disease. MHC II deficient mice, displaying reduced numbers of CD4+ T-cells showed increased survival following IN infection with *S. pneumoniae* D39, associated with a reduced inflammatory response (Lemessurier et al., 2010). Inhibition of T-cell function with cyclosporine or antibody depletion produced a similar protective effect in this model (Lemessurier et al., 2010).

### 1.4.4.2 Role of γδ T-cells

T-cells expressing γδ T-cell receptors (γδ T-cells), may have a role in protection from lung infection with *S. pneumoniae*. Following *S. pneumoniae* infection in mice numbers of γδ T-cells increase in the lungs (Kirby et al., 2007). γδ T-cells expressing the T-cell receptor variable gene segment Vγ4[+] are enriched within the lung. TCR-Vγ4 KO mice display increased susceptibility to *S. pneumoniae* infection and reduced inflammatory responses, including a reduced neutrophil influx in response to challenge (Nakasone et al., 2007). Additionally, TCR-γ KO mice displayed reduced levels of IL-17 following primary *S. pneumoniae* infection (Ma et al., 2010).

### 1.4.4.3 Role of CD8+ T-cells

CD8+ cytotoxic T-cells are thought to be important in immunity to intracellular pathogens. CD8+ cells may also have a role in protection from *S. pneumoniae* infection. CD8 KO mice are more susceptible to primary infection with ST3 *S. pneumoniae* than their WT controls (Weber et al., 2011). However it is not clear if CD8+ T-cells have any role in the development of adaptive immunity to *S. pneumoniae* infection.

### 1.4.4.4 Cellular immunity to carriage

Acquired immunity to pneumococcal carriage in mice is dependent upon IL-17 expressing CD4+ T-cells (Th17) (Zhang et al., 2009, Lu et al., 2008). CD4+ cell depletion or IL-17A neutralisation prevented pneumococcal clearance in mouse models of carriage (Zhang et al., 2009). Protection from colonisation was associated with enhanced neutrophil recruitment in previously colonised mice. Neutrophil depletion abrogated the protective effect of previous colonisation, and IL-17A depletion reduced neutrophil recruitment into the nasopharynx. (Zhang et al., 2009). Importantly acquired protection from colonisation still occurred in antibody deficient mice (McCool and Weiser, 2004, Basset et al., 2007a), indicating that cellular rather than humoral immunity may be the predominant mechanism mediating acquired immunity to colonisation in murine models. In humans stimulation of PBMCs and adenoidal mononuclear cells *in vitro* with the *S. pneumoniae* antigens pneumolysin and CbpA led to proliferation of CD4+ T-cells and the release of IFN-$\gamma$ and TNF-$\alpha$ (Zhang et al., 2007). CD4+ T-cell proliferation and cytokine release was higher in mononuclear cells from children without detectable nasopharyngeal carriage of *S. pneumoniae*, than those who were culture positive for *S. pneumoniae* (Zhang et al., 2007), suggesting a potentially protective role for these responses against carriage. Th17 type cytokine responses were not investigated in this study however polymorphisms in the IL-17A gene have separately been associated with higher levels of *S. pneumoniae* carriage in patients with bronchiolitis (Chen et al., 2010).

**1.4.4.5 Cellular immunity to invasive *S. pneumoniae***

In murine models of disease, experimentally colonised mice were protected from subsequent lung infection (Cohen et al., 2011, Richards et al., 2010). As previously discussed, this protection has been shown to depend on the development of antibody responses and does not require CD4+ cells at the time of challenge (Cohen et al., 2011). However, these models use *S. pneumoniae* D39, which rapidly invades into the blood, and does not necessarily allow an assessment of the protective immune responses specifically within the lung, which may involve the development of cellular immunity. T-cells accumulate in the lung following *S. pneumoniae* challenge in mice, and the entry of T-cells corresponded temporally with reduced bacterial numbers in the lungs post-infection (Kadioglu et al., 2000). Experimentally colonised mice have increased numbers of CD4+ cells and IL-17 in their lungs following *S. pneumoniae* challenge (Richards et al., 2010, Cohen et al., 2011). Mice deficient in IL-23 production displayed increased susceptibility to *S. pneumoniae* challenge (Kim et al., 2013). Isolated lung mononuclear cells from mice deficient in IL-23 demonstrated reduced IL-17A and IFN-$\gamma$ responses to heat killed *S. pneumoniae* upon stimulation *ex vivo* (Kim et al., 2013). Furthermore, morphine treated mice displayed increased susceptibility to *S. pneumoniae* lung infection which could be partially reversed by administration of recombinant IL-17 (Ma et al., 2010). Overexpression of IL-17 in the lung also resulted in enhanced neutrophil recruitment and improved bacterial clearance from the lung following *Klebsiella pneumoniae* challenge in mice (Ye et al., 2001).

In models of experimental human carriage*,* experimental colonisation with *S. pneumoniae* 6B led to increased numbers of CD4+ T- cells expressing IL-17-A and TNF-α in BALF from colonised subjects (Wright et al., 2013). Additionally, the percentage of CD4+ T-cells expressing IL-17A or TNF-α when stimulated with pneumococci *ex vivo*, was increased in cells from BALF or blood of colonised individuals when compared to non-colonised controls (Wright et al., 2013). In humans hospitalised with pneumococcal pneumonia higher numbers of IL-17A+ and IL-22A+ CD4 T-cells have been detected in lavage samples, compared to healthy controls (Paats et al., 2013), suggesting a role for

these cells in the immune responses to *S. pneumoniae* pneumonia. These data indicate that Th17

cells may have a role to play in protective immunity to *S. pneumoniae* within the lung.

## 1.5 Vaccines

### 1.5.1 Serum therapy for *S. pneumoniae*

In the late 19th century it was demonstrated that the serum from rabbits immunised with heat killed *S. pneumoniae* protected against subsequent infection with *S. pneumoniae* (Watson et al., 1993). Following this much work focussed on the development immune serum as a therapy for pneumococcal disease (Bull, 1915b, Young and Huntoon, 1926). A number of different preparation methods for anti-*S. pneumoniae* immune sera have been reported (Young and Huntoon, 1926, Bullowa and Wilcox, 1936), most involving the inoculation of live or dead pneumococci or pneumococcal polysaccharide into and animal, and collection of sera after the development of a humoral immune response. Treatment with immune sera resulted in the agglutination of *S. pneumoniae* both *in vitro* and in the blood of rabbits experimentally infected with *S. pneumoniae* (Bull, 1915a, Bull, 1915c). Such agglutination reactions were reported to only occur following treatment with type-specific (homologous) sera, failing to occur with heterologous sera raised against different *S. pneumoniae* serotypes. 'Clumping' of pneumococci in the sputum of pneumonia patients following serum therapy is reported to be related to their ability to recover from infection (Frisch, 1939). Serotype-specific pneumococcal antiserum was previously used in the treatment of lobar pneumonia. Treatment with immune sera reduced death rate of patients suffering from lobar pneumonia (Kyes, 1918), reduced the spread of *S. pneumoniae* within the lungs (Armstrong and Johnson, 1932), and improved survival of patients with bacteraemic pneumococcal pneumonia (**Figure 1.6**). Passive protection against *S. pneumoniae* with type-specific immune serum highlights the ability of antibodies against the pneumococcal polysaccharide capsule to protect against invasive disease. Demonstration that anti-CPS antibodies were induced following the administration of purified pneumococcal polysaccharide led to the development of purified polysaccharides as vaccines. (Felton et al., 1941, Finland and Ruegsegger, 1935). However due to the widespread use of antibiotics against *S. pneumoniae* it was not until the late 1970's, following the work of R. Austrian,

that the first polyvalent purified polysaccharide vaccines for *S. pneumoniae* were developed and

licensed for widespread use (Austrian, 1977).



**EFFECT OF THERAPY ON % SURVIVAL IN PNEUMOCOCCAL BACTEREMIA**
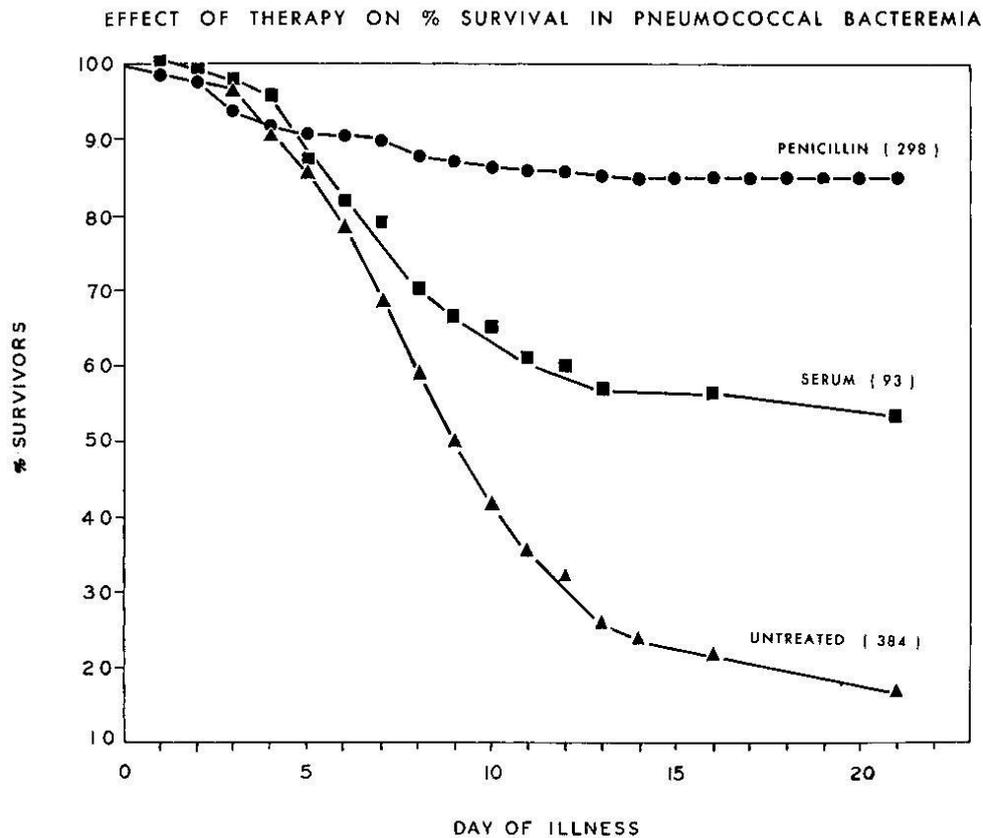
**Figure 1.6: Survival following antibiotic treatment and serum therapy for *S. pneumoniae* infection.**

Results from a 10-year study assessing survival of hospitalised patients with bacteraemic pneumococcal pneumonia treated with either; penicillin, pneumococcal antisera or untreated (Austrian and Gold, 1964).

### 1.5.2    Current vaccines

There are currently two types of vaccine that offer protection against invasive pneumococcal disease; both are based on the polysaccharide capsule. A pneumococcal polysaccharide vaccine (PPV) consisting of the purified capsular polysaccharide of 23 serotypes has been in use since 1983. PPV is immunogenic and protective in immunocompetent adults (Cornu et al., 2001). Vaccines based on purified polysaccharides rely on the development of antibodies from B-cells without T-cell help (Defrance et al., 2011). Infants lack the ability to make T-independent antibody responses; therefore PPS is poorly immunogenic in this age group (Barrett, 1985). This lack of immunogenicity led to the development of pneumococcal conjugate vaccines (PCV). Pneumococcal conjugate vaccines consist of pneumococcal polysaccharide conjugated to a protein carrier. The first pneumococcal conjugate vaccine to be licensed for use in Europe, in 2001, was PCV-7 (Pebody et al., 2005). This vaccine consists of purified polysaccharide from 7 serotypes conjugated to a diphtheria toxoid carrier protein (Murray and Jackson, 2002). Conjugation to a carrier protein facilitates T-cell-B-cell interactions and subsequently the development of T-dependent antibodies against the capsular polysaccharide. Pneumococcal conjugate vaccines are immunogenic and protective in children against the serotypes included in the vaccine (Dominguez et al., 2011).

Despite the success of both PPV and PCV in preventing pneumococcal disease, both vaccines have a number of limitations. Polysaccharide vaccines only protect against included serotypes. At the time of introduction PCV protected against serotypes responsible for up to 80% of invasive pneumococcal disease in the USA (Hausdorff et al., 2000b). However variation in the geographical distribution of serotypes means that the protection afforded by PCV-7 is not globally uniform, and the vaccine coverage is low in areas with a high burden of pneumococcal disease (Gordon et al., 2003). Another limitation of the current vaccines is the phenomenon of 'serotype replacement' (Guevara et al., 2009), this term is used to describe the increase in the prevalence of non-vaccine serotypes following vaccine introduction. To help overcome the limitations of dissimilar serotype distribution

and serotype replacement, newer conjugate formulations containing polysaccharide of up to 13 serotypes have been developed (Reinert et al., 2010). However, serotype replacement may also occur with serotypes not contained within these new vaccine formulations (Flasche et al., 2011). Pneumonia accounts for the highest burden of pneumococcal disease in adults, however PPV used in this age group is poorly protective against pneumonia without bacteraemia (Jackson et al., 2003, Dear et al., 2003). As well as limitations in efficacy and serotype coverage, the cost of developing conjugate vaccines is high, limiting their use in developing countries. The limitations associated with capsular polysaccharide based vaccination have stimulated the investigation for vaccine strategies that may protect against all serotypes of *S. pneumoniae*.

### 1.5.3    Prospective vaccines

A number of new approaches to vaccination against *S. pneumoniae* have been considered, these include; killed whole cell, live recombinant and protein-subunit vaccines (Ferreira et al., 2011). Killed unencapsulated *S. pneumoniae* delivered IN is protective against invasive challenge with encapsulated *S. pneumoniae* in animal models (Malley et al., 2001). Attenuated *Salmonella* based vaccines expressing the pneumococcal protein PspA produce a serum antibody response and protected against pneumococcal challenge in mice (Kang et al., 2002). Pneumococcal proteins or protein epitopes that are highly conserved across serotypes offer the possibility of developing cross-protective vaccines. Since the protective immune response to *S. pneumoniae* is at least partly dependent upon the clearance of opsonised bacteria (Bogaert et al., 2004), the search for protein antigens has focussed on the identification of surface proteins, conserved across all pneumococcal serotypes. Antibodies to conserved surface antigens would be expected to opsonise all *S. pneumoniae* strains and thereby induce broadly protective responses. Numerous pneumococcal proteins have been identified as possible vaccine candidates and shown to be protective in mouse models of disease. Antigens in development include, but are not limited to; the choline binding protein PspA, lipoproteins PsaA and Piu/Pia, histadine triad protein PhtD, detoxified pneumolysin

and the cell wall metabolism and cell signalling proteins PcsB and StkP (Ferreira et al., 2011). Phase I safety and immunogenicity trials in humans have demonstrated that the protein vaccine candidates PhtD and PlyD (a detoxified pneumolysin derivative) are safe and induce specific-IgG responses (Seiberling et al., 2012, Kamtchoua et al., 2012). Although phase I trials are underway, none of these vaccine candidates have yet demonstrated protection against pneumococcal disease in humans.

As well as vaccines aimed at developing protective antibody responses, vaccines capable of stimulating protective cellular responses to *S. pneumoniae* (in-particular Th17 responses) are also being developed. Screening an expression library of pneumococcal proteins has identified a number of antigens capable of inducing CD4+ T-cell dependent IL-17 responses that protected mice from experimental colonisation, and induced IL-17 production in isolated human in CD4+ T-cells (Moffitt et al., 2011). This approach has allowed the identification of a number of pneumococcal T-cell antigens (Moffitt et al., 2011). Interestingly the major *S. pneumoniae* protein antigens recognised by Th17 cells appear to differ from the major antigens associated with humoral immunity (Cohen et al., 2011, Roche and Weiser, 2010, Richards et al., 2010).

## 1.6 Murine models of *S. pneumoniae* colonisation and lung infection

Inoculation of *S. pneumoniae* into the nares of mice under light anaesthesia in a small volume (10μl) can result in nasopharyngeal colonisation without lung infection or bacteraemia (Wu et al., 1997, Cohen et al., 2011). In this model bacteria were not detectable in BALF of colonised mice 2 or 11 days post-challenge with *S. pneumoniae* D39 (Cohen et al., 2011). Nasal colonisation with *S. pneumoniae* D39 in CBA/Ca can be cleared by 21 days post-inoculation (Cohen et al., 2011). IN inoculation of $1\times10^5$ CFU *S. pneumoniae* D39 into MF1 mice resulted in stable nasopharyngeal colonisation, detectable for up to 28 days post-inoculation (Richards et al., 2010). MF1 mice colonised with an isogenic pneumolysin deficient mutant of D39 (PLN-A) clear bacteria from the nasopharynx after 14 days (Richards et al., 2010). Both WT D39 and a D39 PLN-A strain induced polysaccharide-specific IgM responses and IgG to PspA, in the sera of colonised mice. Mice colonised with D39 PLN-A were protected against a subsequent IN lung infection with $1\times10^6$ CFU WT D39, 28 days after initial colonisation (Richards et al., 2010). Mice colonised with WT D39 were protected from subsequent IN infection with $1\times10^7$ CFU of D39 (Cohen et al., 2011). In this challenge model *S. pneumoniae* was detectable in the blood by 9hrs post-infection, and previous colonisation completely protected against bacteraemia. Previously colonised mice also displayed reduced CFU in the lungs post-challenge with *S. pneumoniae* D39, although this effect was more modest. Importantly protection in this model of disease depended upon the development of antibody, as no protection was seen in B-cell deficient (μMT) mice (Cohen et al., 2011). An unencapsulated mutant of *S. pneumoniae* TIGR4 was detectable in the nares of mice 9 days following experimental colonisation and resulted in the development of *S. pneumoniae* specific antibody responses (Roche et al., 2007). Colonisation with an unencapsulated mutant of *S. pneumoniae* TIGR4 improved survival following subsequent IN infection with serotype 6A *S. pneumoniae* (Roche et al., 2007). In another model of lung infection, sera from humans experimentally colonised with *S. pneumoniae* 6B passively protected mice from IN challenge with $1\times10^6$ CFU of *S. pneumoniae* D39 (Ferreira et al.,

2013). In this model bacterial numbers were significantly reduced in the blood of mice treated with sera from colonised individuals, with a more modest effect on bacterial numbers in the lungs. A number of different bacteria can inhabit the upper respiratory tract, and the nasopharyngeal microbiome may have an effect on immune responses to *S. pneumoniae* colonisation. For example, it has been demonstrated that *Haemophilus influenzae* can outcompete *S. pneumonie* within the murine nasopharynx (Lysenko et al., 2005). Additionally the nasopharynx of children colonised with *S. pneumoniae* is often co-colonised with *Staphylococcus aureus*, to which individuals develop antibody responses (Lebon et al., 2011).

As previously discussed, current understanding of the mechanisms of protection from *S. pneumoniae* lung infection following colonisation are based on challenge with strains that rapidly invade into the blood following IN administration. *S. pneumoniae* D39 is detectable in the blood 9hrs following IN challenge with $1\times10^7$ CFU(Cohen et al., 2011). Due to this rapid invasion it is not easy to interrogate the immune responses that may protect specifically within the lung, independent of protection from bacteraemia. A number of strains of *S. pneumoniae* have been used in murine models of primary *S. pneumoniae* lung infection (Chiavolini et al., 2008). The relative invasiveness of strains following IN infection may vary depending on both strain background and capsular serotype (Kelly et al., 1994). *S. pneumoniae* EF3030 does not invade into the blood following IN challenge. Infection of CBA/N mice with $1\times10^{6.8}$ CFU of *S. pneumoniae* EF3030 (serotype 19F) resulted in established pneumococcal disease within the lung (Briles et al., 2003). Bacteria were detectable in the lungs and BALF of mice 5 days following EF3030 challenge, but no bacteria are recovered from the blood. In this model of disease immunisation with purified pneumococcal proteins (PspA and PdB) induced an IgG response and protected against EF3030 lung challenge. Protection from lung infection in this model therefore appears to be as a result of direct protection within the lung, rather than indirectly thorough protection against bacteraemia. Intratracheal infection of BALB/c mice with $3\times10^7$ CFU of *S. pneumoniae* EF3030 similarly led to established pneumococcal disease within the lung, with bacteria detectable 3 days following challenge and 60% mortality at this time point (Winter et al., 2007). It

may therefore be possible to use EF3030 as a model of non-invasive pneumonia, to interrogate the mechanisms of protective immunity in the lung following colonisation. Mice challenged IN with $1x10^7$ CFU EF3030 had detectable bacteria in nasal washes at least 20 days following challenge (Briles et al., 2003), and IN inoculation of CBA/N mice with $1x10^7$ CFU of a serotype 19 strain of *S. pneumoniae* (L82013), resulted in colonisation that is detectable in the nares 7 days post-challenge (Wu et al., 1997). Therefore as well as causing non-invasive lung infection EF3030 may also be used to experimentally colonise mice.

## 1.7 Summary

*S. pneumoniae* exists both as an asymptomatic commensal colonising the nasopharynx and as a cause of severe invasive disease. Nasopharyngeal colonisation with *S. pneumoniae* leads to a prolonged interaction with the immune system of the host. This interaction is likely to be important for the development of natural immunity that may protect the host from subsequent invasive disease. However, the mechanisms of naturally-acquired immunity to *S. pneumoniae* are not fully defined. Pooled human intravenous immunoglobulin (pooled IgG) preparations used to treat individuals with hypogammaglobunemia can partially reverse the risk of respiratory tract infections, suggesting an important role for IgG in protection from *S. pneumoniae* lung infection. However the target antigens of naturally-acquired IgG and the mechanisms by which it may protect against *S. pneumoniae* infection are not well described.

There are some data that suggest individuals may acquire antibodies to either capsular polysaccharide or surface proteins of *S. pneumoniae* following nasopharyngeal colonisation. Epidemiological data demonstrate a parallel decrease in the incidence of invasive disease with increasing age in children across all *S. pneumoniae* serotypes. This suggests the development of a common rather than serotype-specific mechanism of immunity, contradicting the assumption that naturally-acquired immunity to *S. pneumoniae* is dependent upon the development of serotype-specific antibody to the pneumococcal capsular polysaccharide. Further, murine models of colonisation indicate that the development of antibody to non-capsular targets may be important in protection from future invasive disease.

There is therefore a need to understand the major targets of naturally-acquired IgG against *S. pneumoniae* in humans, and to investigate the relative importance of naturally-acquired antibody to the capsular polysaccharide or non-capsular targets in protection from invasive *S. pneumoniae* infection. Investigating the *S. pneumoniae* antibody targets in human sera may be important for the rational design of vaccines aimed at preventing lung infection *with S. pneumoniae*. Additionally,

insights into the mechanisms of naturally-acquired immunity to *S. pneumoniae* may help in understanding the increased risk of *S. pneumoniae* infection in certain patient groups.

The anti-*S. pneumoniae* antibody repertoire in healthy adults will reflect the antibodies acquired during colonisation with *S. pneumoniae*. Similarly IVIG pooled from >1000 adult donors will reflect the naturally-acquired antibody responses within a population that may be protective against *S. pneumoniae* lung infection. Understanding the functionally important *S. pneumoniae* target antigens for IVIG and the mechanisms by which IgG may protect against lung infection will provide new insight into the natural development of protective immunity against *S. pneumoniae* lung infection. Additionally, exploring the major antigen targets in pooled and individual sera from different geographical locations would allow the effects of location and associated changes in pneumococcal ecology on the development of humoral immunity to *S. pneumoniae* to be investigated.

In addition to the development of humoral immunity, colonisation with *S. pneumoniae* also induces a cell-mediated immune response, and cell-mediated Th17 responses are required for protection from re-colonisation. Models of lung infection following colonisation have used highly invasive strains of *S. pneumoniae* that rapidly progress to bacteraemia. In these models antibodies provide protection from the rapid onset of sepsis. However the mechanisms of acquired immunity that protect against *S. pneumoniae* infection within the lung in the absence of bacteraemia are not clearly defined. Developing a model of *S. pneumoniae* colonisation and lung challenge, with an *S. pneumoniae* serotype that does not invade into the blood would allow assessment the mechanisms of acquired immunity following colonisation which may be protective against *S. pneumoniae* pneumonia specifically within the lung. Non-bacteraemic pneumonia represents the largest burden of pneumococcal disease. It is therefore important to understand the mechanisms of naturally-acquired immunity, in order to better develop vaccines that may protect against *S. pneumoniae* within the lung.

## 1.8   Aims of thesis

### 1.8.1   General aim

The aim of this thesis is to understand the naturally-acquired adaptive immune responses to *S. pneumoniae*, and to determine the mechanisms by which these immune responses are protective against *S. pneumoniae* lung infection.

### 1.8.2   Specific aims

1. To assess the immunodominant *S. pneumoniae* antigens targeted by naturally-acquired IgG in adult human sera and IVIG products.

2. To assess the targets of naturally-acquired IgG across different populations and against different strains of *S. pneumoniae*.

3. To investigate the functionally important antigen targets of naturally-acquired human IgG against *S. pneumoniae*.

4. To determine the mechanism(s) by which naturally-acquired human IgG can protect against *S. pneumoniae* lung infection in a mouse model.

5. To develop a murine model of non-bacteraemic pneumonia and colonisation.

6. To assess the effect of prior colonisation on immune responses to non- bacteraemic lung infection with *S. pneumoniae*.

7. To determine the mechanisms by which *S. pneumoniae* colonisation may protect against *S. pneumoniae* challenge within the lung.

## 2 Methods

## 2.1 Sources of sera and intravenous immunoglobulin

### 2.1.1 Sera samples

A number of different sources of sera were used in the experiments described. Firstly, sera were obtained from the whole blood of healthy volunteers within the Centre for Inflammation and Tissue Repair at UCL who had not been vaccinated against *S. pneumoniae*. Whole blood (15ml) was allowed to coagulate for 1hr at room temperature, followed by centrifugation (4800rpm, 10min). The top layer of sera was removed and stored at -80° C until use.

Sera, both pooled and individual were also obtained from a Malawian population through collaboration with Dr Stephen Gordon at the Liverpool School Tropical Medicine (LSTM). Sera were pooled from 20 HIV negative donors aged between 19 and 49 (mean: 29yrs, 16 male 4 female). Individual sera were obtained from 6 HIV negative donors aged between 21 and 36 (mean: 29yrs, 3 male 3 female).

### 2.1.2 Intravenous immunoglobulin preparations

Five different commercially available IVIG preparations were used in this thesis and are summarised in **Table 2.2**. The IgG concentration in each of the products was calculated by nephelometry at the clinical pathology laboratory, Great Ormond Street Hospital. The distribution of IgG subclasses was available from the product data sheets. Intratect from Biotest was used for *in vivo* experiments and functional studies, unless otherwise stated. The concentration of capsular polysaccharide specific IgG in Intratect was measured by multiplexed ELISA at Papworth Hospital, Cambridge (**Table 2.1**).The safety and efficacy in of Intratect in patients has been described previously (Kreuz et al., 2010).

| | Manufacturer | IgG g/L | Sub-class distribution | | Geographical region sourced from |
|---|---|---|---|---|---|
| **Intratect** | Biotest | 46.6 | IgG1 57%, IgG3 3% , | IgG2 37%, IgG4 3% | Germany, Austria, Switzerland |
| **Pentaglobin** | Biotest | 41.2 | IgG1 63%, IgG3 4%, | IgG2 26%, IgG4 7% | USA |
| **Vigam** | BPL | 51.2 | IgG1 64%, IgG3 6%, | IgG2 29%, IgG4 1% | USA |
| **Gammaplex** | BPL | 43 | IgG1 62%, IgG3 6%, | IgG2 31%, IgG4 1% | USA |
| **Flebogammadif** | Grifols | 58.1 | IgG1 66.6%, IgG3 2.7%, | IgG2 28.5%, IgG4 2.2% | Germany |

**Table 2.2**: Summary of pooled IgG products (IVIG) used in this thesis, including the concentration of IgG in each and the population from which each product was sourced.

| Serotype | 1 | 4 | 5 | 6B | 7F | 9V | 14 | 18C | 19F | 23F |
|---|---|---|---|---|---|---|---|---|---|---|
| **ng/ml IgG** | 22.3 | 10.4 | 23.4 | 57.5 | 33.1 | 25.4 | 132.7 | 36.4 | 41 | 40.2 |

**Table 2.1:** Concentration of anti-polysaccharide IgG in IVIG (Intratect) used for in vitro and in vivo experiments in this thesis, measured by multiplexed ELISA.

## 2.2  *S. pneumoniae* culture

### 2.2.1  Bacterial strains and growth conditions

*S. pneumoniae* was grown in Todd-Hewitt medium (THY, Oxoid, UK) containing 5% yeast extract, and on Colombia blood agar plates containing 5% defibrinated horse blood (TCS Biosciences, UK) at 37° C 5% $CO_2$. Growth in medium was assessed by measuring the Optical Density (OD) at $580^{nm}$ with a spectrophotometer (Amersham Pharmacia). Bacterial stocks were grown to mid-log phase ($OD_{580nm}$ 0.4-0.5) before freezing in 10% glycerol at -80° C. **Table 2.3** summarises the bacterial strains used throughout this thesis. *S. pneumoniae* TIGR4 is a well characterised laboratory strain of *S. pneumoniae* that has previously been used in models of invasive lung infection and colonisation (Cohen et al., 2013). This strain was therefore used for *in vivo* and *in vitro* experiments to assess the role of antibody in protection from *S. pneumoniae* infection. The *S. pneumoniae* 19F strain EF3030 does not progress quickly to bacteraemia (Briles et al., 2003) and was consequently used in experiments assessing protection against non-invasive pneumonia. EF3030 was a kind gift from Professor David Briles (University of Alabama), the *S. mitis* strains were kind gifts from Dr Fernanda Peterson (University of Oslo). *S. pneumoniae* mutant strains were selected based on their resistance to the relevant antibiotic (**Table 2.3**). Before each experiment antibiotic resistant strains were grown on blood agar plates containing Erythromycin (0.2μg/ml), Kanamycin (500μg/ml) or Chloramphenicol (4 μg/ml), as appropriate.  The absence of capsular polysaccharide from unencapsulated strains was confirmed by their morphology as 'pinprick' colonies on blood agar.

### 2.2.2  Fluorescent labelling of *S. pneumoniae*

*S. pneumoniae* was grown to OD $^{580}$ 0.6-0.7 in 15mls THY followed by washing in bicarbonate buffer (0.1M $NaHCO_3$), and re-suspension in 1ml bicarbonate buffer. 50μl 6-carboxyfluorescein succinimidyl ester (FAM-SE, Molecular Probes)  solution (10mg/ml in DMSO) was added to the suspension, before incubation for 1hr at 37° C,  5% $CO_2$.The suspension was then washed in phosphate buffered saline

(PBS) until no free dye remained in the supernatant. Labelled stocks were frozen at -80 ° C in PBS +

10% glycerol.

| | Strain | Serotype | Mutation | Phenotype | Antibiotic resistance | References |
|---|---|---|---|---|---|---|
| **S. pneumoniae wild-type strains** | TIGR4 | 4 | | | | |
| | D39 | 2 | | | | |
| | EF3030 | 19F | | | | (Briles et al., 2003) |
| | 23F | 23F | | | | |
| | 0100093 | ST3 | | | | |
| **S. pneumoniae mutants** | D39ΔPspC | 2 | *pspC* | Deletion of surface protein PspC | Erythromycin | (Yuste et al., 2010) |
| | D39ΔPspA | | *pspA* | Deletion of surface protein PspA | Erythromycin | |
| | ST3ΔPpmA | 2 | *ppmA* | Deletion of surface protein PpmA | Trimethoprim | |
| | D39Δlgt | 2 | *lgt* | Deletion of lipoproteins | Chloramphenicol | (Cohen et al., 2012) |
| | D39ΔPhtD | 2 | *phtD* | Deletion of surface protein PhtD | | |
| | D39ΔPly | 2 | | | Erythromycin | |
| | D39ΔPiaA | 2 | *piaA* | | Chloramphenicol | (Brown et al., 2002a) |
| | TIGR4Δ*cps* (P1672) | 4 | *cps* locus | Unencapsulated | Kanamycin | (Trzcinski et al., 2003) |
| | D39ΔD | 2 | *cpsD* | Unencapsulated | Erythromycin | (Morona et al., 2004) |
| | ST3Δ*cps* | 3 | *cps* locus | Unencapsulated | Kanamycin | |
| | 23FΔcps | 23F | *cps* locus | Unencapsulated | Kanamycin | |
| | TIGR4Δ*pab* | 4 | *pabB* | deletion of PABA synthase | Kanamycin | (Chimalapati et al., 2011) |
| **S. mitis strains** | *S. mitis* | | | | | |
| | *S. mitis*Δ*cps* | | *cps* locus | Unencapsulated | Kanamycin | (Rukke et al., 2012) |
| | *S.mitis*T4*cps* | | *cps* locus | *S. mitis* expressing capsule from *S. pneumoniae* TIGR4 | Kanamycin | (Rukke et al., 2012) |

**Table 2.3:** Summary of the bacterial strains used throughout this thesis.

## 2.3    Serological assays

### 2.3.1    Whole cell ELISA

*S. pneumoniae* was inoculated into 20ml THY and grown until OD$^{580}$ 0.4-5. Bacteria were then washed twice in PBS and re-suspended (in PBS) to an OD$^{580}$ of 1.0. 50μl of bacterial suspension was added per well to a 96-well plate (Maxisorb, Nunc) and left to coat overnight at 4° C. Plates were washed 4 times (to remove unbound bacteria) with 200μl wash buffer (PBS+ 0.05% tween). 100μl blocking buffer (PBS+0.05% tween+ 1% BSA) was then added per well, plates were incubated for 1hr at 37° C.  Test sera or pooled Immunoglobulins were serially diluted in dilution buffer (PBS+0.05% tween+ 1% BSA) and added to wells in duplicates (50μl per dilution); plates were then incubated for two hours at room temperature. After this incubation plates were washed 4 times and secondary antibody diluted 1:10,000 (**Table 2.4**) was added in dilution buffer (100μl per well). The plate was then incubated for two hours at room temperature before being washed 4 times in 200μl wash buffer. For alkaline phosphatase (AP) conjugated secondary antibodies substrate para-nitrophenylphosphate (pNPP) (Sigma) was prepared in substrate buffer (1mg/ml), 100μl substrate was added per well. The plates were then incubated in the dark for 20min, after which 100μl 3N sodium hydroxide (NaOH) was added to each well to terminate the reaction. The absorbance was read at 405nm, subtracting readings at 630nm (Versamax). For Horseradish Peroxidase (HRP) conjugated secondary antibodies 100μl TMB substrate was added per well and the reaction stopped by the addition of 1M hydrochloric acid (HCL). The absorbance was read at 450nm subtracting readings at 550nm. For some experiments serial dilutions of sample were analysed and ELISA titre calculated. ELISA titre represents the theoretical sample dilution that would result in an OD of 0.1.

| Antibody | Conjugate | Manufacturer |
|----------|-----------|--------------|
| Anti-human IgG | HRP | Biosource Int. |
| Anti-mouse IgG | AP | Sigma |
| Anti-mouse IgM | AP | Sigma |
| Anti-mouse IgA | AP | Sigma |

**Table 2.4:** Antibodies used in whole cell ELISAs, including the conjugate and the manufacturer.

### 2.3.2    Competition ELISA (Cell Wall Polysaccharide)

Cell Wall Polysaccharide (CWPS) competition ELISAs were performed with pooled sera (1:800) or IVIG (1:10,000) diluted in ELISA dilution buffer and incubated for 30 min at 37° C in purified CWPS (Staten's Serum Institut), CPS (Staten's Serum Institut) or whole cell lysate (*S. pneumoniae* T4), at the following concentrations; 100, 10, 1, 0.1 and 0.01 µg/ml. A whole cell ELISA against *S. pneumoniae* TIGR4 (as described above) was then developed with the pre-incubated sera.

### 2.3.3    Competition ELISA (protease-treated lysates)

20µl of *S. pneumoniae* TIGR4 whole cell lysate (1500 µg/ml) was treated with the addition of 10µl trypsin (2.5mg/ml, Gibco, Invitrogen). As controls 20µl T4 lysate was treated with 10µl PBS and 20µl PBS treated with 10µl trypsin. Lysates were incubated overnight, before the addition of 10µl 25X complete protease inhibitor (Roche). Pooled sera were diluted to a final concentration of 1:3000 in ELISA dilution buffer. Lysate (or trypsin-treated PBS) was added to pooled sera in serial 10-fold dilutions from $1 \times 10^{-4}$ to $1 \times 10^{2}$µg/ml. A whole cell ELISA of IgG binding to T4 was then performed (as described above).

### 2.3.4    Purified protein ELISA

Purified PspC (TIGR 4), SP1633 and SP1651 were a kind gift from Professor Tim Mitchell, University of Birmingham.  Pneumococcal proteins were diluted to 5µg/ml in coating buffer (3.03gNa2CO3, 6.0g NaHCO3 in IL ddH2O); 50µl protein suspension was added per well to 96-well microtitre plate

(Maxisorb, NUNC).  Plates were coated overnight at 4° C , then washed  4 times with 200µl wash buffer (PBS+ 0.05% tween) per well. Plates were then blocked with 100µl blocking buffer for 1hr at 37° C. Test sera was serially diluted in dilution buffer (PBS+0.05% tween+ 1% BSA), and added to wells in duplicates (50µl per dilution) before  incubation for 2hrs at room temperature. Plates were then washed 4 times and secondary antibody (anti-human IgG-alkaline phosphatase conjugate or anti-human IgM-alkaline phosphatase conjugate -1:10,000) added in dilution buffer, followed by incubation for two hours at room temperature. After incubation plates were washed 4 times in 200µl of wash buffer. Substrate (pNPP) was prepared in substrate buffer to 1mg/ml, 100µl substrate was added per well. The plate was then incubated in the dark for 20min, after which 100µl NaOH (3N) was added to each well to terminate the reaction. The absorbance was read at 405nm, subtracting reading at 630nm (Versamax).

### 2.3.5    Preparing whole cell lysates of *S. pneumoniae*

*S. pneumoniae* was grown to mid-log phase in THY, 6mls of culture were pelleted by centrifugation at 13,000 rpm for 5min. Bacterial pellets were washed twice in PBS and re-suspended in 400µl PBS. Bacterial suspensions were sonicated on ice at output level 2, 50% cycle, for 2mins (probe sonicator, Branson S250). Samples were centrifuged for 5min at 13,000rpm to remove cell debris and the clarified supernatant recovered. The protein concentration of lysates was measured with the bicinchoninic acid (BCA) assay (Thermo Scientific, US). Briefly, 100µl BCA working solution was added to 5µg lysate in duplicates, absorbance at 562nm was measured (Versamax plate reader) and compared to a bovine serum albumin (BSA) standard prepared in PBS. Lysates were normalised to a total protein concentration of 1mg/ml.

### 2.3.6 Immunoblotting

10μl lysate was added to 2.5μl loading dye (10% 1M DTT + 1X Laemelli buffer: 3.125 ml 1M tris HCl, 1g Sodium dodecyl sulphate (SDS), 2ml glycerol, bromophenol blue (trace), made up to 10ml ddH$_2$O). Samples were heated to 95° C for 5min before loading into polyacrylamide gels (10%). Proteins were separated by SDS-PAGE at 120mv for 2hrs (Novex mini-cell, Invitrogen, USA).The protein was then transferred onto a nitrocellulose membrane (GE healthcare) by semi-dry blotting at 20 mv for 1hr (Invitrogen, USA). Nitrocellulose membranes were blocked overnight in TBS + 0.1% tween (TBST) + 5% milk powder. Nitrocellulose membranes were probed with; human serum (1:1000), IVIG (1:3300) in 5mls TBST+ 5% milk for 1hr rolling incubation at room temperature, followed by three washes in TBST for 15min each. Membranes were then probed with the secondary antibody; anti-Human IgG-HRP conjugate (1:5000), diluted in TBST+5% milk, for 1hr at room temperature. Blots were developed with HRP substrate (GE healthcare) and imaged in the dark using hyperfilm (GE healthcare).

### 2.3.7 Multiplex binding assay (Luminex)

Multiplex (Luminex) bead assay of pneumococcal proteins. 18 recombinant pneumococcal proteins from a range of strain backgrounds were conjugated to seroMAP beads (**Table 2.5**). The coupling reaction has been described previously (Verkaik et al., 2009). In brief, 25μg of each recombinant protein was covalently attached to 5x10$^6$ microspheres (xMAP). The Luminex panel was then validated by comparing binding on a singleplex basis to multiplexed binding. For relative quantification of antibody levels in serum samples, mixtures of equal numbers of each bead type were made (1.1μl of each bead per well), in a final volume of 55μl per well (diluted in PBS+1% BSA). Assays were performed in 96 well plates. Human serum samples were diluted 1:1000 in PBS+1% BSA. Murine sera and BALF samples were diluted 1:100 and 1:1 respectively.  50μl bead mix was added per well, followed by 50μl diluted serum. Plates were then covered and incubated at room

temperature for 35min shaking at 800rpm. The buffer was then aspirated by suction, after which beads were washed twice (PBS+1% BSA). After washing beads were re-suspended in 50μl buffer. 50μl secondary antibody (anti-human or anti-mouse IgG, conjugated to PE, 1:50) was then added per well. Plates were incubated for 35min at room temperature, shaking at 800rpm. Following incubation the buffer was aspirated from wells and beads were washed once (PBS+1%BSA) prior to reading. The fluorescence of each antigen-couple bead was measured using a Bio-Plex machine (Bio-Rad).

| Pneumococcal protein | | Location | Notes | Strain |
|---|---|---|---|---|
| PhtD | Pneumococcal histadine triad protein D | CW | Zinc binding protein | TIGR4 |
| PspC | Pneumococcal surface protein C | CW | Binds factor H | TIGR4 |
| Ply | Pneumolysin | EX | Number of roles in virulence (adhesion and lysis of host immune cells) | TIGR4 |
| PsaA | Pneumococcal surface adhesin A | M | Component of the ABC transport system, which is involved in resistance to oxidative stress and transport of Mn2+ | TIGR4 |
| PspA | Pneumococcal surface protein A | CW | Inhibition of complement deposition binding of apolactoferrin | TIGR4 |
| Hyal | Hyaluronidase | CW | Breaks down ECM components | TIGR4 |
| PhtE | Pneumococcal histadine triad protein E | CW | Metal binding protein | TIGR4 |
| PpmA | Putative proteinase maturation protein A | M | Role in colonisation, adherence to epithelial cells. | D39 |
| SP0189 | Hypothetical protein | | | TIGR4 |
| IgA1ase | IgA1 protease | CW | Cleaves human IgA1 | TIGR4 |
| CbpD | Choline binding protein D | CW | Competence induced cell lysis (fratricide) | TIGR4 |
| SP1633 | | I | Response regulator | TIGR4 |
| NanA | Neuraminidase | CW | Biofilm formation, endothelial invasion | TIGR4 |
| SlrA | Streptococcal lipoprotein rotamase A | M | Modulates biological function of virulence factors | D39 |
| Eno | Enolase | CW | Binds to plasminogen | D39 |
| SP1651 | | I | Thiol peroxidase | TIGR4 |
| SP0376 | | I | Response regulator | TIGR4 |
| PilusA | Pneumococcal pilus | | Role in adhesion | TIGR4 |

**Table 2.5:** Pneumococcal proteins conjugated to xMAP beads for Luminex assay, including the strain of *S. pneumoniae* from which each purified protein was isolated and their cellular location (CW=cell wall, EX=extracellular, M=membrane, I=intracellular,  (Lebon et al., 2011).

## 2.4   *S. pneumoniae* growth and agglutination

### 2.4.1   *S. pneumoniae* growth assay

$1\times10^6$ CFU of *S. pneumoniae* were inoculated into Todd-Hewitt medium (THY, Oxoid, UK) containing 5% yeast extract (Oxoid, UK). Growth of *S. pneumoniae* was assessed by measuing the $OD_{580}$ of cultures incubated at $37^\circ$ C 5% $CO_2$ over an 8 hour period, using a spectrophotometer. Growth in the presence of 10% IVIG  (Intratect, 40mg/ml IgG) or PBS was assessed. Following 8 hour growth, cultures were methanol-fixed on to polylysine slides (VWR), and stained with rapid romanowsky staining (Diff-Quick). Stained *S. pneumoniae* were immaged with a light microscope (Olympus, BX40) using Qcapture pro software.

### 2.4.2   *S. pneumoniae* agglutination assay

To directly assess agglutination, FAM-SE labelled *S. pneumoniae* were diluted in PBS to a concentration of $1\times10^6$ CFU/ml and incubated at 37°C for 1 hr with 0%, 1%, 5%, 10%, IVIG (Intratect) in a volume of 100μl. Following incubation bacteria were fixed by the addtion of 100μl neutral buffered formallin. Bacteria were identified as a fluorescent (FL-1) positive population by flow-cytometrey and particle size was asessed as a change in forward-scatter (FSC). Analysis of cells was performed on a FACSCalibur flow-cytometer using CellQuest and FlowJo software (BD Bioscience, UK).

## 2.5    Surface targets of IgG

### 2.5.1    IgG deposition on surface of *S. pneumoniae*

IgG binding to the surface of *S. pneumoniae* was assessed by flow-cytometry. $5\times10^5$ CFU of *S. pneumoniae* were washed in PBS and incubated with varying concentrations of IVIG diluted in PBS, at room temperature for 30min. Bacteria were then washed twice in PBS and incubated with anti-human IgG secondary antibody (1:200), conjugated to PE (Sigma-Aldrich) at 4° C for 20mins. Finally bacteria were washed twice in PBS to remove unbound secondary antibody and fixed in 4% PFA. FACS analysis of bacterial cells was performed on the FACSCalibur, CellQuest and FlowJo software (BD Bioscience, UK). Gating of bacteria was based on FSC and SSC, and fluorescent controls incubated with no primary antibody.

### *2.5.2    Protease shaving of S. pneumoniae*

 *S. pneumoniae* TIGR4$\Delta pab$ ($1\times10^7$ CFU) were washed in PBS and re-suspended in 500µl PBS with or without 100µg/ml of pronase (Roche). Bacteria were incubated for 20min at 37° C, shaking at 150rpm. Following incubation 20µl of complete-mini protease inhibitor (25X, Roche) was added to each tube. Bacteria were then washed twice in PBS and re-suspended in PBS+10% glycerol. IgG binding to pronase treated TIGR4$\Delta pab$ incubated in 1% IVIG or 10% polyclonal anti-type 4 rabbit serum (Staten's Serum Institute, Pool A) was assessed as described above.

### 2.5.3    Absorption of IVIG (depletion of CPS-specific IgG)

*S. mitis* expressing a TIGR4 capsule (*S. mitis*T4*cps*) was grown to OD$_{580}$ 0.4 washed in PBS and re-suspended to OD 1.0 in PBS. 4mls of suspension was pelleted by centrifugation and re-suspended in 1.8mls of IVIG (Intratect). The suspension was incubated for 1hr at 37° C, shaking at 100rpm. Following incubation the bacteria were removed by centrifugation and IVIG recovered. This was repeated once. Mock absorbed IVIG was prepared by following the absorption steps without the addition of bacteria.

## 2.6 Phagocytosis and killing assays

### 2.6.1 Culture of murine macrophages

RAW 264.7 macrophages were grown in Roswell Park Memorial Institute medium (RPMI, Invitrogen UK) supplemented with L-glutamine, Pen-strep (Invitrogen) and fetal calf serum (FCS, Lonza) (RPMI +++). Cells were passaged at 80% confluence. Cells were passaged by washing in PBS, followed by gentle scraping to re-suspend the cells. The cell suspension was then centrifuged (300xg, 5min), and the pellet re-suspended in RPMI+++ and transferred to a cell culture flask (T175) in a final volume of 25ml.

### 2.6.2 Macrophage opsonophagocytosis assay

RAW 264.7 cells were harvested by gentle scraping, then centrifuged at 300xg for 5mins and re-suspended in RPMI without supplements (RPMI---). Cells were counted with trypan blue on a haemocytometer and diluted to $5 \times 10^5$ cells/ml in RPMI (---). 400µl of the cell suspension was added per well to a 24 well plate, followed by 200µl RPMI --- per well. Cells were incubated for 1-3hrs at 37° C, 5% $CO_2$ to allow adherence. $1.4 \times 10^7$ FAM-SE labelled bacteria were opsonised with 25% pooled sera, 10% IVIG, or RPMI alone in a final volume of 400µl RPMI ---, for 30min at 37° C. Opsonised bacteria were then suspended in 1.4 ml RPMI (final volume) and 200µl added to each well as required (MOI 10). After addition of FAM-SE labelled bacteria, plates were centrifuged at 1800rpm for 3mins before incubation at 37° C in 5% $CO_2$. After 45mins the medium was removed and cells were washed twice in PBS. 300µl trypsin was then added per well, and plates incubated for 7 min at 37° C. The cells were re-suspended by gentle scraping. 50µl FBS added to quench the trypsin, then 100µl 3% paraformaldehyde (PFA) added to each well to fix the cells. The plates were kept in the dark at 4° C before analysis using a FACSCalibur, and CellQuest and Flowjo software (BD Bioscience, UK). Gates were based on dot plots of uninfected controls. Fluorescence was detected in FL-1 and mean fluorescent intensity (MFI) of macrophages used as a measure of bacterial uptake.

### 2.6.3    Neutrophil Isolation

Granulocytes were isolated from heparinised whole blood by dual layer Histopaque separation using Histopaque 1077 and 1119 (Sigma-Aldrich, UK). Briefly, 3ml histopaque 1077 was carefully layered on top of 3ml histopaque 1119 in a 15ml centrifuge tube. 6ml heparinised whole blood was carefully layered on top. Tubes were then centrifuged (25min 700xg, brakes off). The granulocytes were recovered from a layer above the red cell-debris, washed twice in PBS to remove platelets, and re-suspended in RPMI ---. Cell numbers were counted on a standard haemocytometer following trypan blue staining to exclude dead cells.

### 2.6.4    Neutrophil phagocytosis assays

Isolated human neutrophils were washed in PBS and re-suspended in Hanks buffered salt solution with calcium and magnesium to a concentration of $1x10^6$cells/ml, 100µl of this suspension was added to wells of a 96-well plate. FAM-SE labelled *S. pneumoniae* were opsonised for 30min at 37° C in different concentrations of IVIG. $2x10^6$CFU of FAM-SE labelled *S. pneumoniae* were added per well, in a volume of 25µl (MOI 20). Neutrophils were co-incubated with bacteria for 30min at 37° C then fixed by the addition of 100µl PFA. Cells were analysed on a FACSCalibur, CellQuest and Flowjo software (BD Bioscience, UK), as described above for macrophage phagocytosis assays.

### 2.6.5 Neutrophil killing assays

*S. pneumoniae* were opsonised in PBS with and without 10% IVIG, for 30min at 37° C, shaking at 150rpm. 400 CFU were added per well to a 96 well plate, followed by the addition of $1x10^5$ neutrophils in 100μl of Hanks buffered salt solution (HBSS) with calcium and magnesium. The plates were incubated for 45mins at 37° C, 150rpm. After 45mins well contents were diluted and plated onto blood agar plates for CFU enumeration, after overnight incubation at 37° C and 5% $CO_2$.

## 2.7 *In vivo* experiments

### 2.7.1 Animals

All *in vivo* experiments were performed in mice at the biological services unit, UCL. For adoptive transfer experiments (presented in chapter 5) for which no mutant mice were required, experiments were performed on outbred CD1 mice. For experiments assessing the protective immune response to colonisation (presented in chapter 6), experiments were performed on inbred C57/BL6 mice, to match the genetic background of antibody deficient (μMT) mice.

| Strain | Age (weeks) | Source/Supplier | Short Description |
|--------|-------------|-----------------|------------------|
| CD1 | 6-8 | Harlan | Outbred |
| C57/BL6 | 6-8 | Harlan | Inbred |
| μMT (C57/BL6) | 8-10 | Provided by Dr Clare Notley UCL. (JAX ) | μMT mice contain a mutation in the IgM heavy chain; therefore do not produce mature B-Cells or antibody. |

**Table 2.6** gives an overview of the different mouse strains used throughout this thesis.

**Table 2.6**: Strains and suppliers of mice used for *in vivo* experiments throughout this thesis.

### 2.7.2 Murine pneumonia challenge model

C57/BL6 or CD1 mice were anesthetised with aerosolised halothane (Vet-Tech) (4%) and challenged via IN installation of *S. pneumoniae*, suspended in 50μl of PBS. At designated time points post-infection mice were anaesthetised with pentobarbitol and culled by severing the femoral artery. Blood was collected into heparinised tubes (Sigma-Aldrich) to prevent clotting. In some experiments blood was also collected into non-heparinised tubes and allowed to clot, to obtain sera by centrifugation for 5min at 13,000 rpm. To perform bronchoalveolar lavages (BAL) an incision in the diaphragm was made to allow lung expansion, followed by a small (1mm) incision in the trachea,

into which a 20-gauge cannula was passed. 1ml PBS was injected through the cannula and aspirated repeatedly for a total of three times. Lungs or spleens were harvested into 500µl of sterile PBS and homogenised by passing through a 40µM filter in 3mls PBS. CFU in lung homogenates, BALF and blood were counted by serial dilution in PBS and plating on blood agar plates. Total cell numbers in BALF were enumerated with a standard haemocytometer after trypan blue staining (Sigma-Aldrich). 100µl BALF was spun on to polysine microscope slides (Shandon Cytopsin) and stained by rapid Romanowsky staining (Diff-quick, Thermo Scientific), for differential cell counts under a light microscope (Zeiss). Supernatants of lung homogenates, BALF and sera were stored at -80$^{o}$C for cytokine measurement.

### 2.7.3 Murine colonisation model

C57/BL6 or CD1 mice were anesthetised with aerosolised isoflurane (4%) (MiniRad) and challenged via IN instillation of *S. pneumoniae* suspended in 10µl of PBS. At designated time points post-infection the nares were washed with 400µl PBS. *S. pneumoniae* CFU in nasal washings were enumerated by plating onto blood agar, and blood agar containing optochin (100µg/ml, Sigma-Aldrich). *S. pneumoniae* was discriminated from other bacteria that may colonise the murine nasopharynx by alpha-haemolysis and optochin sensitivity.

### 2.7.4 Pre-colonisation and pneumonia challenge

Mice were colonised with 1x10$^{7}$ CFU *S. pneumoniae* or PBS as control. 30 days following colonisation mice were challenged with 1x10$^{7}$ CFU as described. At designated time points post-infection lungs, BALF, blood and sera were collected as described.

### 2.7.5 IVIG treatment model

Mice were pre-treated with IVIG prior to pneumonia challenge. 13mg IVIG (Intratect) was administered as two separate IP injections, 3hrs prior to and immediately before pneumonia

challenge or colonisation. Human IgG concentration in the sera, BALF and nasal washings of treated

mice was quantified by ELISA (Cambridge Bioscience).

### 2.7.6    Passive transfer of immune sera

CD1 mice were vaccinated by IP administration of $5\text{x}10^4$ CFU of S. *pneumoniae* TIGR4Δ*pab*, in a volume of 100µl. Mice were re-vaccinated after a week. 28 days following the first vaccination mice were culled with a lethal dose of pentobarbitol and blood collected from the femoral artery to obtain sera for measuring antibody responses. Immune serum from mice vaccinated with TIGR4Δ*pab* was transferred into naïve CD1 mice by IP injection. 400µl of sera was administered as two separate IP inoculations 6hrs apart. 24hrs following transfer mice BALF was collected, nasal washes performed and serum collected.

### 2.7.7 Cellular and cytokine depletion

To deplete ly6G+ neutrophils, 600µg anti-ly6G mAb (1A8, BioxCell) was administered by IP injection 24hrs prior to challenge in a volume of 200µl. Neutrophil depletion was confirmed by counting cells in the BALF following challenge. To deplete CD4+ T-cells cells 250µg anti-CD4 mAb (GK 1.5, BioxCell) was administered by IP injection 48 and 24hrs prior to challenge. CD4+ T-cell depletion was confirmed by flow-cytometry as a reduction in CD4+ cells or a reduction in CD3+ CD8- cells in the spleens and lungs of mice. Depletion of splenic macrophages was achieved by IV administration of 100µl 5mg/ml liposomal clodronate (or PBS liposomes) as described previously (van Rooijen and van Nieuwmegen, 1984). Macrophage depletion was confirmed using flow-cytometry as a reduction in F4/80+ splenocytes. IL-17 was neutralised by IP administration of 100µg of anti-mouse IL-17A (BioXcell) 24hrs and immediately prior to challenge.

## 2.7.8 ELISAs on murine tissue

The concentration of IL-17 in samples was measured using a Quantikine ELISA kit (R&D systems). 50μl of sample or standard, diluted in 50μl assay diluent was added to wells pre-coated with capture antibody. Samples were incubated for 2hrs at room temperature, before wells were washed 4 times with wash buffer (PBS+0.01% tween). 100μl detection antibody, conjugated to HRP, was added to each well and incubated for 2hrs at room temperature. Plates were washed 4 times in washing buffer before the addition of TMB substrate. After 15min the reaction was stopped by the addition of Hydrochloric acid. The colour change in each well was assessed by reading the OD at 450nm, with wavelength correction set at 550nm (Versamax). The cytokine concentration in samples was calculated from a standard curve plotting OD against standards of known concentration. Concentrations of TNF-$\alpha$ and IL-10 were assessed using a Duo Set ELISA (R&D systems). 96-well plates (Nunc, Maxisorb) were coated overnight with capture antibody. Wells were washed and blocked in PBS+1% BSA. After washing 100μl sample or and standard was added per well and the assay performed as described above for the Quantikine ELISA. IL-23 was measured using a CytoSet ELISA (Invitrogen), and KC using a KC ELISA set (Insight Biotechnology) following the same procedure.

Albumin concentration was assessed using a murine albumin ELISA kit (Bethyl Laboratories). 96-well plates were coated overnight with capture antibody. Due to the sensitivity of the ELISA samples were diluted 1:10,000. 100μl of sample or standard was added per well and the assay performed as described above. The concentration of total human or mouse IgG in samples was also assessed by ELISA. Briefly, 96-well plates were coated overnight with the relevant capture antibody. Samples were diluted 1:100. 100μl of sample or standard was added per well and the assay performed as described above.

### 2.7.9 Histology of murine lung tissue

Lungs from mice were collected into 4% PFA, in PBS and left to fix for 4hrs. Lungs were then incubated overnight in 15% sucrose then transferred to 70% ethanol for storage at 4° C until processing. Lungs were processed in paraffin wax overnight using an automated tissue-processor (Leica). Samples were then embedded into paraffin blocks. 3μm lung sections were prepared on a rotary microtome (Shandon), and mounted on to glass slides. Slides were stained with haematoxylin and eosin using a Tissue-Tek automated stainer (Sakura), then overlaid with a glass coverslip. Slides were imaged using a NanoZoomer digital pathology system (Hammatsu).

## 2.8 Analysis of murine cells by flow-cytometry

### 2.8.1 Cell preparation and staining

Murine lungs and spleens were collected into ice cold PBS and single cell preparations prepared by homogenisation though a 40μM filter. Red blood cells were lysed by re-suspension of cells in 5mls red blood cell lysis buffer for 5mins (Santa-Cruz Biotechnology). Cells were then washed in PBS and re-suspended in 1ml PBS. Cell numbers were determined by crystal violet staining and counting on a standard haemocytometer. $1x10^6$ cells were added per wells to a 96-well plate. Plates were spun at 1400rpm for 2min, to pellet and cells re-suspended in 50μl PBS+ 1% BSA. 50μl of specific antibodies (**Table 2.7**) diluted 1:50 in PBS+1% BSA were added to each well. For multiple antibody staining single stained controls and 'fluorescent minus one' (FMO) controls were included. Plates were incubated for 20min at 4°C, and then washed twice in 200μl PBS, and the cells re-suspended in 100μl of 4% PFA. Plates were kept in the dark at 4°C before analysis by flow-cytometry.

### 2.8.2 Flow-cytometry lymphocytes (lungs)

Lung or spleen cells were analysed on a FACSCalibur (BD) flow-cytometer, using CellQuest acquisition software and analysed using FlowJo software. At least 10,000 lymphocytes were acquired per sample on the basis of size (FSC) and granularity (SSC). Lymphocyte populations were identified by staining for CD4, CD8 and CD3 (**Table 2.7**). Gates were based on FMO controls of samples stained with all antibodies excluding the target of interest. Single stained controls were used to set compensation gates. Total lymphocyte numbers were calculated based on the percentage of each subset as a proportion of all cells in each sample.

### 2.8.3 Flow-cytometry macrophages (Spleen)

Splenocytes were analysed on a FACSCalibur (BD) flow-cytometer, using CellQuest acquisition software and FlowJo analysis software. At least 10,000 individual cells were acquired per sample. Macrophages were identified by staining for F4/80 and gates based on unstained controls. Total macrophage numbers were calculated based on the percentage of F4/80 positive cells as a proportion of all splenocytes in each sample.

| Antibody | Fluorochrome | Supplier | Clone | FL channel |
|----------|--------------|----------|-------|------------|
| Anti-mCD4 | FITC | BD Bioscience | RM 4-5 | 1 |
| Anti-mCD3 | PE | E Bioscience | 145-2c11 | 2 |
| Anti-mCD8 | PerCP | BD Bioscience | 53-6.7 | 3 |
| Anti-F4/80 | PE | Caltag | MF 48000 4 | 1 |

**Table 2.7:** Antibodies used in this thesis for phenotyping of cells by flow-cytometry.

## 2.9  Statistics

Data were presented as group means +/- standard deviation (SD). Student's T-test was used to compare the mean of two groups, or analysis of variance (ANOVA) for comparison between multiple groups, with Bonferonni post-test to compare selected groups. Parametric tests were used for *in vivo* experiments due to their greater sensitivity; this reduces the number of animals required for experimentation which is preferred for ethical and practical reasons. For depletion studies, where the absence of a difference indicates a biological effect the greater sensitivity of parametric tests is also favourable, as any true differences between groups should be identified. F-tests were performed to assess if the slope of linear regression lines were statistically different from 0. All statistical tests were performed using Graph Pad Prism statistical software.

## 2.10  Ethics

All sera samples from individuals at the Centre for Respiratory Research at UCL were obtained with ethical consent from UCL Research Ethics Committee (Ref: 3076/001). All Malawian sera and bronchoalveolar lavage samples were obtained with ethical consent from Liverpool School of Tropical Medicine (Ref: 00.54) and the University of Malawi (Ref: P.99/00/102). All animal experiments were approved by the UCL Biological Services Ethical Committee and the Home Office (UK) under project licence (PPL70/6510).

# Results

# 3 Results (1): Serology

Nasopharyngeal colonisation with *S. pneumoniae* is an immunising event that results in a host antibody (IgG) response. However, there remains some controversy over which *S. pneumoniae* antigens are dominant targets for this naturally-acquired IgG. Host and bacterial variation may also alter which antigens are dominant in different individuals and across populations. A number of *in vitro* assays were therefore performed to assess the dominant antigen targets recognised by IgG in different sources of adult human sera and pooled IVIG products.

### 3.1.1 IgG to whole *S. pneumoniae* and purified capsular polysaccharide

To assess the levels of IgG in sera from different Malawian individuals to whole pneumococci, a whole cell ELISA of IgG binding to 4 different strains of *S. pneumoniae* (ST4, ST14, ST9V, and ST1) was used. All sera contained detectable levels of IgG to each strain of *S. pneumoniae* with some variation in the extent of binding to different strains (**Figure 3.1**). There was also some variation in the extent of IgG binding to each *S. pneumoniae* strain between sera from different individuals (**Figure 3.1**). For example, sera 'B' demonstrated considerably greater binding to *S. pneumoniae* TIGR4, whereas sera 'F' displayed the greatest binding to *S. pneumoniae* ST9V.

The concentration of serotype-specific IgG to different pneumococcal polysaccharides in these individual Malawian sera was determined by multiplexed ELISA. Results indicated varying levels of IgG to a range of pneumococcal polysaccharides and between sera from different individuals for each specific serotype (**Figure 3.1**). Overall the highest responses were to type-14 polysaccharide.

**A**
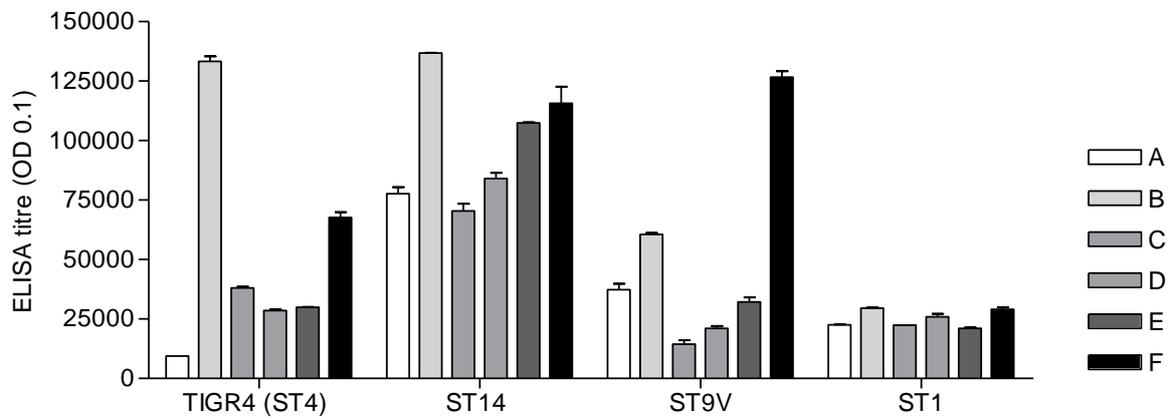
**B**
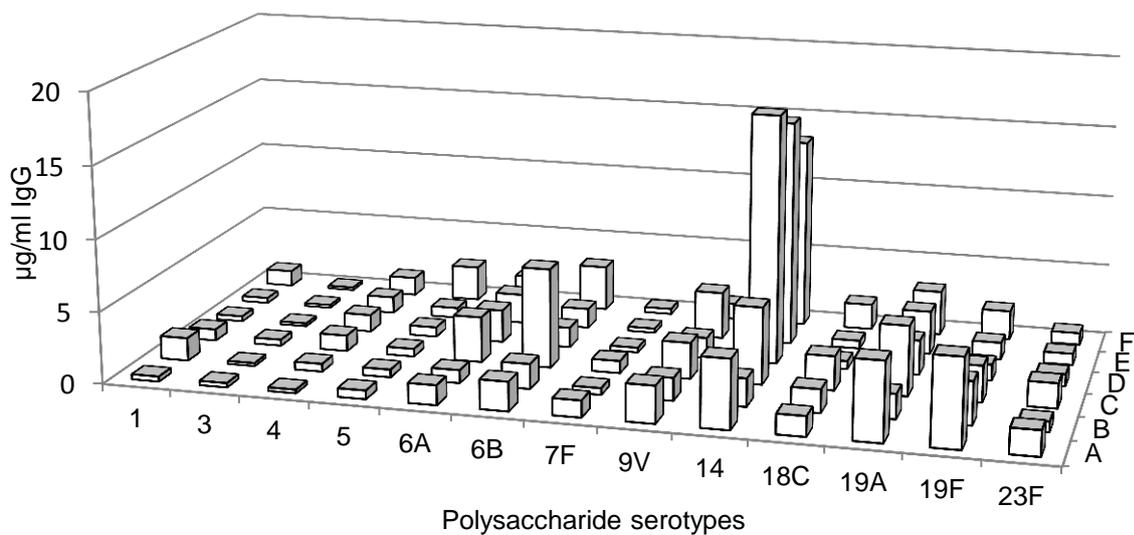
**Figure 3.1: IgG binding in different human sera.**

(A) Whole cell ELISA IgG binding titre to *S. pneumoniae* serotypes 4, 14, 9V and 1 in individual Malawian sera (A-F) (B) Concentration of IgG to 13 different *S. pneumoniae* polysaccharide types in different individual Malawian sera, as assessed by multiplexed ELISA. (Bars represent mean, error bars represent SD). Multiplexed polysaccharide ELISAs were kindly performed at the Institute of Child Health, UCL by Polly Burbidge. Results in figure A are representative of two independent experiments, antibody quantification presented in figure B was performed once, in duplicate.

### 3.1.2 Correlation of anti-CPS IgG concentration and whole cell ELISA titre

Different individual Malawian sera varied both in their binding to different *S. pneumoniae* serotypes by ELISA and their concentration of serotype-specific anti-capsular polysaccharide (CPS) antibody. Linear regression was performed to correlate serotype-specific anti-CPS IgG levels in different individual sera to the level of IgG binding to different *S. pneumoniae* serotypes (as determined by ELISA). This allowed assessment as to whether the concentration of serotype specific anti-capsular IgG in individual sera was the dominant determinant of IgG binding (**Figure 3.2**). Levels of anti-serotype 4 CPS IgG failed to correlate with binding of individual sera to whole type-4 pneumococci (**Figure 3.2**), this persisted for IgG to anti-serotype14 or anti-serotype 9V polysaccharide. In contrast levels of IgG to the type-1 polysaccharide did correlate with IgG binding to whole ST1 pneumococci (**Figure 3.2**). These data suggest that with the exception of ST1, levels of IgG to specific capsular polysaccharides do not accurately predict the ability of IgG in different individual sera to bind to *S. pneumoniae*, by whole cell ELISA.
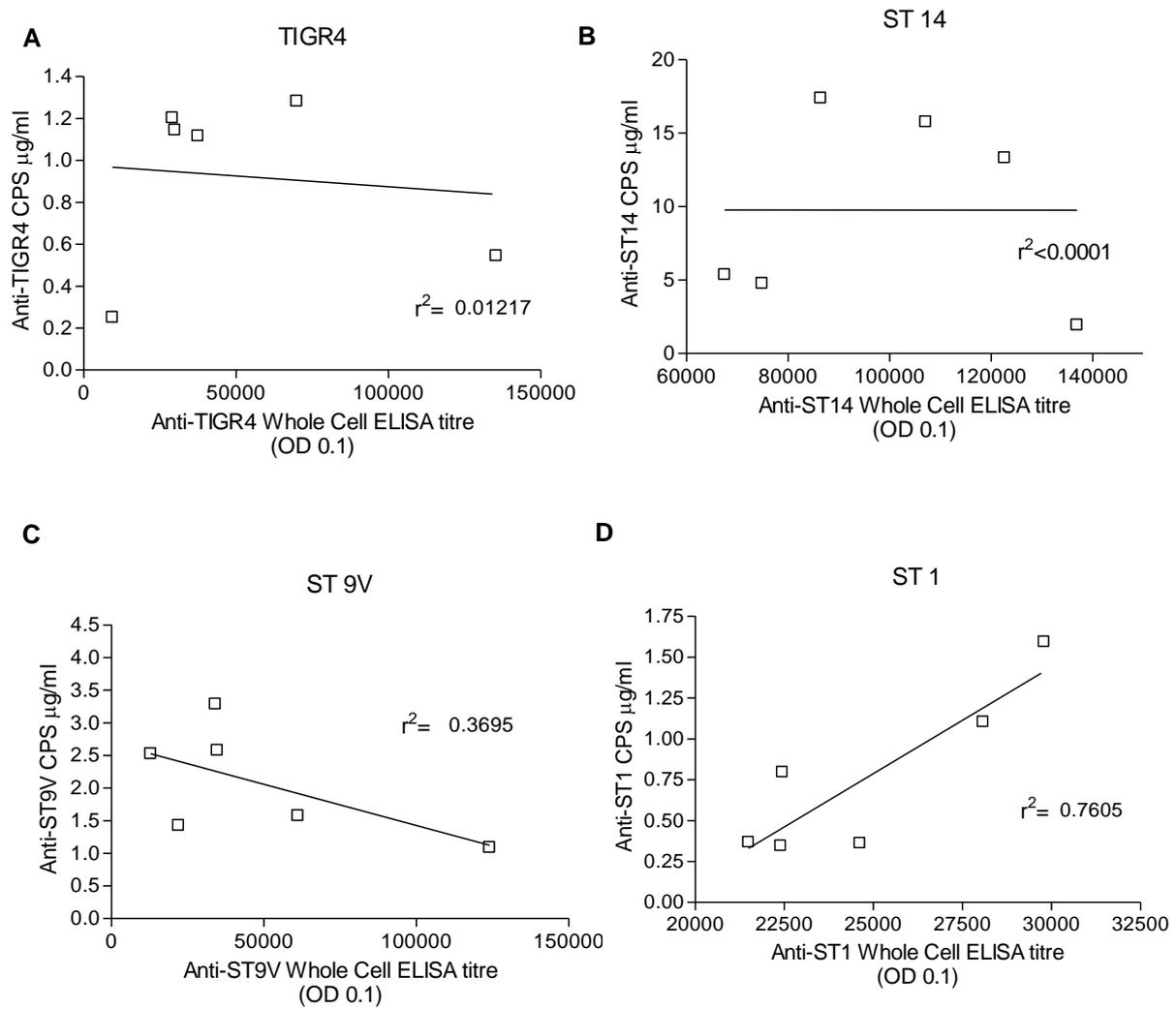
**Figure 3.2: Correlation of anti-polysaccharide IgG with IgG binding to whole *S. pneumoniae.***

(A-D)Whole cell ELISA IgG binding titre to *S. pneumoniae* serotypes 14, 1, 4 and 9V, compared with specific anti-capsular IgG concentrations in individual Malawian sera by linear regression. ELISA titres are representative of two independent experiments.

### 3.1.3    IgG binding to TIGR4Δ*cps* (ELISA)

Since levels of anti-CPS IgG broadly failed to correlate with IgG binding to whole pneumococci, the ability of different individual sera to bind to *S. pneumoniae* TIGR4 lacking the polysaccharide capsule (TIGR4Δ*cps*) was investigated to assess if individual adult sera contained IgG to non-capsular antigens. In the absence of the polysaccharide capsule binding of IgG in different individual human sera to *S. pneumoniae* was maintained (**Figure 3.3**), demonstrating that IgG to the capsular polysaccharide of *S. pneumoniae* was not necessarily required for IgG binding to whole pneumococci, at least if assessed by whole cell ELISA.
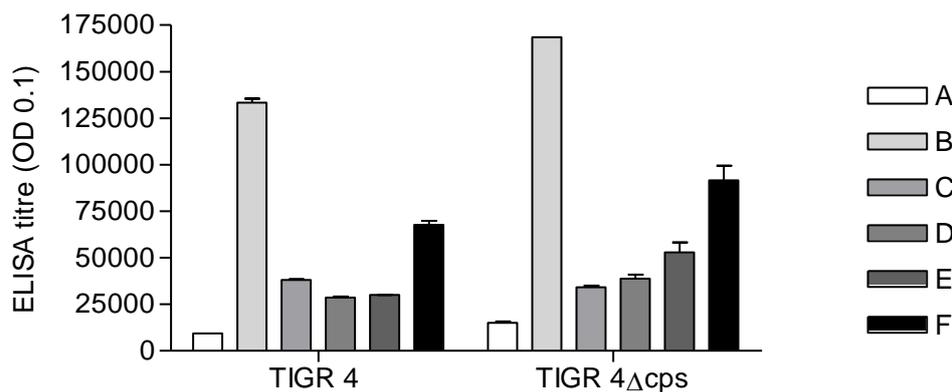


**Figure 3.3: IgG binding to TIGR4Δ*cps*.**

Whole cell ELISA IgG binding titre to *S. pneumoniae* TIGR4 and TIGR4Δ*cps* in different individual Malawian sera. (Columns represent mean, error bars represent SD). Results are representative of two independent experiments.

### 3.1.4 Correlation of whole cell ELISA and anti-CWPS IgG concentration

Another antigen of *S. pneumoniae* that may be an important target for IgG binding is the cell wall polysaccharide (CWPS). However, the concentration of IgG to the pneumococcal CWPS (measured by ELISA) in individual sera, failed to correlate with IgG binding of each serum to *S. pneumoniae* TIGR4 by whole cell ELISA (**Figure 3.4**). This indicates that anti-CWPS IgG in not a major determinant of naturally-acquired human IgG in human sera binding to *S. pneumoniae*, by ELSA.
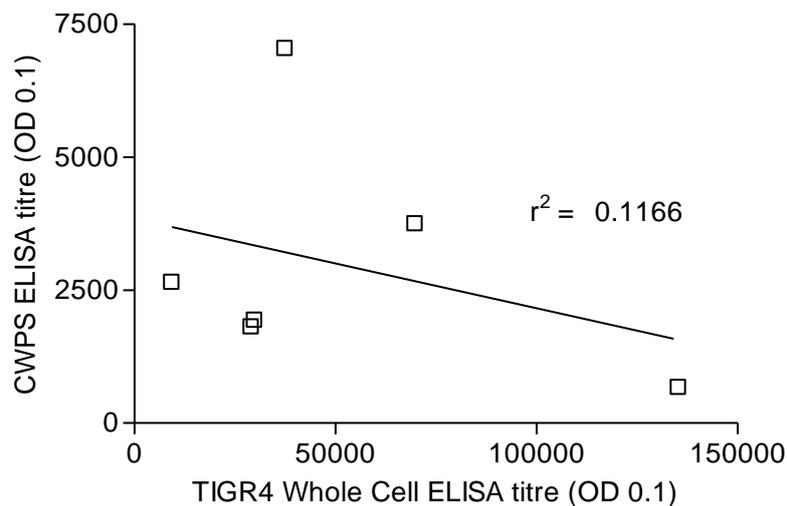


**Figure 3.4: Correlation of anti-CWPS IgG and binding to whole *S. pneumoniae***

Whole cell ELISA IgG binding titre to *S. pneumoniae* TIGR4 compared with anti-CWPS IgG binding titre, in individual Malawian sera compared by linear regression. Results are representative of two independent experiments.

### 3.1.5 Competition ELISAs

Pooled sera and IVIG contain IgG representative of the population from which they are pooled. To directly assess the targets of IgG binding to *S. pneumonia*, in pooled adult sera and IVIG competition ELISAs were performed. This involved pre-incubation of IVIG or pooled sera in different pneumococcal antigens including; capsular polysaccharide, cell wall polysaccharide and whole cell lysates of *S. pneumoniae*. Pre-incubation of pooled sera with increasing concentrations of CWPS, failed to reduce binding of IgG to *S. pneumoniae* TIGR4, as measured by ELISA (**Figure 3.5**). Similarly incubation of IVIG in increasing concentrations of CWPS or purified type-4 or 19F polysaccharide failed to inhibit binding to *S. pneumoniae* TIGR4 and a serotype 19F strain respectively. In comparison binding was inhibited by pre-incubation of sera or IVIG in whole cell lysates of the respective serotype (TIGR4 or ST19F) (**Figure 3.5**). These data indicate that the CWPS or capsular polysaccharide of *S. pneumoniae* is not necessarily important for the observed binding of IgG in human sera or IVIG to whole pneumococci, as measured by ELISA.
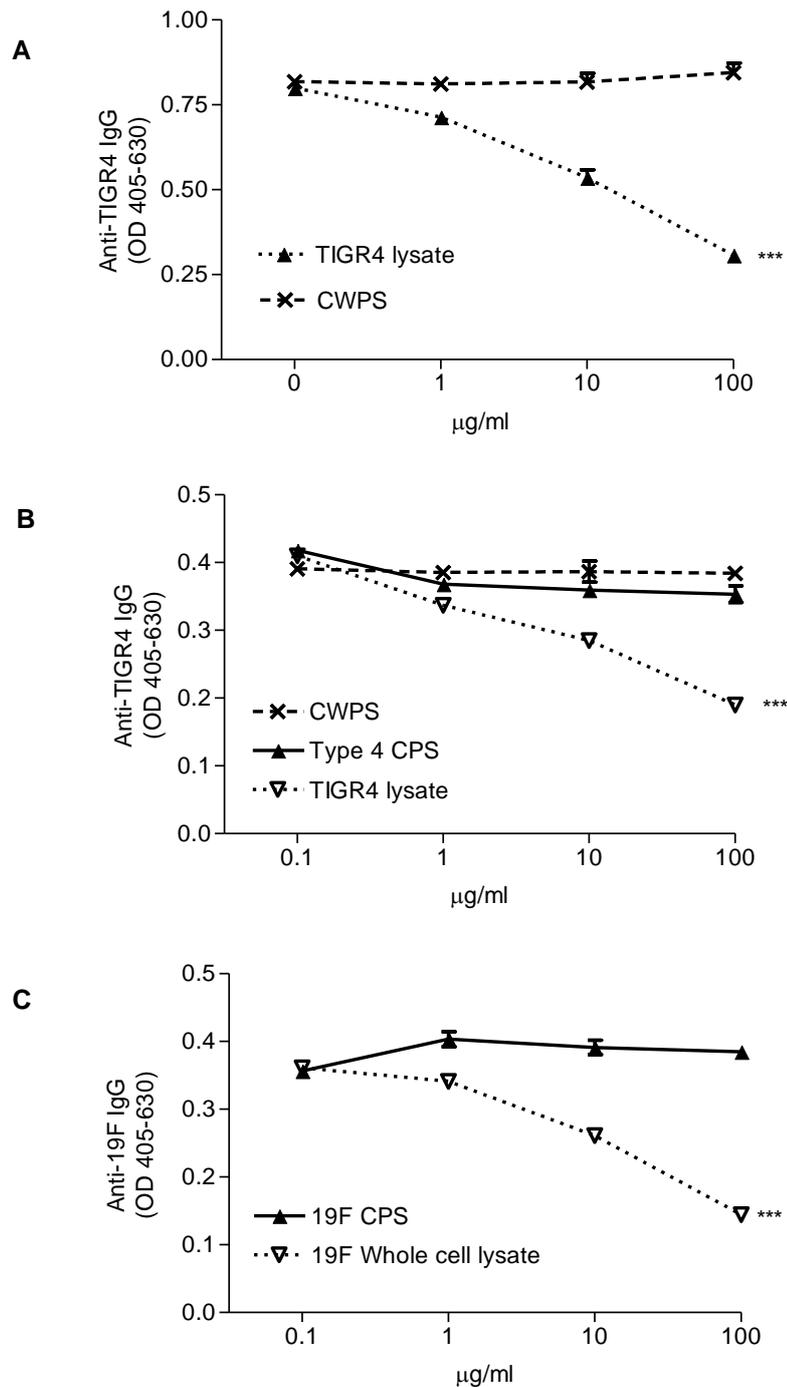
**Figure 3.5: Competition ELISAs IgG binding to *S. pneumoniae.***

(A) IgG binding in pooled human sera to *S. pneumoniae* TIGR4 following pre-incubation in increasing concentrations of *S. pneumoniae* lysate or purified *S. pneumoniae* CWPS. (B) IgG binding in IVIG (Intratect) to *S. pneumoniae* TIGR4 following pre-incubation in increasing concentrations of whole *S. pneumoniae* lysate, purified *S. pneumoniae* CWPS or type-4CPS. (C) IVIG binding to *S. pneumoniae* 19F following pre-incubation in whole cell lysate or type-19F CPS. (***= *P* < 0.001 representative of two way ANOVAs comparing change in OD following incubation in whole lysate versus CPS or CWPS, error bars represent SD). Results are representative of two independent experiments.

To investigate if IgG to pneumococcal proteins could be responsible for the observed binding to *S. pneumoniae* by whole cell ELISA, competition ELISAs were repeated. Pooled sera and IVIG were pre-incubated in either whole cell lysates of *S. pneumoniae* TIGR4 or lysates treated with trypsin to digest pneumococcal proteins. Pre-incubation of sera or IVIG in whole cell lysates reduced IgG binding to TIGR4. Prior trypsin treatment of these lysates partially diminished this effect (**Figure 3.6**), though didn't fully restore IgG binding

Overall these whole cell ELISA data suggest that protein targets, rather than anti-CWPS IgG or anti-CPS specific IgG, are at least partially responsible for IgG binding to *S. pneumoniae* in adult human sera and IVIG.
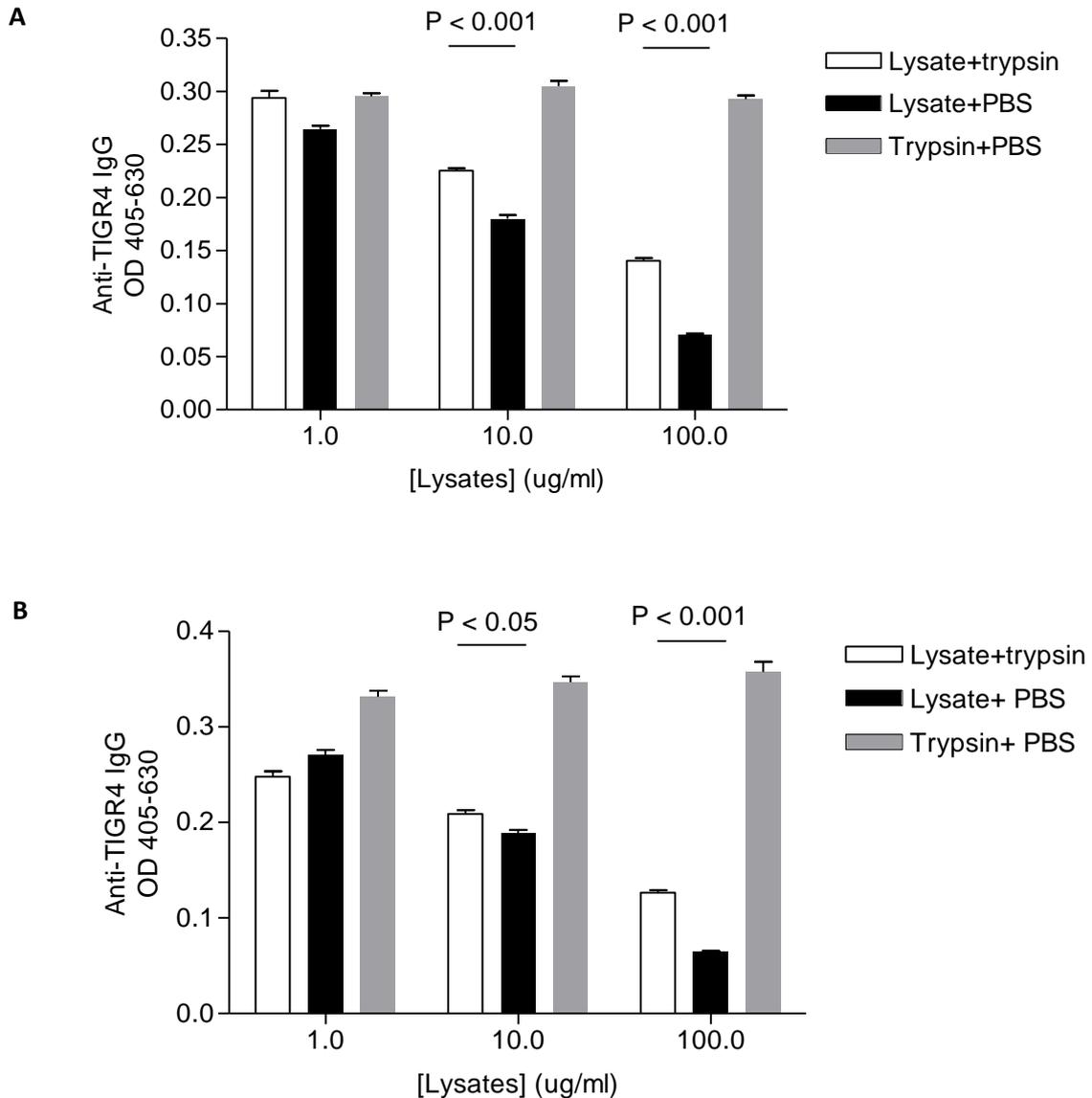
**Figure 3.6: Competition ELISAs protease treated lysate.**

(A) IgG binding in IVIG (Intratect) to *S. pneumoniae* TIGR4, by whole cell ELISA, following pre-incubation in different concentrations of whole *S. pneumoniae* lysates, with and without pre-treatment of lysates with trypsin. (B) IgG binding in pooled sera to *S. pneumoniae* TIGR4, by whole cell ELISA, following pre-incubation in different concentrations of whole *S. pneumoniae* lysates, with and without pre-treatment of lysates with trypsin. (*P* values represent two-way ANOVAs with Bonferonni post-test to compare columns, error bars represent SD). Results are representative of two independent experiments.

### 3.1.6    Luminex assay

The above data indicated that non-capsular antigens, including pneumococcal proteins are likely to be major targets for naturally-acquired IgG binding to *S. pneumoniae*. To assess the range of anti-protein IgG targets in human sera and pooled immunoglobulin preparations, multiplex (Luminex) bead assays were performed. This involved simultaneous detection of antibody binding to recombinant pneumococcal proteins coated on to fluorescent (xMAP) beads. Binding was quantified by an increase in fluorescence, using an anti-human IgG secondary conjugated to PE. Importantly, this allowed a semi-quantitative analysis of antibody levels to a range of pneumococcal proteins in different sources of sera and IVIG.

To confirm that the Luminex assay allowed a quantitative assessment of antibody binding, and to determine appropriate dilutions for subsequent assays, IgG binding in serially-diluted IVIG was assessed (**Figure 3.7**).  Binding of IgG to all proteins was concentration dependent, with a reduction in fluorescence intensity at increasing dilutions of IVIG (**Figure 3.7**). A dilution of 1:1000 represented a point with a large dynamic range of IgG binding to different purified proteins; this dilution (1:1000) was therefore used in subsequent experiments.
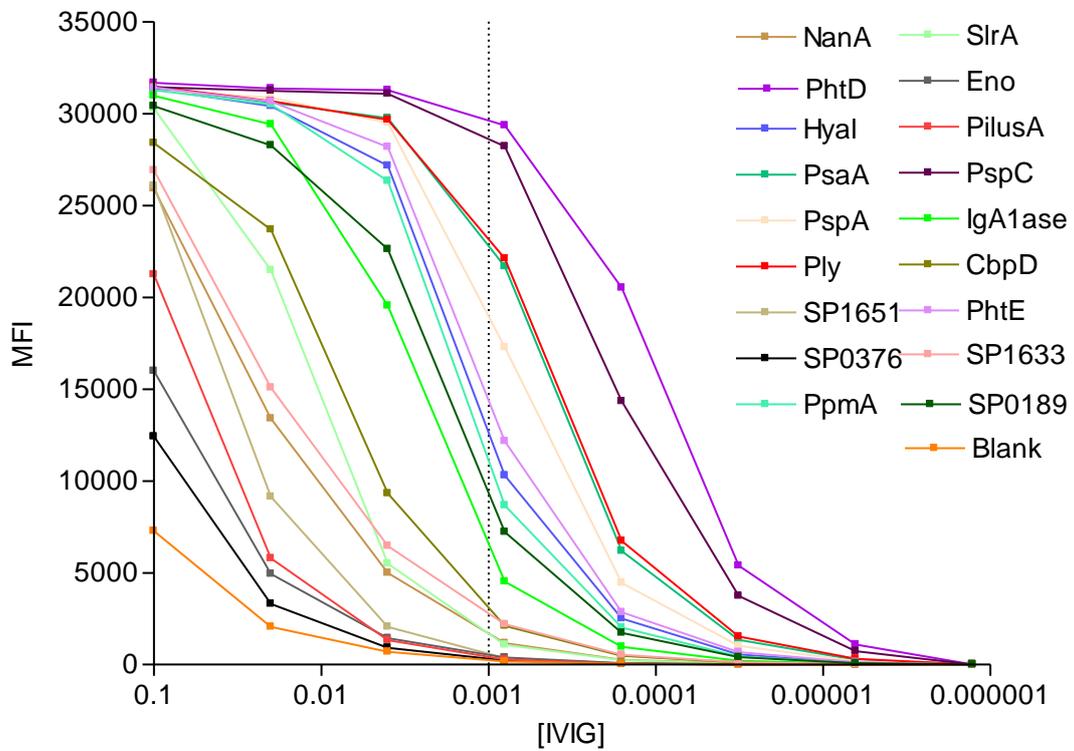
**Figure 3.7: Dilution series of IgG binding to pneumococcal proteins.**

MFI of anti-human IgG binding to xMAP beads coated with different purified pneumococcal proteins, following incubation in increasing dilutions of IVIG (Intratect). The dashed line indicates the dilution (1:1000) used in subsequent assays.

The Luminex assay was used to assess the major protein antigen targets for IgG in different pooled IVIG preparations. Levels of IgG to pneumococcal protein antigens, in different sources of IVIG, appeared to show a consistent pattern of responses (**Figure 3.8**), with high levels of anti-PhtD and anti-PspC antibodies, intermediate responses to the proteins PspA, PsaA and Ply, slightly inferior responses to PpmA, PhtE, Hyal and Sp0189 and low or absent responses to all other proteins.

The targets of anti-protein IgG in individual human sera, both from Malawi and the UK, were also assessed by Luminex. A similar general pattern of dominance was seen to the results for IVIG (**Table 3.1**) with strong responses to PhtD, PspC, PspA, PsaA and PpmA. However there was significant variation in the level of IgG to certain pneumococcal protein antigens between sera from different individuals (**Figure 3.8**). For example, sera 'B' and 'F' from Malawian individuals demonstrated markedly higher binding to PspC than the remaining 4 individual Malawian sera, and sera 'B' and 'C' had poorer responses to purified PsaA (**Figure 3.8**).
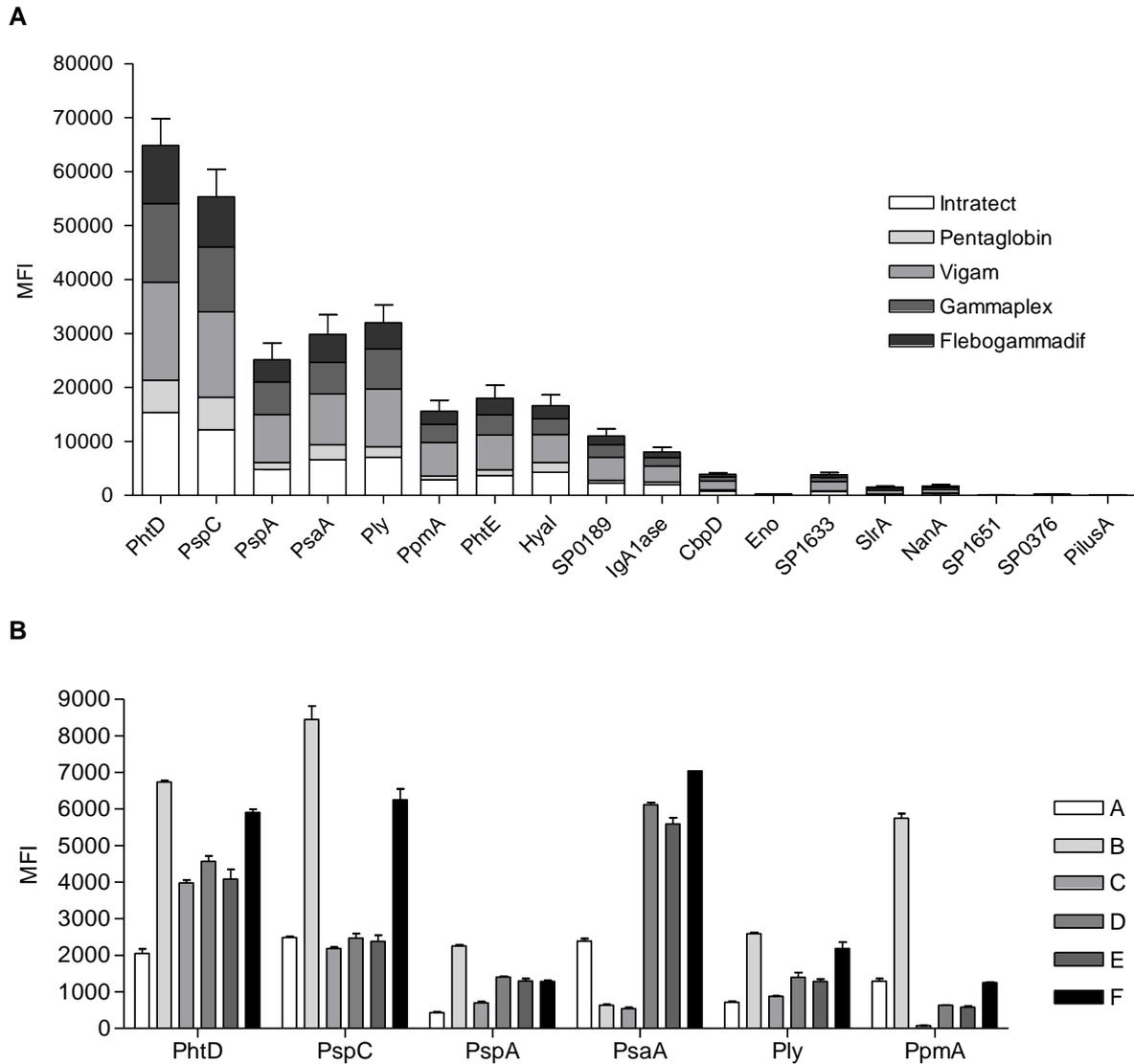
**Figure 3.8: IgG binding to purified pneumococcal proteins.**

(A) Stacked bars representing MFI of IgG in five different IVIG preparations binding to purified *S. pneumoniae* proteins conjugated to fluorescent (xMAP) beads (Luminex). (B) MFI of IgG in 6 different individual sera (Malawi) binding to purified *S. pneumoniae* proteins (Luminex). (Error bars represent SD). Results in Figure A and B are representative of one experiment, performed in duplicate.

|        | UK |   |   |   | Malawi |   |   |   |   |   |
|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|        | A | B | C | D | A | B | C | D | E | F |
| PhtD   | 3  | 2  | 1  | 5  | 3  | 2  | 1  | 2  | 2  | 3  |
| PspC   | 6  | 3  | 4  | 1  | 1  | 1  | 2  | 3  | 3  | 2  |
| PspA   | 4  | 1  | 2  | 4  | 7  | 6  | 4  | 4  | 4  | 5  |
| PsaA   | 2  | 5  | 9  | 3  | 2  | 10 | 6  | 1  | 1  | 1  |
| Ply    | 8  | 4  | 3  | 7  | 6  | 5  | 3  | 5  | 5  | 4  |
| PpmA   | 1  | 7  | 10 | 2  | 5  | 3  | 13 | 7  | 7  | 6  |
| PhtE   | 7  | 8  | 6  | 6  | 4  | 4  | 5  | 8  | 8  | 8  |
| Hyal   | 5  | 10 | 5  | 11 | 9  | 8  | 7  | 6  | 6  | 7  |
| SP0189 | 11 | 11 | 8  | 12 | 12 | 11 | 8  | 13 | 13 | 10 |
| IgA1ase| 14 | 6  | 7  | 9  | 8  | 7  | 11 | 9  | 9  | 9  |
| CbpD   | 9  | 9  | 13 | 8  | 11 | 9  | 14 | 10 | 10 | 13 |
| Eno    | 15 | 15 | 15 | 15 | 16 | 15 | 16 | 15 | 15 | 15 |
| SP1633 | 10 | 12 | 11 | 13 | 13 | 13 | 10 | 14 | 14 | 11 |
| SlrA   | 16 | 17 | 16 | 10 | 14 | 14 | 9  | 16 | 17 | 14 |
| NanA   | 12 | 13 | 12 | 14 | 10 | 12 | 12 | 11 | 12 | 12 |
| SP1651 | 16 | 17 | 14 | 17 | 18 | 17 | 17 | 18 | 18 | 18 |
| SP0376 | 13 | 16 | 16 | 16 | 17 | 18 | 15 | 17 | 16 | 17 |
| PilusA | 16 | 14 | 16 | 17 | 15 | 16 | 18 | 12 | 11 | 16 |

**Table 3.1:** Rank of IgG binding (MFI) to different purified protein antigens of *S. pneumoniae* (measured by Luminex) in individual sera from the UK or Malawi. The same ranking score was assigned if differences between proteins were less than blank controls.

### 3.1.7 Luminex assay validation

To determine if the Luminex assay was reliable for the quantification of antibody responses to different pneumococcal proteins, antibody levels in individual Malawian sera to two of the purified pneumococcal proteins included on the Luminex panel (PspC, PsaA) were also assessed by ELISA. ELISA titres were then compared against MFI (Luminex) values for these proteins by linear regression (**Figure 3.9**). A strong correlation was seen between the two methods; where individual sera had a high MFI to PspC or PsaA by Luminex they also had a relatively high ELISA titre to that same protein (**Figure 3.9**). IgG binding in Malawian sera to the purified proteins SP1633 and SP1651 to which no antibody binding was detected by Luminex, was also assessed by ELISA, again low or absent IgG binding was detected to these proteins (**Figure 3.9**). In comparison IgG levels to PspC against which high responses were detected by Luminex, were also relatively high by ELISA (**Figure 3.9**). In addition to individual sera IgG binding in different IVIG preparations was also assessed by ELISA, and binding to PspC was higher than to PhtE when measured by both ELISA and Luminex (**Figure 3.9**). These data confirm that the Luminex assay appears as reliable as ELISAs for the quantification of antibody responses to different *S. pneumoniae* protein antigens.
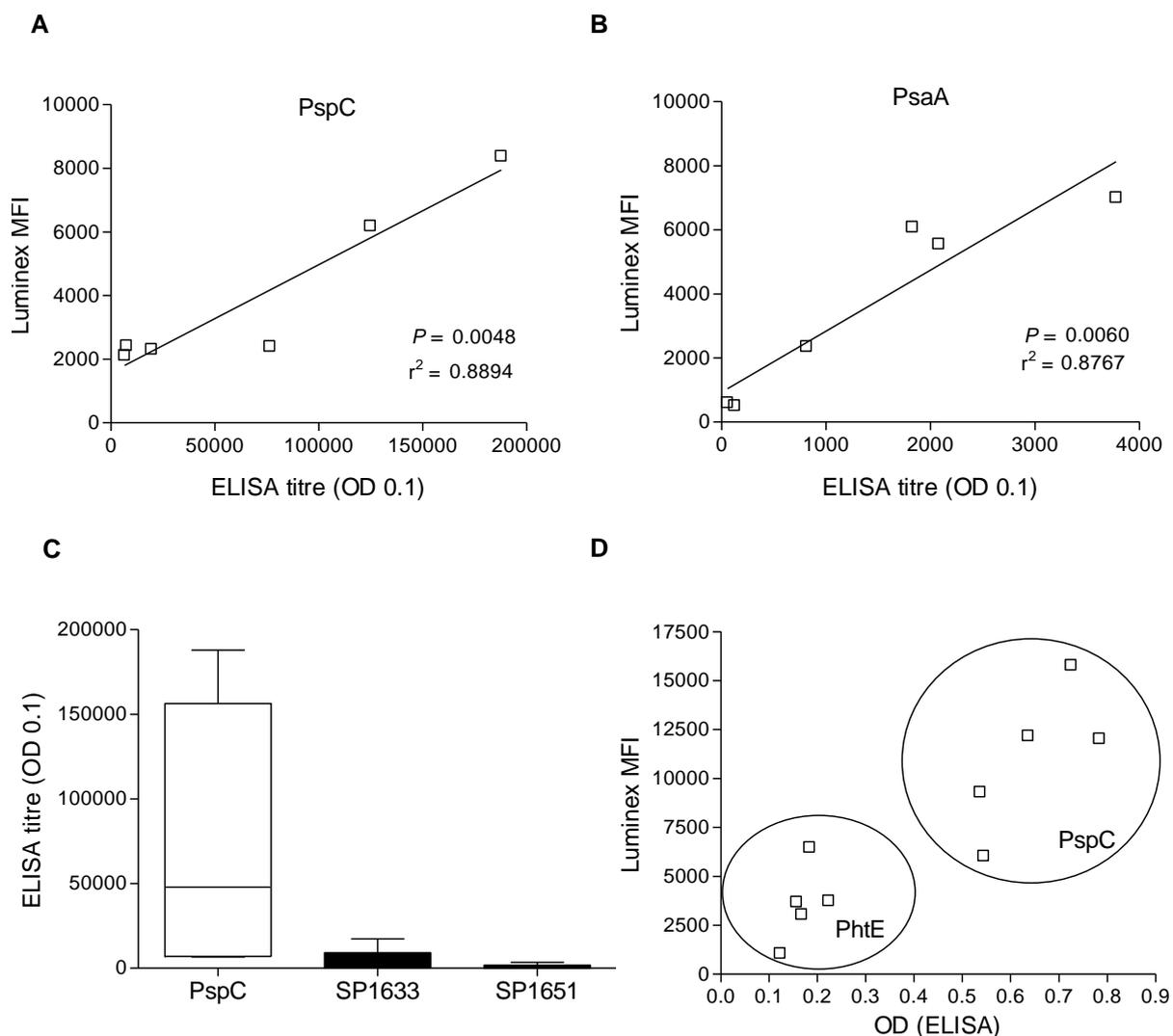
**Figure 3.9: Validation of Luminex assay.**

(A) Linear regression of IgG in different individual sera binding to purified PspC by ELISA (titre) compared to Luminex (MFI). (B) Linear regression of IgG in different individual sera binding to purified PsaA by ELISA (titre) compared to Luminex (MFI) (C). ELISA titre of IgG binding to purified pneumococcal proteins PspC, SP1633, SP1651 in different individual sera (Malawi). (D) Comparison of IgG binding in different IVIG products to purified PhtE and PspC by ELISA (OD 405-630) and Luminex (MFI). (*P* values represent F-test assessing difference of slope compared to zero, error bars represent SD). Luminex and ELISA quantification was performed once, in duplicate.

### 3.1.8   Correlation between IgG to protein antigens and whole cell ELISA titre

The Luminex assay provided a quantitative assessment of antibody responses to protein antigens in different individual sera. This allowed comparison between the quantity of IgG to pneumococcal protein antigens in different individual sera and IgG binding to whole *S. pneumoniae* as measured by whole cell ELISA. The IgG responses to all proteins on the Luminex system were summed for each individual Malawian serum sample; the cumulative anti-protein IgG response was then compared by linear regression to IgG binding to *S. pneumoniae* (as previously assessed by whole cell ELISA, **Figure 3.1**). There was a good correlation between IgG levels to these pneumococcal proteins in a particular individuals sera and the level of IgG binding to *S. pneumoniae* TIGR4, ST14 and ST1 (**Figure 3.10**). ST9V demonstrated a similar trend, but this was not significant (**Figure 3.10**).

These correlations could be due to differences in total IgG concentration between sera samples, rather than levels of pneumococcal-specific IgG. However, there was no correlation between Luminex measurements of IgG binding to *S. pneumoniae* protein antigens and total IgG concentration as measured by ELISA (**Figure 3.10**). Additionally, IgG binding *to S. pneumoniae* protein antigens did not correlate with binding to another streptococcus species *S. pyogenes* (**Figure 3.10**).

These data indicate that the quantity of IgG to specific    *S. pneumoniae* protein antigens may be important in determining IgG binding to whole *S. pneumoniae*, as measured by whole cell ELISA. Therefore *S. pneumoniae* proteins could be major targets of naturally-acquired IgG.
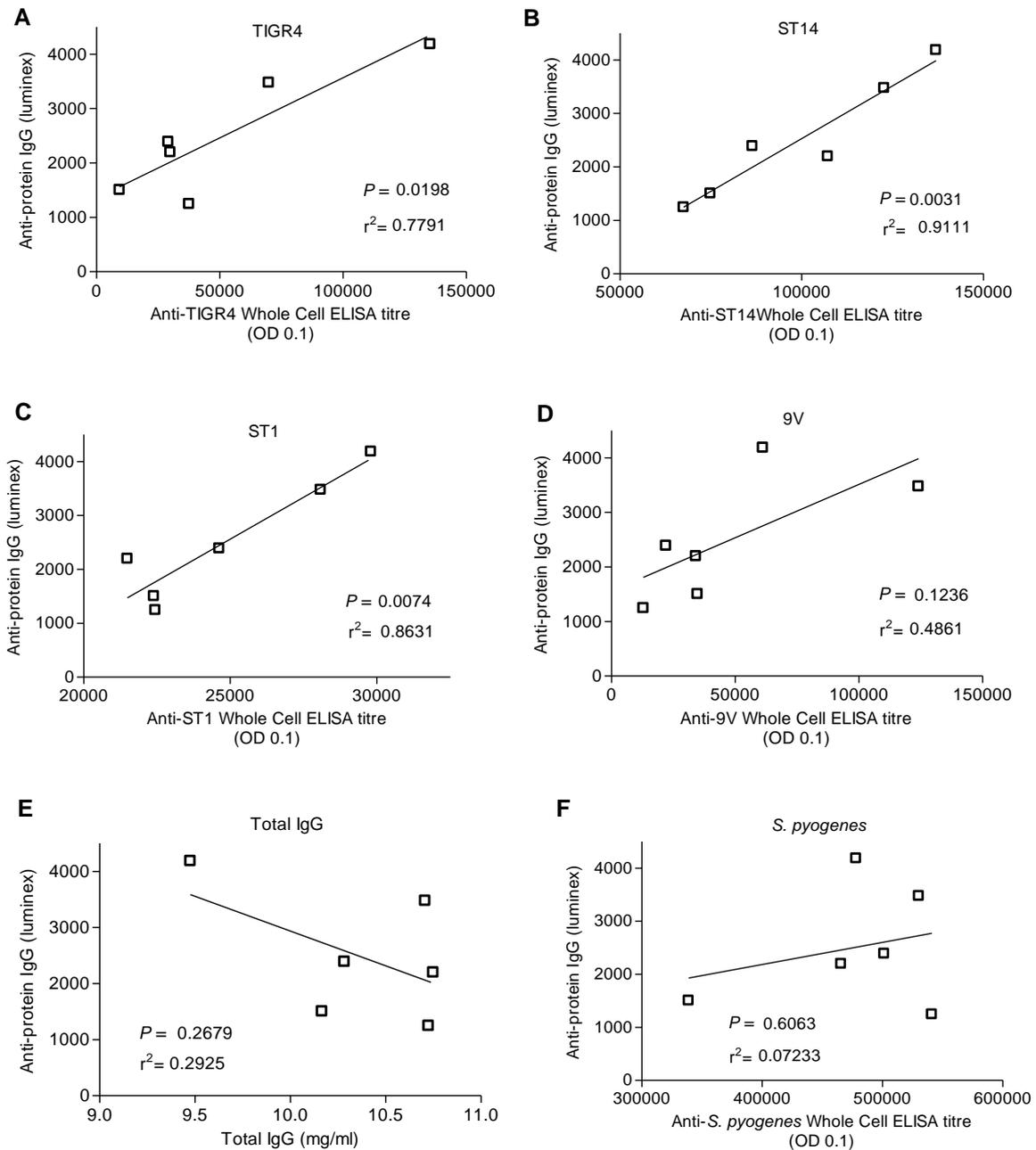
**Figure 3.10: Correlation of anti-protein IgG with IgG binding to whole *S. pneumoniae*.**

(A-D) Linear regression of IgG binding to different serotypes of *S. pneumoniae* by whole cell ELISA compared to anti-protein IgG concentration in different individual sera (as assessed by Luminex). (E) Linear regression of total IgG concentration in each individual sera (quantified by ELISA) compared to cumulative anti-protein IgG (as assessed by Luminex). (F) Linear regression of IgG binding to *S. pyogenes* by whole cell ELISA compared to cumulative anti-protein IgG concentration in different individual sera (as assessed by Luminex). (*P* values represent F-test assessing difference of slope compared to zero). ELISA titres are representative of two independent experiments.

### 3.1.9 IgG protein targets in different geographical sources of sera and IVIG

To determine if antibodies from distinct populations recognise a similar range of *S. pneumoniae* protein antigens, the range of pneumococcal protein targets for IgG binding in pooled sera or IVIG from 3 geographically distinct locations (Europe, USA, and Malawi) were compared. This was achieved by comparing the MFI for each protein obtained from the multiplex binding assays by linear regression (**Figure3.11**).

There was a very strong correlation in the order of anti-protein antibody responses in IgG pooled from three populations (**Figure3.11**) with consistently high responses to PhtD, PspC and PsaA. Slightly more variation in the order of anti-protein antibody responses was seen when comparing pooled sera from Malawi, to IVIG products sourced from Europe or USA. This may be due to the greater number of donors that make up IVIG (N=>1000) which could reduce the variability of responses to protein antigens, compared to pooled Malawian sera, which was pooled from relatively few donors (N=20).
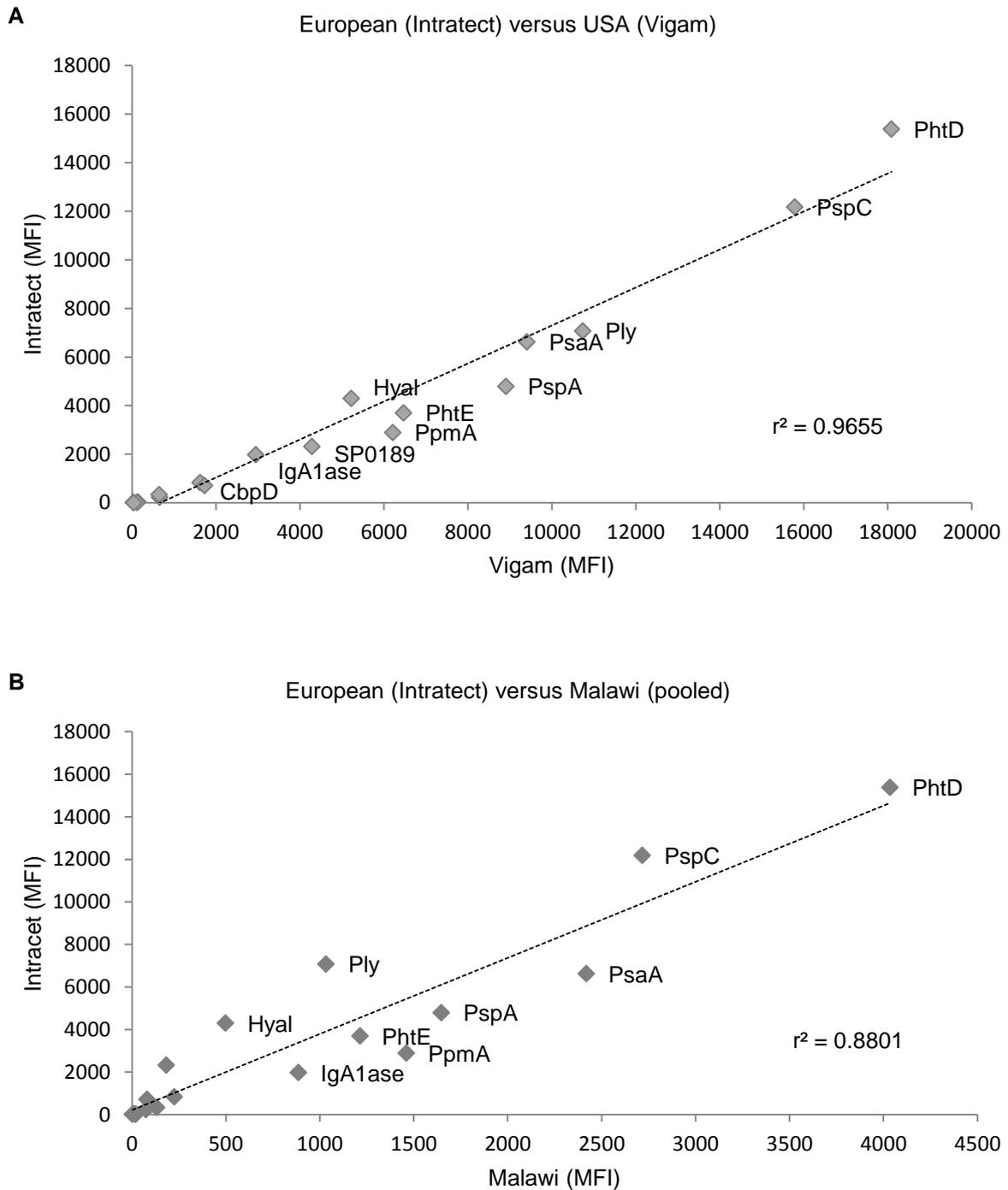
**Figure3.11: IgG targets in different sources of pooled immunoglobulin.**

(A) Linear regression of IgG binding (MFI) to different purified *S. pneumoniae* protein antigens in IVIG pooled from a European population compared to IVIG pooled from USA. (B) Linear regression of IgG binding (MFI) to different purified *S. pneumoniae* protein antigens in IVIG pooled from Europe or pooled Malawian sera (pooled from 20 donors). Luminex assays were performed once, in duplicate.

### 3.1.10   Identification of IgG protein targets by Western blot

The Luminex assay assesses antibody responses to a pre-selected panel of protein antigens. Therefore Western blotting was used for an overall assessment of the potential number of protein targets for IgG in different sources of sera and IVIG. Probing lysates of the *S. pneumoniae* D39 strain with IVIG indicated responses to a number of different protein targets, identified as bands on immunoblots (**Figure 3.12**). Probing mutants *S. pneumoniae* D39 lacking known surface proteins allowed the identification of some of these protein targets, including PiaA, PspA, PspC and PhtD (**Figure 3.12**). Importantly (where included on the panel) the targets identified by Western blotting were also previously identified as immunodominant targets of IgG binding by Luminex. Immunoblots against lysates of different *S. pneumoniae* serotypes demonstrated a consistent pattern when probed with IVIG (**Figure 3.12**), though responses to certain protein were absent in some strains. This suggests, with some exceptions, the major protein targets of IVIG are generally conserved between strains of *S. pneumoniae*.  Immunoblots of *S. pneumoniae* TIGR4 probed with pooled IgG from different geographical locations (Europe, USA, and Malawi) demonstrated a similar pattern of responses (**Figure 3.12**). This supports data from the Luminex assay, indicating that IgG pooled from these different geographical locations recognises a consistent pattern of dominant pneumococcal protein antigens.
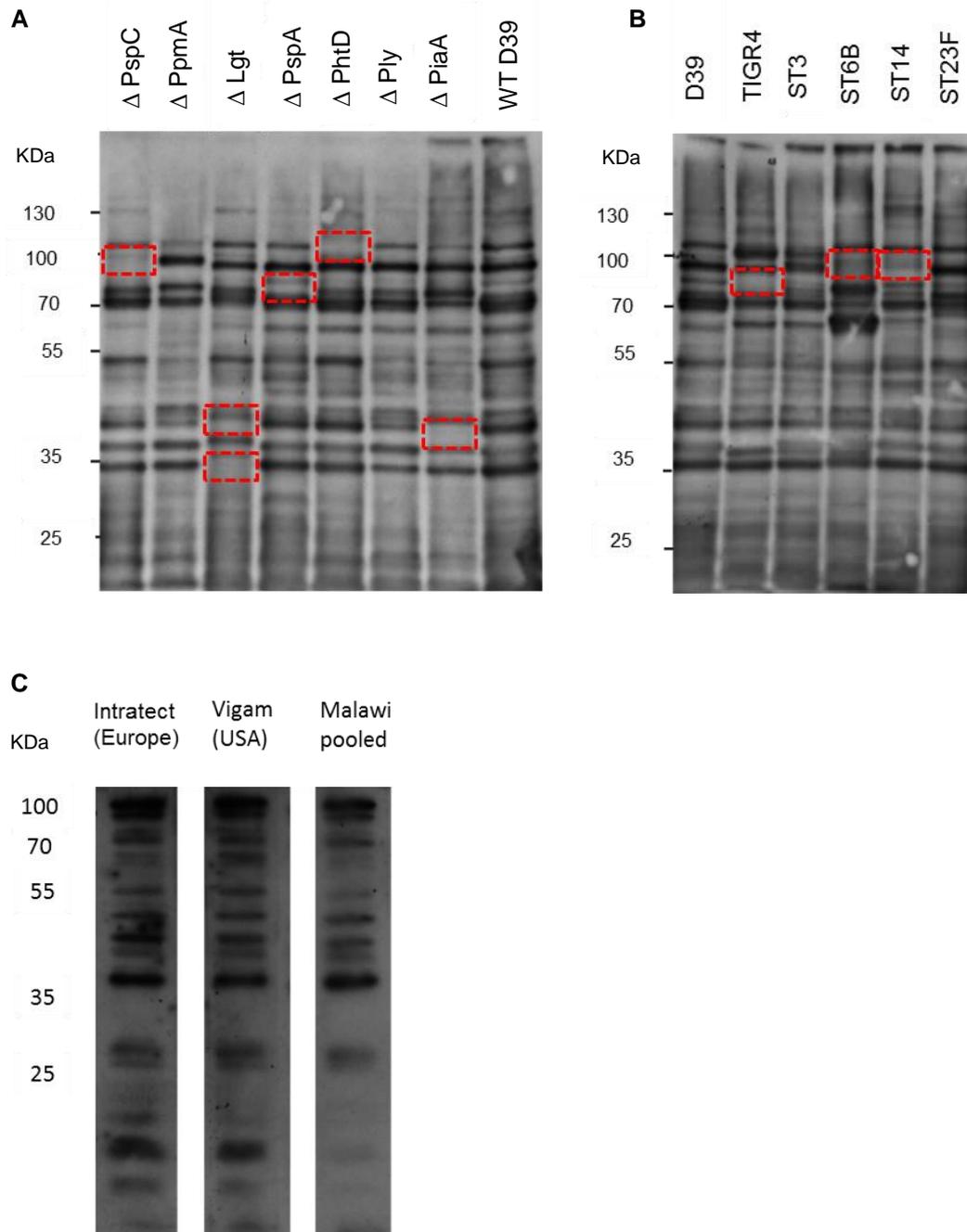
**Figure 3.12: Targets of IgG in pooled sera binding by immunoblotting.**

(A) Immunoblots of deletion mutants of *S. pneumoniae* D39 lacking known surface proteins (indicated above lanes) probed with IVIG (Intratect). Red boxes highlight absent bands at correct molecular weight for deleted protein. (B) Immunoblots of whole cell lysates of different *S. pneumoniae* serotypes probed with IVIG (red boxes indicate absent bands in certain serotypes). (C) Immunoblots of *S. pneumoniae* TIGR4 probed with different pooled sera (indicated above lanes). Western blots are representative of two independent experiments.

### 3.1.11 Pattern of IgG responses in individual sera by Western blot

To comprehensively assess the range of protein antibody targets in different individual sera, immunoblots of lysates of *S. pneumoniae* D39 and TIGR4 were probed with sera from 6 individuals (from the UK). The pattern of responses was then compared. There were broadly similar patterns of bands between different individual sera, probed against the same lysates (**Figure 3.13**), indicating that the dominant IgG responses to protein antigens were relatively consistent across different individual sera. However, for both D39 and TIGR4 there was some variation between individuals, with Western blots of sera from certain individuals having reduced intensity of bands at particular protein sizes. For example, against *S. pneumoniae* D39 sera 'A' and 'B' had low responses to a band at approximately 70KDa, and sera 'C' and 'F' had low responses to a band at approximately 35KDa in *S. pneumoniae* TIGR4. These data support the Luminex data for different individual sera, where the pattern of antigen dominance was broadly similar between all individuals, but selected sera demonstrated low or absent IgG responses to certain *S. pneumoniae* protein antigens.
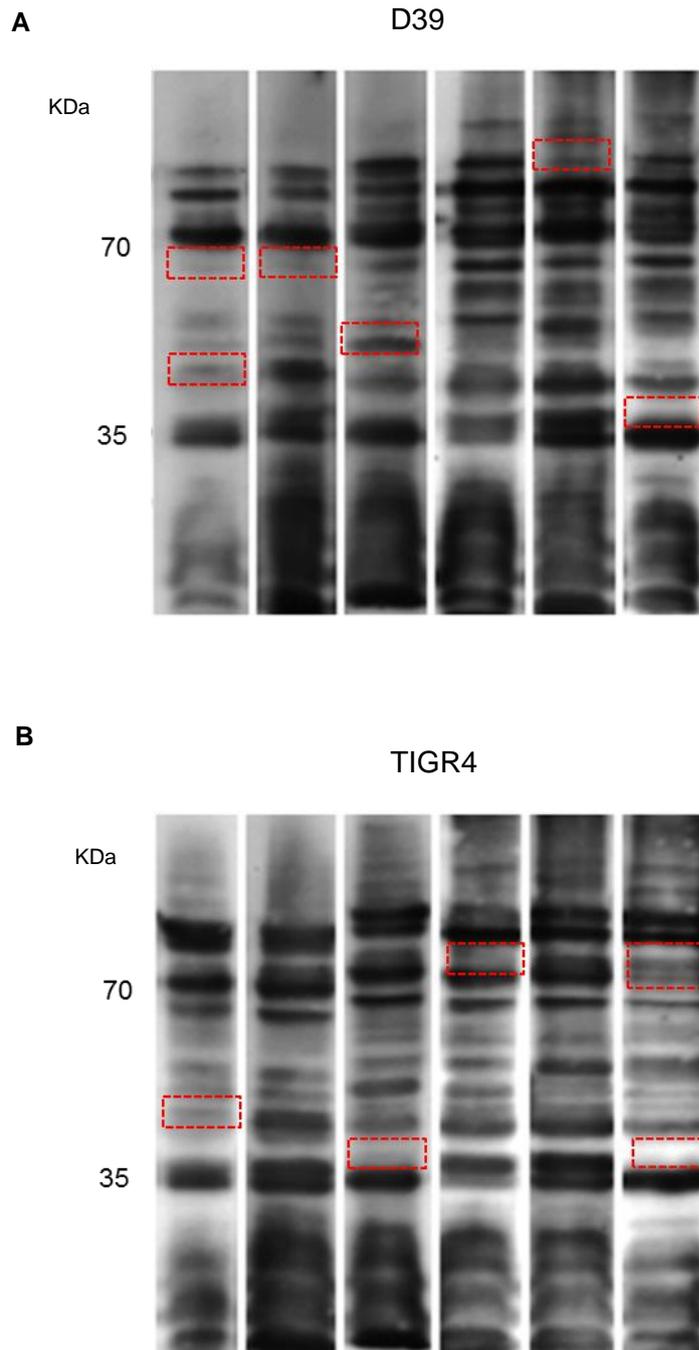
**Figure 3.13: Targets of IgG binding in individual sera by immunoblotting.**

(A) Immunoblots of whole cell lysates of *S. pneumoniae* D39 probed with different sera from UK individuals, developed with anti-human IgG. (B) Immunoblots of whole cell lysates of *S. pneumoniae* TIGR4 probed with different individual human sera, developed with anti-human IgG (Red boxes highlight areas of variation between individual sera). Western blots are representative of two independent experiments.

### 3.1.12   Anti-*S. pneumoniae* IgG in human lavage samples

Adult human sera contain IgG to whole *S. pneumoniae*, as well as to a range of pneumococcal surface proteins. To determine if anti-pneumococcal IgG is present within the lung of adult humans, IgG binding in BALF from different Malawian individuals was investigated. BALF was obtained from volunteers in Malawi through collaboration with Dr Stephen Gordon (Liverpool School of Tropical Medicine). IgG binding to whole *S. pneumoniae* was assessed by whole cell ELISA. Due to limitations with sample quantity, titres were not calculated; instead ELISA data is represented as an OD. BALF samples contained IgG binding to whole *S. pneumoniae* TIGR4 by ELISA, with variation in the amount of anti-pneumococcal IgG binding between different individuals (**Figure 3.14**). The highest responses were comparable to pooled sera from the same population diluted 1:1000. However, as the process of collecting lavage fluid involves considerable dilution of the epithelial lining fluid, it is difficult to directly estimate the concentration of anti-pneumococcal IgG within the human lung.

Due to limited quantities of the multiplex beads Luminex assays were not performed on these samples, however BALF IgG levels to the purified pneumococcal proteins PspC and PhtE was assessed by ELISA. BALF fluid contained detectable IgG to each of these proteins (**Figure 3.14**). Binding of IgG to whole *S. pneumoniae* by ELISA correlated with levels of IgG to either PspC or PhtE in different lavage samples.  There was no significant correlation between total IgG concentration in BALF and binding to *S. pneumoniae*TIGR4 by whole cell ELISA (**Figure 3.14**). These data demonstrate that human BALF contains IgG to *S. pneumoniae* including to purified pneumococcal proteins, and the concentration of IgG to specific antigens may determine the binding of IgG in BALF to whole *S. pneumoniae*. The concentration of IgG to pneumococcal capsular polysaccharide was not assessed in these BALF samples.
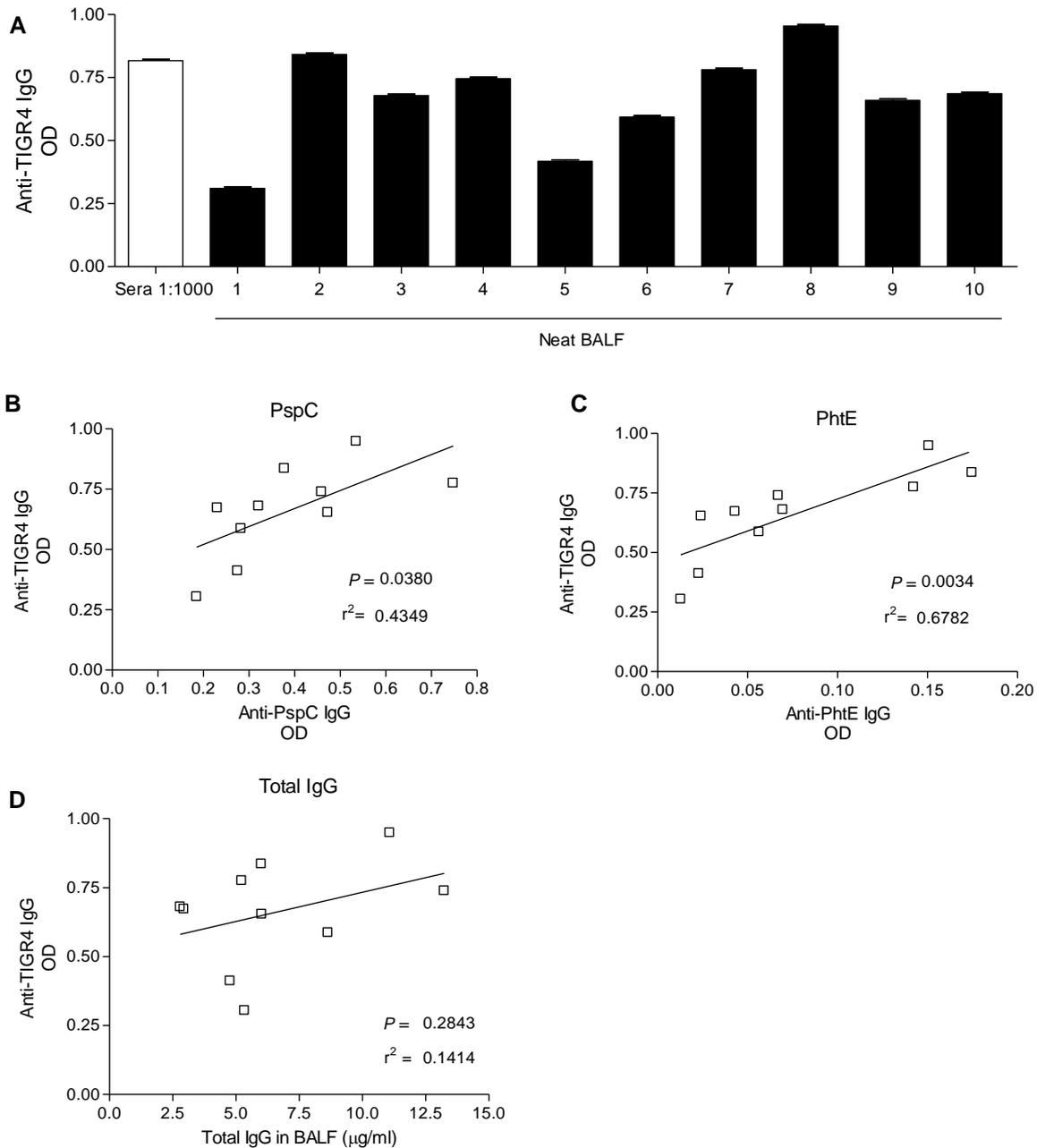
**Figure 3.14: Anti-*S. pneumoniae* IgG in human bronchoalveolar lavage fluid.**

(A) IgG binding in different BALF samples from 10 Malawian individuals to *S. pneumoniae* TIGR4 by whole cell ELISA (OD 450-550), alongside IgG binding in pooled sera (Malawi). (B) Linear regression of IgG binding in different individual BALF samples to TIGR4 by whole cell ELISA compared to binding to purified PspC by ELISA. (C) Linear regression of IgG binding in different individual lavage samples to TIGR4 by whole cell ELISA compared to binding to purified PhtE by ELISA. (D) Linear regression of IgG binding in individual lavage samples to TIGR4 by whole cell ELISA compared to the concentration of total IgG in each sample. (Error bars represent SD, *P* values represent F-test assessing difference of slope compared to zero). ELISA titres are representative of two independent experiments.

### 3.1.13 Chapter Summary

The pneumococcal antigen targets for IgG in adult human sera and IVIG are likely to reflect those acquired following natural exposure to *S. pneumoniae*. Data presented in this chapter indicate that there is a range of different *S. pneumoniae* antigens for IgG, including protein and capsular polysaccharide antigen. The whole cell ELISA results indicate that *S. pneumoniae* proteins were an important target for anti-pneumococcal IgG binding, as the titres in different adult sera correlated with IgG titres to different *S. pneumoniae* proteins but not anti-capsular IgG concentration. Additionally, competition ELISAs indicated that IgG binding to *S. pneumoniae* was not inhibited by pneumococcal capsular or cell wall polysaccharide, hence these antigens are not necessarily the major targets of naturally-acquired IgG binding to *S. pneumoniae*, when assessed by a whole cell ELISA assay.

Luminex assays and Western blots were used to identify potential protein targets for IgG in human sera. The results indicated a small subset *of S. pneumoniae* protein antigens including PhtD, PspC, PspA and PsaA that are targets for IgG binding which appear to be conserved across geographical sources of pooled IgG, and between different strains of *S. pneumoniae*. The number of unidentified bands on Western blots indicates that antigen targets for IgG binding likely also include other proteins that were not assessed here. Different individual sera also had IgG responses to a similar range of protein antigens but with some variation, with certain individuals having low or absent responses to protein antigens that were otherwise dominant in pooled sera/IVIG.

These data suggest that *S. pneumoniae* proteins may be targets for IgG binding. However, thus far the functional relevance of IgG to *S. pneumoniae* protein antigens remains unclear. Data presented in this chapter demonstrate IgG binding to purified *S. pneumoniae* proteins and lysates, or to *S. pneumoniae* by whole cell ELSIA. It has recently been demonstrated that whole cell ELISAs do not

necessarily provide an accurate assessment of antibody binding to the surface of *S. pneumoniae* (Cohen et al., 2013), and therefore are not necessarily a good measure of functional antibody responses.

# 4   Results (2): Functional targets of naturally-acquired IgG

In the previous chapter protein antigens were shown to be an important target for naturally-acquired IgG that recognises *S. pneumoniae*. However, the functional importance of anti-protein IgG is not known. IgG binding to the surface of *S. pneumoniae* is likely to be functional, promoting protective mechanisms including bacterial agglutination, opsonisation and fixation of complement. In this chapter the contribution of protein antigen targets for naturally-acquired IgG dependent immunity was assessed, using a range of *in vitro* functional assays.

### 4.1.1 IgG binding to *S. pneumoniae* and *S. pneumoniae*Δ*cps*

To assess the relative contribution of the *S. pneumoniae* polysaccharide capsule or sub-capsular antigens as targets for naturally-acquired human IgG, flow-cytometry based surface binding assays to whole bacteria were performed. Binding to TIGR4 and an unencapsulated derivative of TIGR4 (TIGR4Δ*cps*) following incubation in 1% or 10% IVIG was assessed (**Figure 4.1**). Significant IgG bound to both strains with increased IgG binding to the surface of unencapsulated bacteria. IgG surface binding assays were repeated in 1% IVIG with the *S. pneumoniae* D39, ST3 and ST23F strains (**Figure 4.1**), and their unencapsulated (Δ*cps*) derivatives. Again there was increased binding of IgG to the surface of unencapsulated ST3 and ST23F *S. pneumoniae* but not the D39 strain. These results demonstrate that the pneumococcal capsular polysaccharide is not a very important antigen target for naturally-acquired IgG binding to the surface of these strains. The presence of a capsule significantly decreased IgG binding for ¾ strains tested, suggesting sub-capsular antigens (proteins or CWPS) are the targets of naturally-acquired IgG, and the presence of a capsule can inhibit IgG binding to these targets.
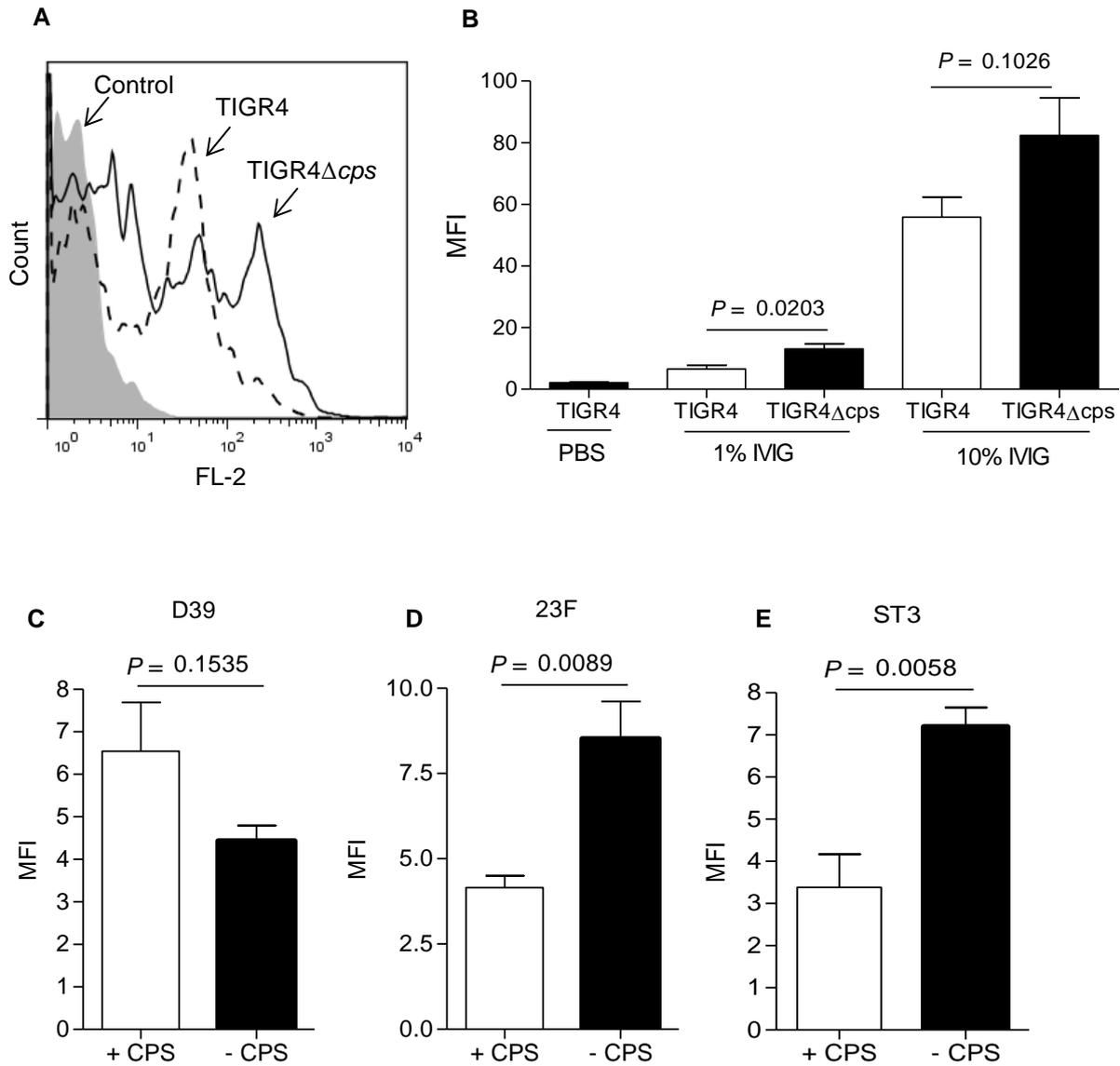
**Figure 4.1: Binding of IgG to the surface of *S. pneumoniae*.**

(A) Histogram of anti-human IgG-PE binding to *S. pneumoniae* TIGR4 and TIGR4Δ*cps* following incubation in IVIG (Intratect) or PBS. (B) MFI of anti-human IgG-PE binding to *S. pneumoniae* TIGR4 and TIGR4Δ*cps* following incubation in 1% or 10% IVIG. (C-E) MFI of anti-human IgG-PE binding to *S. pneumoniae* D39, ST23F, ST3 and their unencapsulated derivatives following incubation in 1% IVIG. (*P* values represent Student's unpaired T-test, error bars represent SD). Experiments presented in figures C-E were performed by Michal Barabas under my supervision, as part of a BSc project. Results are representative of two independent experiments.

### 4.1.2    IVIG binding to *Streptococcus mitis*

To investigate the general effect the pneumococcal capsule had on binding of IgG to the bacterial surface a strain of *S. mitis* that was transformed to express a capsule from *S. pneumoniae* TIGR4 was utilised (*S.mitis*T4*cps*). Binding of IVIG to the surface of *S.mitis*T4*cps* was compared with binding to an unencapsulated strain of *S. mitis*. Instead of providing a target for IgG deposition, the binding of IgG to the surface of *S.mitis*T4*cps* was reduced following incubation in 1% IVIG, relative to unencapsulated *S. mitis* (**Figure 4.2**). These results further indicate that the *S. pneumoniae* capsule is not a major target for IgG binding to *S. pneumoniae* in IVIG, and encapsulation may instead prevent binding of naturally-acquired IgG to sub-capsular antigens. WT *S. mitis* like *S. pneumoniae* also produces a capsule-like structure(Rukke et al., 2012). WT encapsulated *S. mitis* also bound less IgG than its unencapsulated derivative following incubation in IVIG (**Figure 4.2**). This further suggests that encapsulation in general may mask targets for IgG deposition on the surface of bacteria.
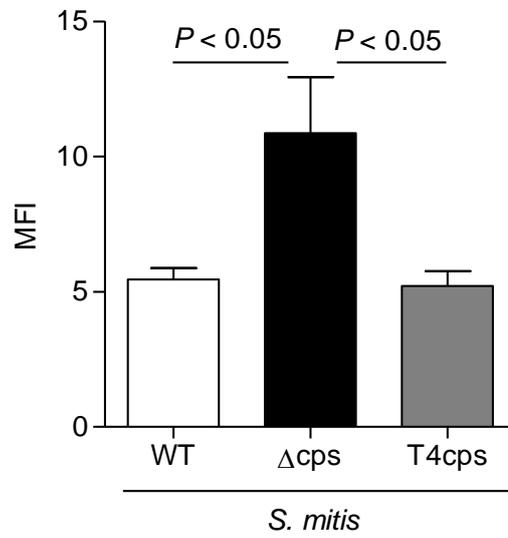
**Figure 4.2: IgG binding to *S. mitis* expressing a TIGR4 capsule**

MFI of anti-human IgG-PE binding to *S. mitis*, *S. mitis*Δ*cps* and *S.mitis*T4*cps*, following incubation in 1% IVIG (Intratect). (*P* value represents one way ANOVA and Tukey's post-test to compare columns, error bars represent SD). This experiment was performed by Helina Marshall under my supervision, as part of a pre-PhD project.  Results are representative of two independent experiments.

### 4.1.3 Specific antibody absorbtion (depletion) of IVIG

Assays of IgG binding to the surface of unencapsulated mutants of *S. pneumoniae* indicate that naturally-acquired IgG in IVIG can target non-capsular antigens. However these assays do not allow the relative contribution of IgG targeting the capsular polysaccharide or sub-capsular antigens to be assessed for encapsulated WT *S. pneumoniae*.  To assess the relative importance of IgG to the capsular polysaccharide for IgG binding to WT *S. pneumoniae* TIGR4, IVIG was depleted of antibodies to the type-4 capsular polysaccharide. This was achieved by absorption of IVIG with *S. mitis* expressing a type-4 capsule. IVIG absorbed against *S. mitis*T4*cps* displayed a 6-fold reduction in IgG binding titre to purified type-4 polysaccharide (**Figure 4.3**), with no effect on antibody binding to *S. pneumoniae* proteins when as assessed by Western blot or quantitatively by ELISA to purified PsaA or PhtE (**Figure 4.3**).
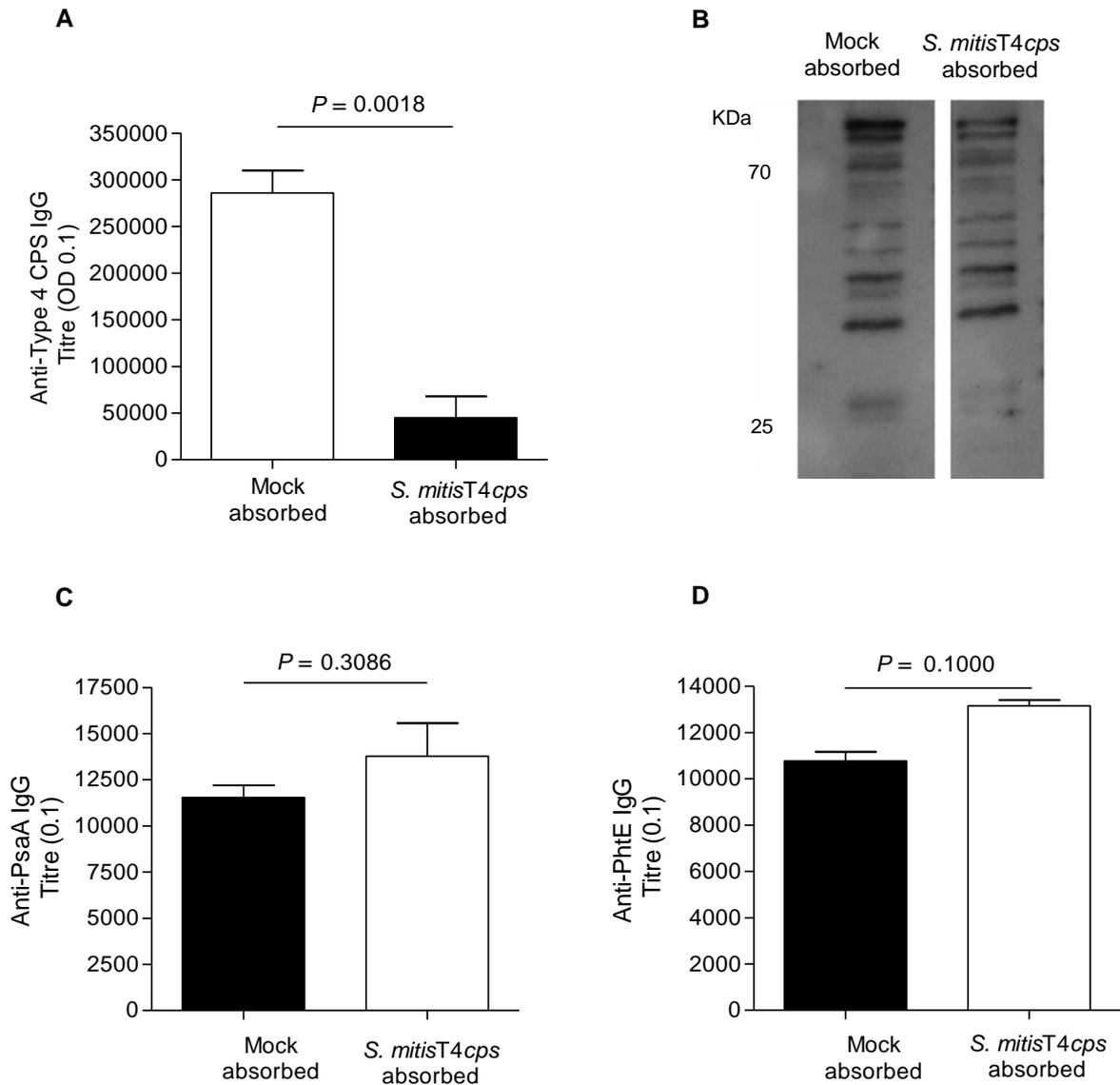
**Figure 4.3: Absorbtion of anti-capsular IgG from IVIG.**

(A) ELISA titre of anti-serotype 4 IgG in IVIG pre-absorbed by incubation with *S. mitis* expressing a TIGR4 capsule or mock absorbed. (B) Western blot of *S. pneumoniae* TIGR4 lysate probed with absorbed or mock absorbed IVIG, developed with anti-human IgG. (C) ELISA titre of IgG binding to purified PsaA in absorbed and mock-absorbed IVIG. (D) ELISA titre of IgG binding to purified PhtE in absorbed and mock-absorbed IVIG. (*P* values represent Student's unpaired T-test, error bars represent SD).

The effect of specific depletion of anti-capsular IgG on the ability of IVIG to bind to the bacterial surface was assessed by flow-cytometry. Specific depletion of anti-serotype 4 IgG from IVIG had no significant effect on IgG binding to the surface of *S. pneumoniae* TIGR4 following incubation in both 1% and 10% IVIG (**Figure 4.4**). These data therefore indicate that naturally-acquired IgG in IVIG predominantly targets non-capsular antigens on the surface of intact *S. pneumoniae*.

**A**
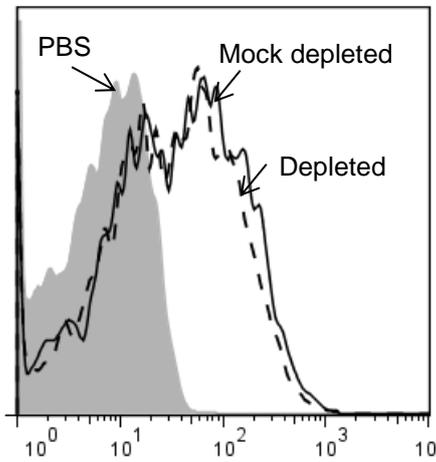


**B**
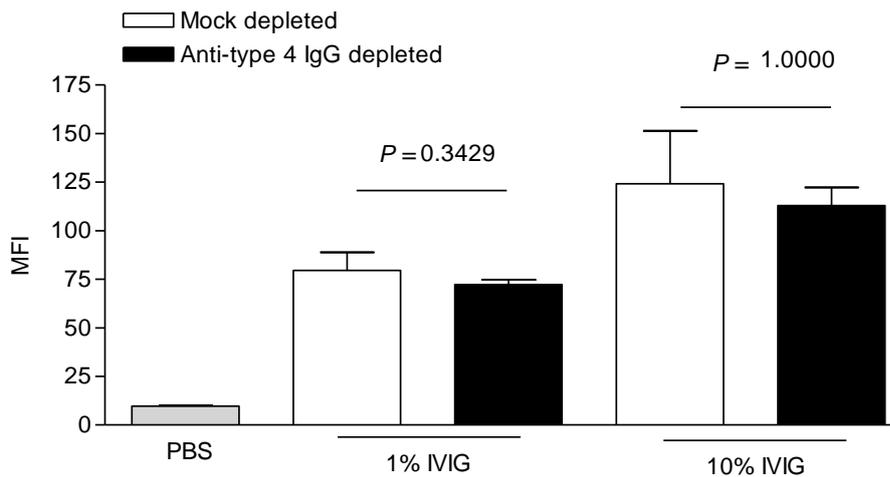


**Figure 4.4: Surface binding of absorbed IVIG.**

(A) Histogram of anti-human IgG-PE binding to *S. pneumoniae* TIGR4 following incubation in 1% IVIG depleted of anti-CPS IgG by in absorbtion against *S. mitis+TIGR4*, or mock depleted IVIG. (B) MFI of anti-human IgG-PE binding to *S. pneumoniae* TIGR4 following incubation in 1% and 10% depleted or mock depleted IVIG. (*P* values represent Student's unpaired T-test, error bars represent SD).

### 4.1.4 Protease shaving of *S. pneumoniae*

Depletion of anti-capsular IgG from IVIG, and IVIG binding to unencapsulated mutants, demonstrated a role for IgG to non-capsular targets in determining binding of naturally-acquired IgG to the surface of *S. pneumoniae*. To assess if these non-capsular targets could be proteins, *S. pneumoniae* was incubated with a protease (pronase) to digest surface-exposed proteins. Pronase treatment of *S. pneumoniae* has previously been used to confirm the surface localisation of pneumococcal proteins (Hammerschmidt et al., 1997). To ensure pronase treated *S. pneumoniae* didn't re-express surface proteins upon division a strain of *S. pneumoniae* TIGR4 lacking PABA synthetase (TIGR4Δ*pab*) was used, which is unable to replicate in sera (Chimalapati et al., 2011). There was no reduction in bacterial CFU following incubation of TIGR4Δ*pab* with pronase, compared to PBS alone. The effect of pronase incubation on the binding of IVIG to the surface of *S. pneumoniae* was assessed by flow-cytometry. Pronase treatment of *S. pneumoniae* reduced the ability of IVIG to bind to the surface of *S. pneumoniae*Δ*pab* (**Figure 4.5**). To confirm that pronase treatment didn't affect the binding of IgG to the *S. pneumoniae* capsule the binding of anti-serotype 4 PPS antisera to pronase treated and untreated *S. pneumoniae*Δ*pab* was assessed. Pronase treatment had no effect on IgG binding to the surface of *S. pneumoniae* Δ*pab* following incubation in 10% type-4 CPS antisera (**Figure 4.5**). Incubation of WT *S. pneumoniae* and *S. pneumoniae*Δ*pab* in type-4 antisera also confirmed that the Δ*pab* mutation did not affect IgG binding to the *S. pneumoniae* TIGR4 capsule (**Figure 4.5**). These data indicate that some of the *S. pneumoniae* targets of IVIG binding to encapsulated *S. pneumoniae*, and therefore targets of naturally-acquired IgG are pneumococcal surface proteins, sensitive to digestion by pronase.
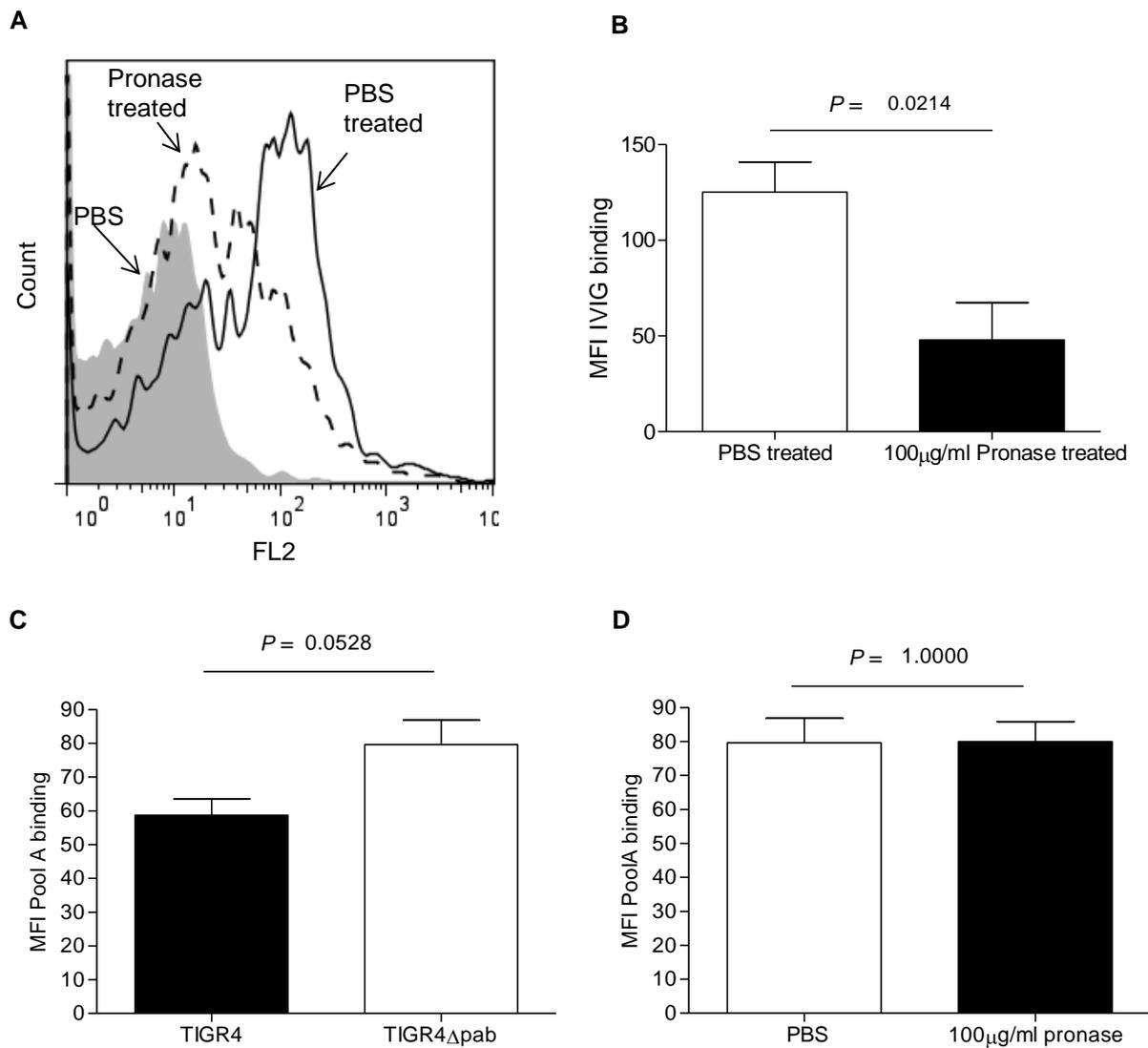
**Figure 4.5: IgG binding to pronase-treated *S. pneumoniae*.**

(A) Histogram of anti-human IgG-PE binding to *S. pneumoniae* TIGR4Δ*pab* pre-treated with 100μg/ml pronase or PBS, incubated in 1% IVIG (Intratect).(B) MFI of anti-human IgG-PE binding to *S. pneumoniae* TIGR4Δ*pab* pre-treated with 100μg/ml pronase or PBS, incubated in 1% IVIG. (C) MFI of anti-rabbit IgG-PE binding to *S. pneumoniae* TIGR4 and TIGR4Δ*pab* following incubation in 10% anti-serotype 4 polyclonal rabbit sera. (D) MFI of anti-rabbit IgG-PE binding to *S. pneumoniae* TIGR4Δ*pab* pre-treated with 100μg/ml pronase or PBS, incubated in 10% anti-serotype 4 polyclonal rabbit sera. (*P* values represent Student's unpaired T-test, error bars represent SD). Results are representative of two independent experiments.

### 4.1.5 Effect of IgG on *in vitro* growth of *S. pneumoniae*

Surface binding assays utilising unencapsulated strains of *S. pneumoniae* indicated a potentially important role for opsonisation with IgG to sub-capsular protein antigens in IVIG. To evaluate if naturally-acquired IgG to non-capsular targets could be functional the effect of IVIG on *in vitro* growth of WT and unencapsulated *S. pneumoniae was investigated*. The addition of 10% IVIG to the growth medium (THY) impaired *S. pneumoniae* TIGR4 growth (**Figure 4.6**) as assessed by a change in $OD_{580}$ over 8hrs. Repeating this assay with the TIGR4Δ*cps* strain indicated that the inhibitory effect of IVIG was greater when the polysaccharide capsule was removed (**Figure 4.6**). The enhanced effect of IVIG on growth inhibition of unencapsulated *S. pneumoniae* was also demonstrated when repeating assays with unencapsulated derivatives of *S. pneumoniae* D39, ST3 and ST23F (**Figure 4.6**). This indicates that naturally-acquired IgG to non-capsular targets is capable of mediating the observed reduction in $OD_{580}$ following the addition of IVIG. Light microscopy of 8 hour cultures demonstrated that the addition of 10% IVIG to the growth medium facilitated bacterial agglutination (**Figure 4.7**). This phenotype was maintained for unencapsulated *S. pneumoniae* TIGR4 following growth in IVIG, indicating that antibodies to the capsular polysaccharide are not necessarily required for bacterial agglutination (**Figure 4.7**).
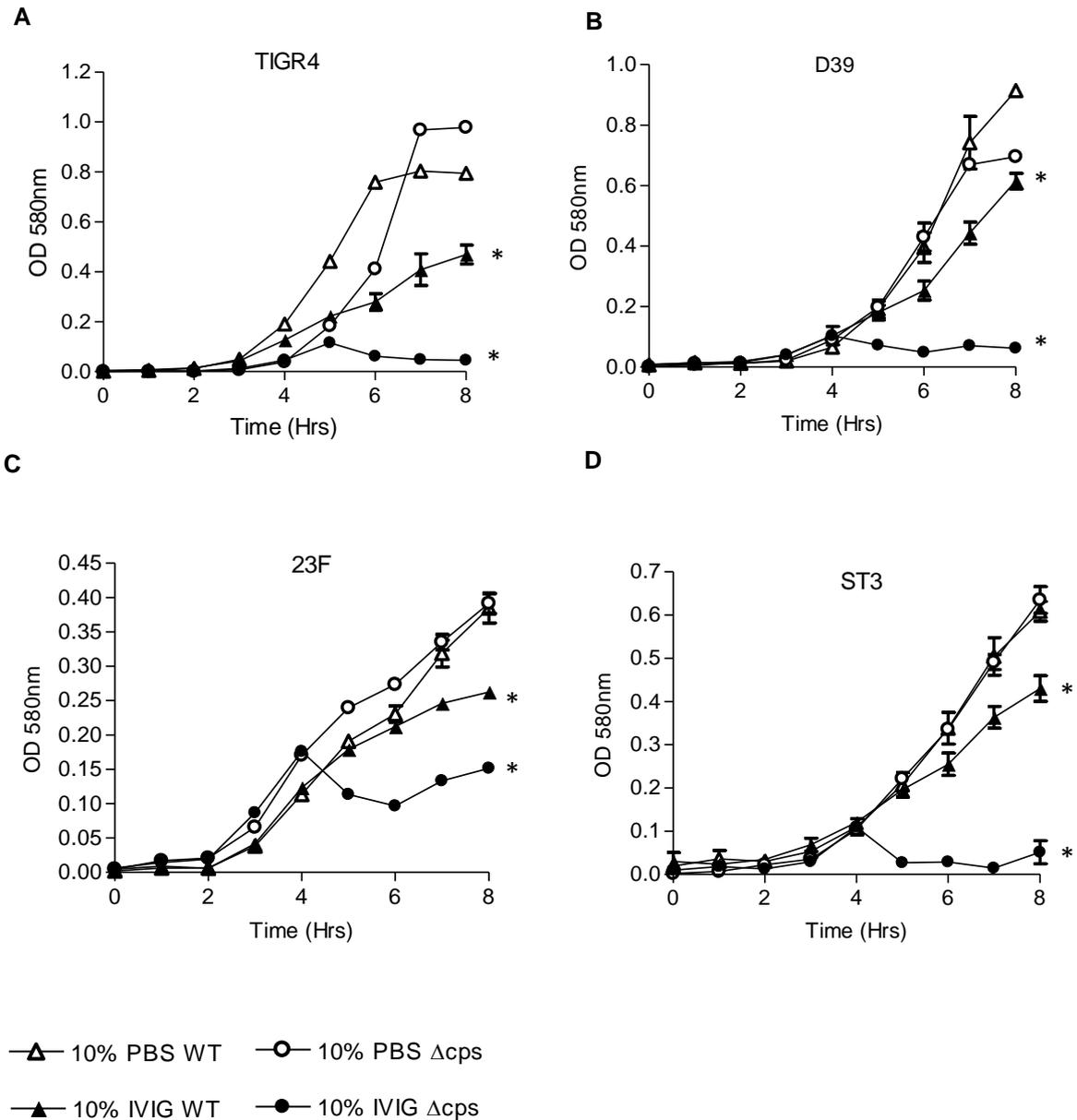
**Figure 4.6: Effect of IVIG on *S. pneumoniae* growth.**

(A-D) 8 hour growth (OD$_{580nm}$) of WT *S. pneumoniae* strains TIGR4, D39, ST23F, ST3 and their unencapsulated (Δ*cps*) derivatives in THY supplemented with 10% IVIG (Intratect) or PBS. (Error bars represent SD). Experiments presented in figures B-D were performed by Michal Barabas under my supervision, as part of a BSc project. (Curves were compared by two-way ANOVA*= P<0.001 compared to PBS controls). Results are representative of two independent experiments.
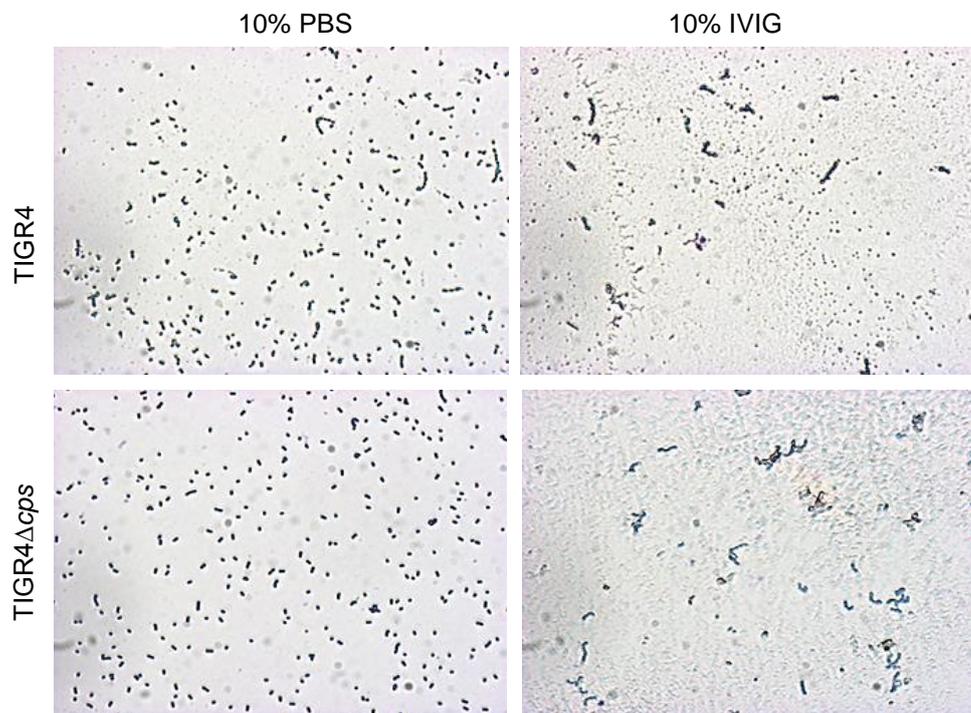
**Figure 4.7: Microscopy of _S. pneumoniae_ grown in the presence of IVIG.**

Light microscopy of _S. pneumoniae_ TIGR4 and TIGR4Δ*cps* after 8hrs growth in THY supplemented with 10% IVIG (Intratect) or PBS. 10µl of culture was air-dried on to glass slides and stained by rapid-Romanowsky staining (Diff-quick). Results are representative of two independent experiments.

### 4.1.6    Effect of IgG on *S. pneumoniae* agglutination

IgG recognition of *S. pneumoniae* may inhibit growth when measured by a change in OD via inhibition of surface protein function (e.g. cation uptake) or by promoting bacterial agglutination. Light microscopy of 8 hour cultures demonstrated that the addition of 10% IVIG to the growth medium did facilitate bacterial agglutination (**Figure 4.7**). This phenotype was maintained for unencapsulated *S. pneumoniae* TIGR4 following growth in IVIG, indicating that antibodies to the capsular polysaccharide are not necessarily required for this effect (**Figure 4.7**).To assess the targets of naturally-acquired IgG that may mediate agglutination independently of growth, a flow-cytometry based approach was used. Forward scatter (FSC) of light indicates the relative size of particles by flow-cytometry. An increase in particle size by FSC was therefore used as a measure of increased bacterial agglutination (**Figure 4.8**). The addition of increasing concentrations of IVIG to a suspension of either $1x10^6$ CFU/ml FAM-SE labelled *S. pneumoniae* TIGR4 or TIGR4$\Delta cps$ led to an increase in particle size for both strains, indicative of agglutination (**Figure 4.8**). This further suggests the target antigen of naturally-acquired IgG mediating agglutination is not necessarily the polysaccharide capsule and sub-capsular antigens can be functional targets.
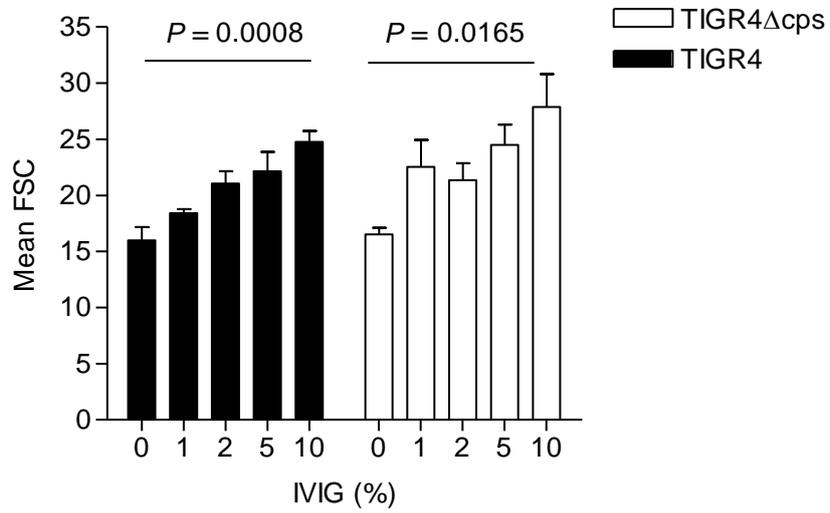
**Figure 4.8: IVIG mediated agglutination of *S. pneumoniae*.**

Mean forward scatter (flow-cytometry) of suspensions of *S. pneumoniae* TIGR4 and TIGR4Δ*cps* pre-incubated in increasing concentrations of IVIG (Intratect). (*P* values represent one-way ANOVA, error bars represent SD). Results are representative of two independent experiments.

### 4.1.7 IgG mediated phagocytosis and killing of *S. pneumoniae*

Enhancing opsonophagocytosis is a primary mechanism by which IgG may be protective against *S. pneumoniae*. Therefore the functional targets of naturally-acquired IgG *in vitro* that could facilitate opsonophagocytosis of *S. pneumoniae* by macrophages and neutrophils were assessed. This was achieved by measuring the association of fluorescently labelled *S. pneumoniae* TIGR4 and TIGR4Δ*cps* with phagocytes by flow-cytometry. Prior opsonisation of *S. pneumoniae* in 10% IVIG facilitated increased association of FAM-SE labelled *S. pneumoniae* with a murine macrophage cell line (RAW 264.7) when co-incubated at an MOI of 10 (**Figure 4.9**). This effect compared to unopsonised bacteria was stronger against unencapsulated *S. pneumoniae* (**Figure 4.9**), indicating that naturally-acquired IgG to non-capsular targets may be functional mediating phagocytosis of *S. pneumoniae*. Prior opsonisation in IVIG also increased the association of FAM-SE labelled *S. pneumoniae* TIGR4 with human neutrophils (MOI 20), in a dose-dependent manner (**Figure 4.9**). Again the effect of increasing concentrations of IVIG was greater for the TIGR4Δ*cps* strain, indicating IgG to sub-capsular targets is functional and mediates enhanced phagocytosis by both a RAW macrophage cell line and isolated human neutrophils. The lower association of encapsulated *S. pneumoniae* with phagocytes indicates that the polysaccharide capsule of *S. pneumoniae* may partially abrogate the opsonic effects of IgG binding to non-capsular targets following incubation in IVIG.

The effect of opsonisation in IVIG on neutrophil killing of *S. pneumoniae* was also assessed. Isolated human neutrophils were incubated with *S. pneumoniae* (MOI 1/250) and bacterial survival assessed by counting CFU. Prior opsonisation in IVIG enhanced the killing of both *S. pneumoniae* TIGR4 and TIGR4Δ*cps* by human neutrophils *in vitro*, relative to unopsonised controls (**Figure 4.9**). This further supports the hypothesis that non-capsular antigens can be functional targets of naturally-acquired IgG in IVIG.
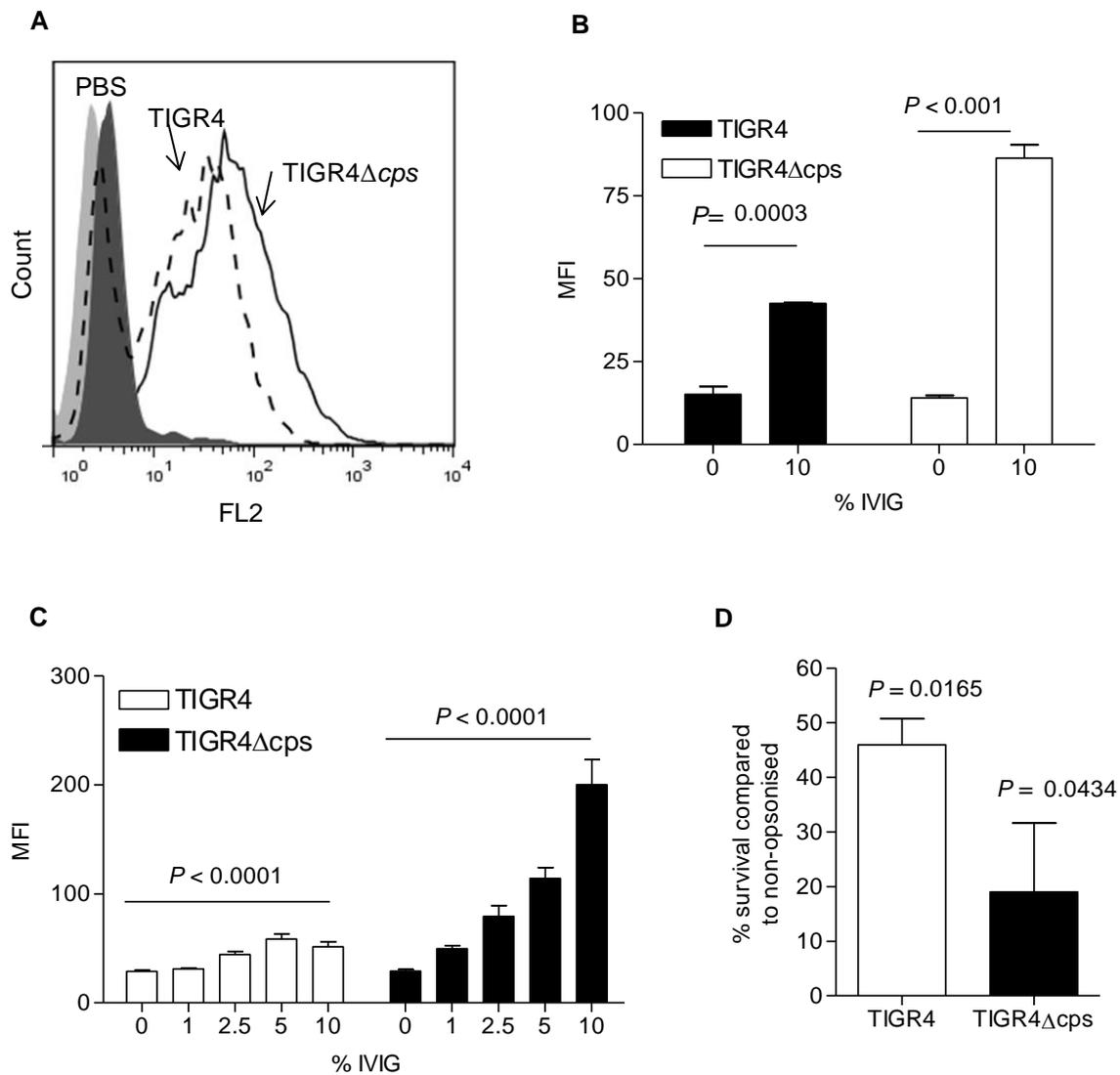
**Figure 4.9: Effect of IVIG on *S. pneumoniae* phagocytosis and killing.**

(A) Histograms showing fluorescence intensity (FL-1) of RAW macrophages, detected by flow-cytometry, following incubation with FAM-SE labelled S. *pneumoniae* TIGR4 and TIGR4Δ*cps*, opsonised in 10% IVIG or PBS. (B) MFI (FL-1) of RAW macrophages incubated with FAM-SE *S. pneumoniae* TIGR4 and TIGR4Δ*cps* opsonised in 10% IVIG or PBS. (C) MFI (FL-1) of human neutrophils incubated with FAM-SE labelled *S. pneumoniae* TIGR4 and TIGR4Δ*cps* opsonised with increasing concentrations of IVIG. (D) Percentage survival of *S. pneumoniae* TIGR4 and TIGR4Δ*cps* opsonised in 10% IVIG, incubated with neutrophils (MOI 1/250), compared to controls opsonised in PBS. (*P* values represent Student's unpaired T-test (B,D) or one-way ANOVA (C), error bars represent SD). All results are representative of two independent experiments.

### 4.1.8 Chapter summary

As previously discussed the pneumococcal targets in IVIG pooled from >1000 donors are likely to reflect the targets of naturally-acquired IgG within a population. IgG binding to unencapsulated *S. pneumoniae* indicated that naturally-acquired IgG in IVIG can opsonise *S. pneumoniae* by binding to non-capsular antigens on the surface of intact bacteria. Specific depletion of anti-capsular IgG in IVIG suggested that IgG to the *S. pneumoniae* capsular polysaccharide may be redundant for IgG binding to the surface of wild-type encapsulated *S. pneumoniae* TIGR4. Furthermore protease treatment of *S. pneumoniae* reduced IgG binding, indicating that surface proteins may be major targets of naturally-acquired IgG binding to *S. pneumoniae*.

Assays of growth and agglutination using unencapsulated mutants of *S. pneumoniae* demonstrated that IgG recognition of protein antigens has a functional effect. Additionally, assays using TIGR4Δ*cps* indicated that antibodies to these targets may be functional, enhancing both macrophage and neutrophil phagocytosis of *S. pneumoniae* and killing by neutrophils. The effect of opsonisation in IVIG was greater for unencapsulated strains of *S. pneumoniae*, both in terms of increased deposition of IgG on the bacterial surface, growth inhibition and enhanced phagocytosis and killing. This suggests that the bacterial effects of naturally-acquired IgG to *S. pneumoniae* are not necessarily mediated by anti-capsular antibody, but are mediated by anti-protein antibody, and that the *S. pneumoniae* capsule protects the bacteria from opsonisation by naturally-acquired IgG to non-capsular antigens.

# 5  Results (3): *In vivo* effects of naturally-acquired IgG

*In vitro* assays indicated that naturally-acquired IgG to *S. pneumoniae* may be protective facilitating agglutination of *S. pneumoniae* and enhancing phagocytosis.  To determine if naturally-acquired IgG could be protective against lung infection *in vivo* a mouse model of experimental *S. pneumoniae* challenge following IVIG treatment was used.  The effect of IVIG treatment on IN lung infection, experimental colonisation and IV challenge with *S. pneumoniae* TIGR4 was assessed. The *S. pneumoniae* TIGR4 strain was used for these experiments as it causes invasive, but not rapidly overwhelming infection in mice (Cohen et al., 2013), thereby allowing protection in different tissue compartments to be assessed. Cellular depletion strategies *in vivo* were used to assess the mechanisms by which naturally-acquired IgG mediated protection.

### 5.1.1 IgG distribution following IVIG treatment

CD1 mice were passively vaccinated with human IgG (IVIG) via intraperitoneal inoculation 3hrs prior to challenge. To assess the distribution of IgG at different time points post-challenge the concentration of human IgG in sera, BALF and nasal washings was quantified by ELISA (**Figure 5.1**). 3hrs following treatment human IgG was readily detectable in the sera of IVIG treated mice, but not the BALF or nasal washings (**Figure 5.1 A,B**). Following experimental colonisation of the nasopharynx with TIGR4 *S. pneumoniae* human IgG remained absent from nasal washes, 5 days post-inoculation (**Figure 5.1 B**). IP injection of IVIG followed by experimental lung infection 3hrs later resulted in detecdable levels of human IgG in BALF (**Figure 5.1 C**). There was a much greater accumulation of human IgG in the BALF 24hrs following infection with $1\times10^7$ CFU *S. pneumoniae* TIGR4 compared to 2.5hrs after inoculation of $5\times10^5$ CFU (**Figure 5.1**). These data indicate that in this model human IgG was only detectable in the alveolar space post-infection following IVIG treatment. Bronchoalveolar lavages were performed 24hrs following IVIG adminisaration to assess if a longer time period post-treatment would allow human IgG accumulation in BALF. 24hrs following treament human IgG remained absent from the BALF of IVIG treated mice without infection.

**Figure 5.1: Distribution of human IgG *in vivo* following IVIG treatment.**

(A) Concentration of human IgG measured by ELISA, in sera of IVIG treated mice pre-challenge. (B) Human IgG concentration in nasal washings of IVIG treated mice pre and 5 days post IN colonisation with $1\times10^7$ CFU *S. pneumoniae* TIGR4. (C) Human IgG concentration in BALF 0, 2.5 (low dose) and 24hrs (high dose) post IN challenge with *S. pneumoniae* TIGR4. (D) Human IgG concentration in BALF 24hrs following IVIG treatment without challenge (Lines represent mean). Results are from one experiment at each time-point, except 24hrs which was performed twice.

To evaluate whether the absence of human IgG from the alveolar space in uninfected mice could be due to a species difference affecting the transit of human IgG across the murine alveolar epithelium, hyperimmune anti-*S. pneumoniae* mouse serum was passively transferred into mice by IP inoculation. A hyperimmune serum was obtained from mice 28 days after vaccination with the replication deficient TIGR4Δ*pab* strain. 24hrs following passive vaccination with murine hyperimmune sera there was an increase in S*. pneumoniae* specific IgG compared to non-vaccinated controls was detected in BALF (**Figure 5.2**), suggesting that murine IgG can enter the alveolar space in the absence of inflammation. From these samples the relative concentration of *S. pneumoniae* specific IgG in the sera compared to BALF of passively vaccinated mice could be calculated. Using this ratio the concentration of human IgG that would be expected in BALF of mice 3hrs following IVIG treatment was estimated, based on the concentration in the sera (**Figure 5.2**).The BALF concentration of human IgG 24hrs following challenge in IVIG treated mice was significantly greater than the estimated concentration, based on the concentration of IgG in the sera at this time point post infection (**Figure 5.2**). This suggests that challenge with *S. pneumoniae* TIGR4 and associated inflammation may facilitate human IgG leak into the alveolar space.  To assess the potential contribution of alveolar permeability to IgG accumulation in the BALF following challenge with *S. pneumoniae*, albumin concentration in BALF from infected mice was measured as marker of epithelial permeability. Human IgG concentration in lavage samples of individual mice following challenge closely correlated with the albumin concentration in the same samples (**Figure 5.2**). These data suggest that in this murine model human IgG accumulates in the alveolar space following *S. pneumoniae* challenge In line with increased alveolar permeability.
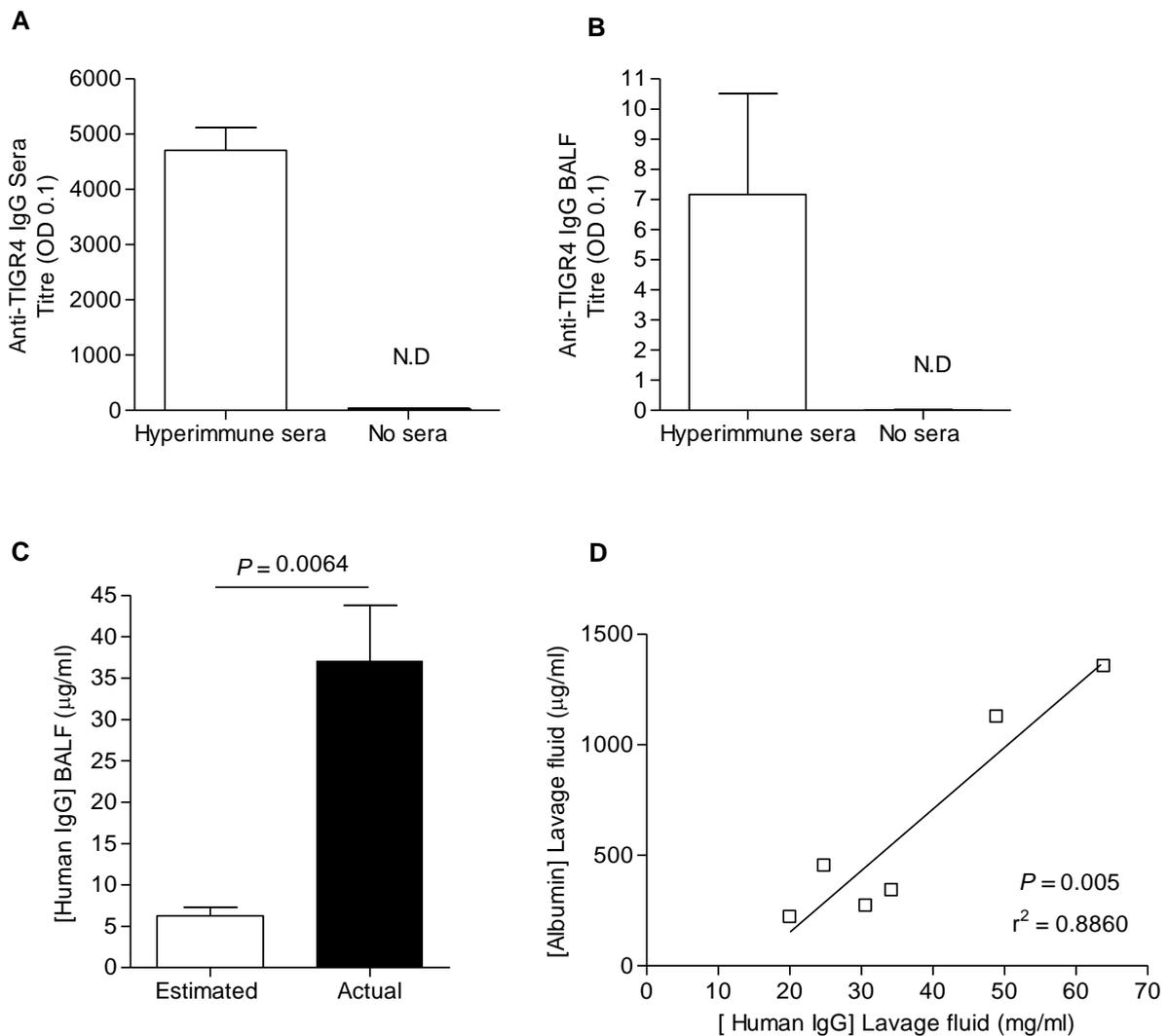
**Figure 5.2: Distribution of IgG following passive transfer of murine immune sera.**

(A) Whole cell ELISA titre of anti-TIGR4 IgG in sera of mice 3hrs following IP administration of hyperimmune anti-pneumococcal sera. (B) ELISA titre of anti-TIGR4 IgG in BALF of mice 3hrs following IP administration of hyperimmune anti-pneumococcal sera. (C) Estimated concentration of human IgG in BALF based on the concentration of IgG in the sera and actual IgG concentration detected in BALF 24hrs post-challenge with *S. pneumoniae* TIGR4. (D) Linear regression of human IgG concentration in BALF and albumin concentration in BALF, measured by ELISA 24hrs following IN infection with *S. pneumoniae* TIGR4. (*P* value represents F-test assessing difference of slope compared to zero, error bars represent standard error). Results are from one experiment.

### 5.1.2 Protective effect of IVIG

To assess at what level passively transferred human IgG (IVIG) may protect against *S. pneumoniae* infection, mice were challenged with different models of infection 3hrs post- IVIG treatment. Passive transfer of human IgG failed to protect against experimental colonisation with *S. pneumoniae* TIGR4; CFU in nasal washes were not reduced 5 days post-experimental colonisation in IVIG treated mice compared to controls (**Figure 5.3**). Similarly, IVIG treatment failed to reduce CFU in the lungs or BALF at an early time point (2.5hrs) following challenge with $5x10^5$ CFU *S. pneumoniae* TIGR4 (**Figure 5.3**). This lack of protection in the nasopharynx and early in the lungs perhaps reflects the low distribution of human IgG at these body compartments and time points post-infection. At 24hrs following IN infection with $1x10^7$ CFU prior IVIG treatment improved clearance of *S. pneumoniae* from the lung, and was powerfully protective against septicaemia compared to PBS treated controls (**Figure 5.3**). IVIG treatment had no effect on bacterial numbers in the BALF of mice 24hrs following challenge (**Figure 5.3**). The ability of human IgG to clear *S. pneumoniae* from the blood following challenge was assessed by IV challenge with *S. pneumoniae* TIGR4. 4hrs following IV challenge with $5x10^6$ CFU *S. pneumoniae,* 80% of mice treated with IVIG had cleared *S. pneumoniae* from the blood, whereas *S. pneumoniae* bacteraemia was detectable in all PBS treated controls (**Figure 5.3**).These data indicate that naturally-acquired human IgG can protect mice from lung infection with *S. pneumoniae*, reducing bacterial numbers in the lung and also strongly inhibits the development of bacteraemia.
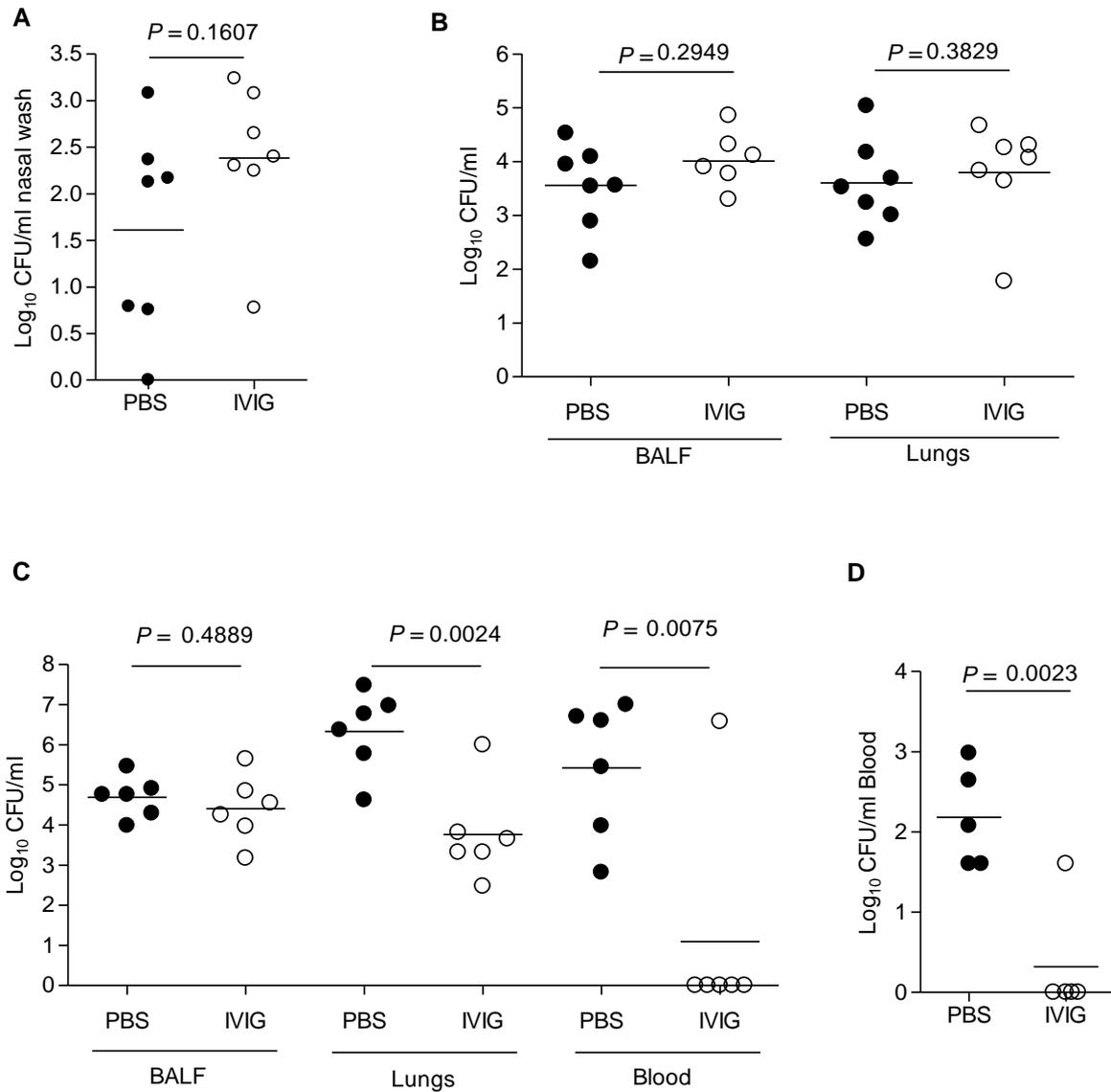
**Figure 5.3: Bacterial CFU in *S. pneumoniae* infected mice, following IVIG treatment.**

(A) CFU in nasal washes 5-days post IN colonisation with $1 \times 10^7$ CFU *S. pneumoniae* TIGR4 in mice treated with IVIG or PBS. (B) CFU in lungs and BALF 2.5hrs post IN challenge with $5 \times 10^5$ CFU *S. pneumoniae* TIGR4. (C) CFU in lungs, blood and BALF of PBS and IVIG treated mice 24hrs post IN challenge with $1 \times 10^7$ CFU *S. pneumoniae* TIGR4. (D) CFU in blood 4hrs post IV challenge with $5 \times 10^6$ CFU *S. pneumoniae* TIGR4 in IVIG or PBS treated mice. (*P* values represent Student's unpaired T-test, lines represent mean). Results are from one experiment at except those presented in Figure C which were performed twice.

### 5.1.3 Inflammatory responses to infection in IVIG treated mice

There are a number of mechanisms by which IVIG may mediate *in vivo* protective effects against *S. pneumoniae* lung infection. IVIG has been demonstrated to have immunomodulatory capacities which may mediate protection from infection separately to improved bacterial opsonisation (Hagiwara et al., 2008). However, in this model of infection IVIG treatment didn't appear to significantly affect the inflammatory response to *S. pneumoniae* lung infection. Cell numbers in the BALF of IVIG treated mice were unchanged compared to PBS controls (**Figure 5.4**), as were levels of the pro-inflammatory cytokine TNF-α in lung tissue 24hrs post-infection with *S. pneumoniae* TIGR4 (**Figure 5.4**). These data therefore suggest that the protective effects of IVIG in this model were probably not due to modulation of the inflammatory response within the lung.
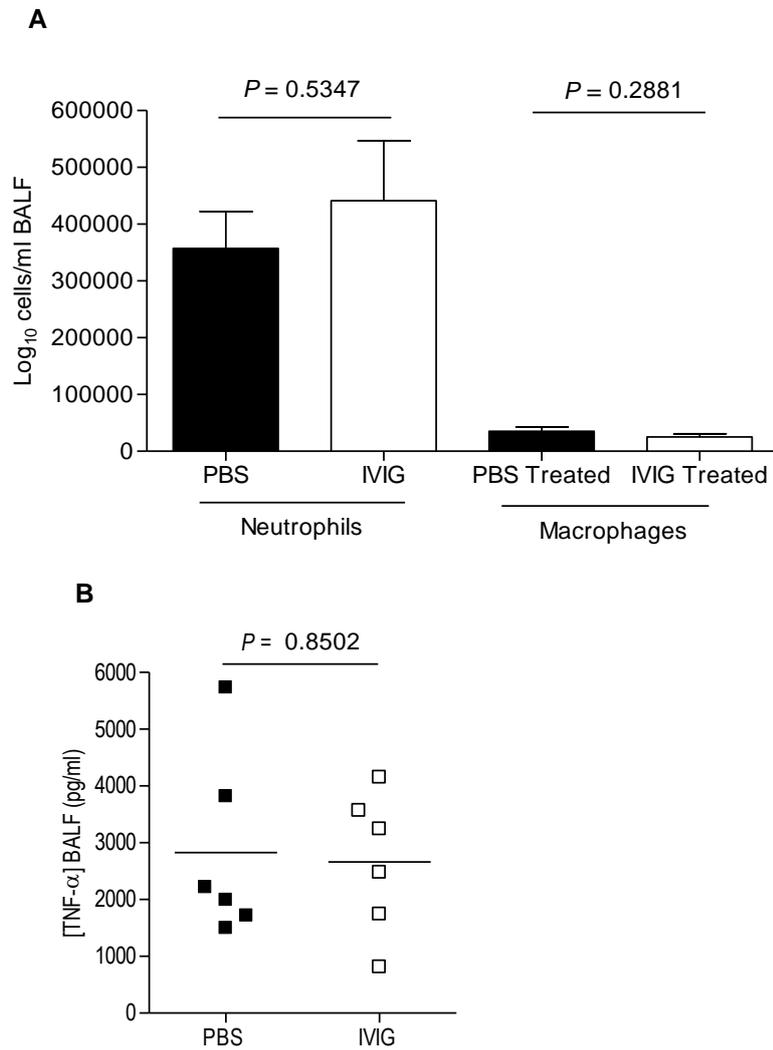
**Figure 5.4: Inflammatory responses to *S. pneumoniae* in IVIG treated mice.**

(A) Total cell counts in BALF 24hrs following IN challenge with $1\times10^7$ CFU *S. pneumoniae* TIGR4 in PBS and IVIG treated mice. (B) Concentration of TNF-α in BALF, measured by ELISA, 24hrs following challenge with $1\times10^7$ CFU *S. pneumoniae* TIGR4 in PBS and IVIG treated mice. (*P* values represent Student's unpaired T-test, error bars represent SD).

### 5.1.4   Role of neutrophils in IVIG mediated protection

Neutrophils were the main cell type in the alveolar space 24hrs following IN infection with *S. pneumoniae* TIGR4 (**Figure 5.4**). To determine if they were important for the protective effect of human IgG neutrophils were depleted prior to challenge in IVIG or PBS treated mice. Administration of a monoclonal antibody against the neutrophil surface-marker Ly-6G, 24hrs prior to challenge, led to a 20-fold reduction in neutrophil numbers in the BALF 24hrs post-infection (**Figure 5.5**). Neutrophil depletion abolished the protective effect of IVIG treatment on *S. pneumoniae* CFU within the lung (**Figure 5.5**), suggesting an important role for neutrophils in protection from lung infection mediated by naturally-acquired human IgG. Interestingly neutrophil depletion also protected control mice from the development of bacteraemia following *S. pneumoniae* TIGR4 challenge (**Figure 5.5**), suggesting that neutrophils may contribute to the invasion of *S. pneumoniae* into the blood which in the absence of human IgG is not cleared in this model.
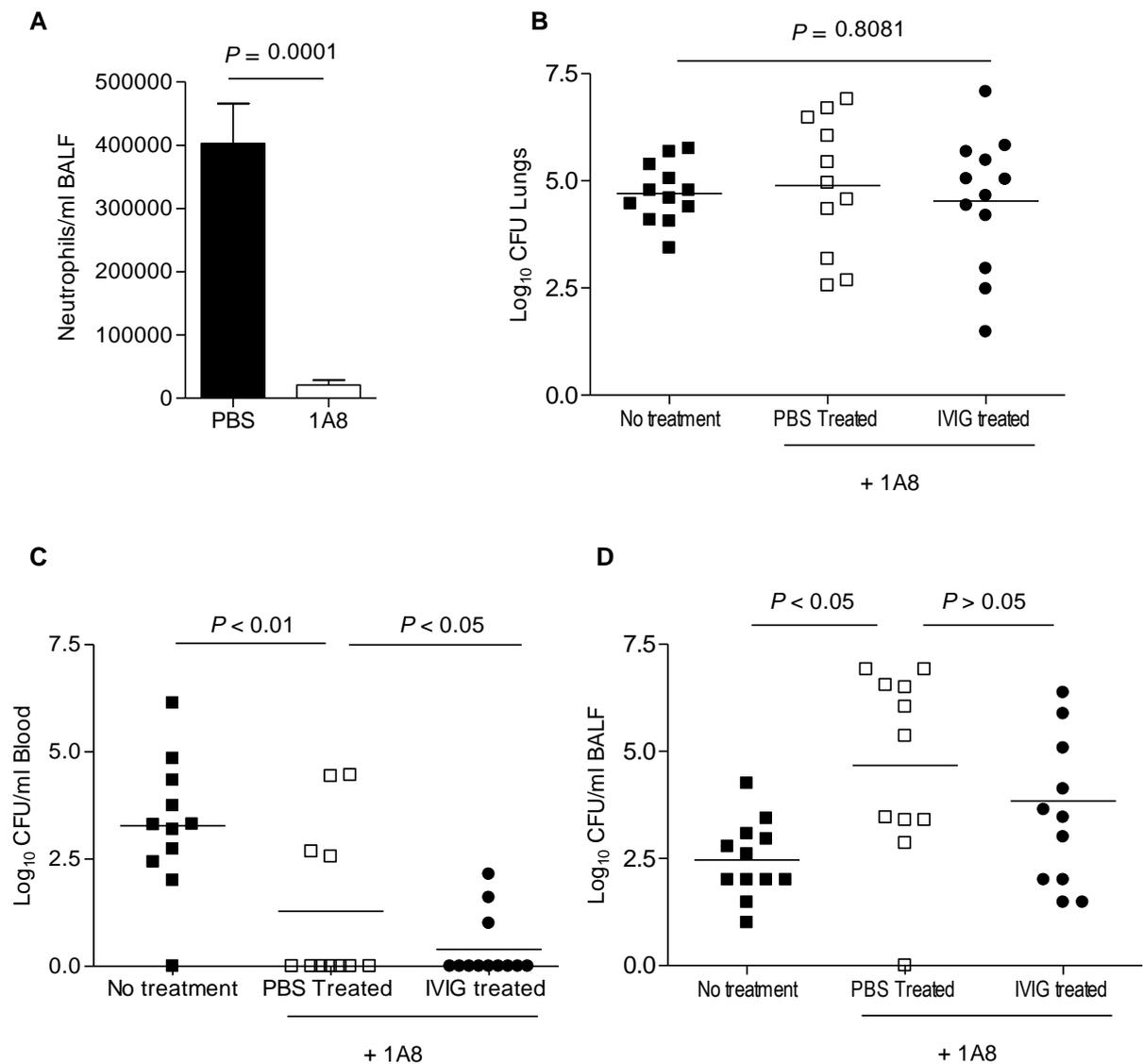
**Figure 5.5: Effect of IVIG *in vivo* following neutrophil depletion**.

(A) Neutrophil numbers in BALF of mice treated with 1A8 anti-Ly6G neutrophil-depleting antibody or PBS, 24hrs after challenge with 1x10$^7$ CFU *S. pneumoniae* TIGR4. (B) CFU in lungs of neutrophil depleted mice treated with PBS or IVIG, 24hrs after challenge with 1x10$^7$ CFU *S. pneumoniae* TIGR4, compared to untreated controls. (C) CFU in blood of neutrophil depleted mice treated with PBS or IVIG, 24hrs after challenge with 1x10$^7$ CFU *S. pneumoniae* TIGR4. (D) CFU in BALF of neutrophil depleted mice treated with PBS or IVIG, 24hrs after challenge with 1x10$^7$ CFU *S. pneumoniae* TIGR4. (*P* values represent Student's unpaired T-test (A) or one way ANOVAs with Tukey's post-test to compare columns (B-D)). Results are representative of two independent experiments.

### 5.1.5 Mechanisms of clearance from blood

Splenic macrophages play an important role in the clearance of bacteria from the blood (Kang et al., 2004, Aichele et al., 2003). Therefore the role of these cells in the enhanced clearance of *S. pneumoniae* TIGR4 from the blood observed in mice treated with IVIG was investigated. IV administration of liposomal clodronate was used to specifically deplete splenic macrophages (van Rooijen and van Nieuwmegen, 1984), reducing the number of F4/80+ve macrophages in the spleen of CD1 mice by 50%, as assessed by flow-cytometry (**Figure 5.6**). Depletion of splenic macrophages by IV administration of liposomal clodronate impaired the ability of IVIG treated mice to clear *S. pneumoniae* TIGR4 from the blood following IV challenge with $5x10^5$ CFU (**Figure 5.6**). IVIG treated mice that received liposomes containing PBS had no detectable bacteria in the blood; whereas 66% of IVIG treated mice that received liposomal clodronate had detectable bacteraemia. These data indicate that naturally-acquired IgG requires intact splenic macrophages for full clearance of *S. pneumoniae* from the blood.
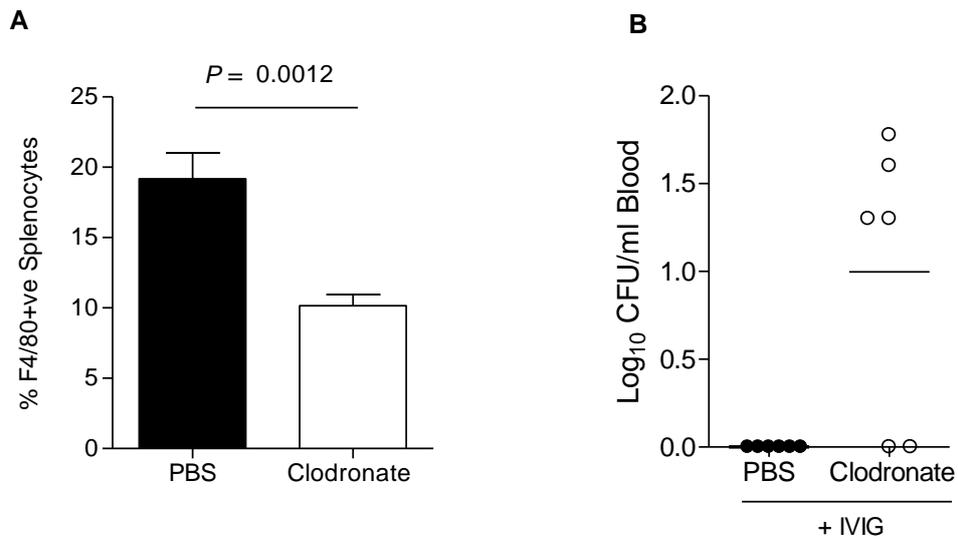
**Figure 5.6: Clearance of *S. pneumoniae* following clodronate depletion of macrophages.**

(A) Percentage of F4/80+ macrophages in the spleens of mice treated IV with liposomal clodronate or liposomal PBS. (B) CFU 4hrs following IV challenge with $5\times10^5$ *S. pneumoniae* TIGR4 in the blood of IVIG treated mice, treated with liposomal clodronate or liposomal PBS. (*P* values represent Student's unpaired T-test, error bars represent SD). Results are representative of two independent experiments.

### 5.1.6    Effect of specific antibody depletion on protection

Previous data (**Figure 4.3**) indicate that incubation of IVIG with *S. mitis* expressing a type-4 capsule (*S.mitis*T4*cps*) can deplete IVIG of anti-capsular antibody.  To assess if IVIG depleted of IgG specific for the serotype 4 capsular polysaccharide was still protective *in vivo*, mice were treated with depleted IVIG or PBS.  3hrs following administration of IVIG mice were challenged IN with $1x10^7$ CFU *S. pneumoniae* TIGR4. The numbers of neutrophils or macrophages in the BALF of mice 24hrs following challenge were not significantly altered by anti-CPS ST4 depleted IVIG treatment (**Figure 5.7**). IVIG depleted of anti-type-4 CPS IgG was still able to significantly enhance bacterial clearance from the blood 24hrs following IN challenge (**Figure 5.7**), and there was also a trend towards reduced *S. pneumoniae* CFU in the lungs post-infection (**Figure 5.7**). These data indicate that naturally-acquired IgG to the polysaccharide capsule is not required for the protective effect of IVIG, at least in the blood, and strongly supports the hypothesis that naturally-acquired IgG to non-capsular targets is protective against *S. pneumoniae* infection.
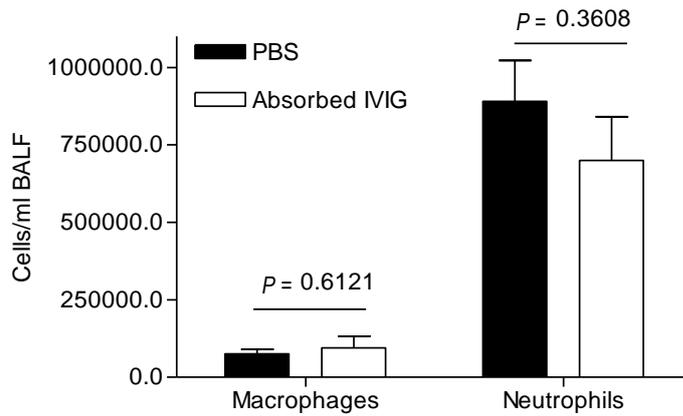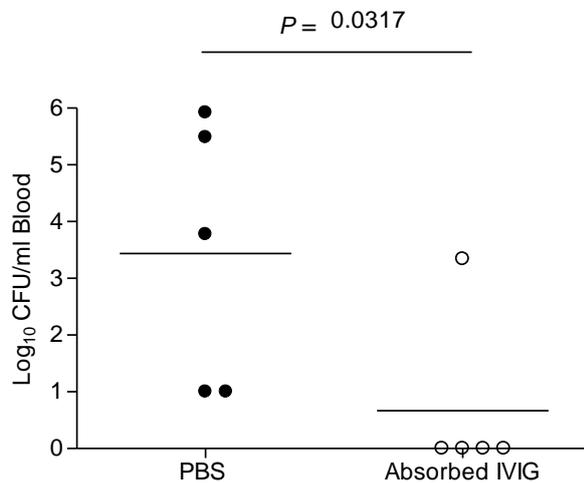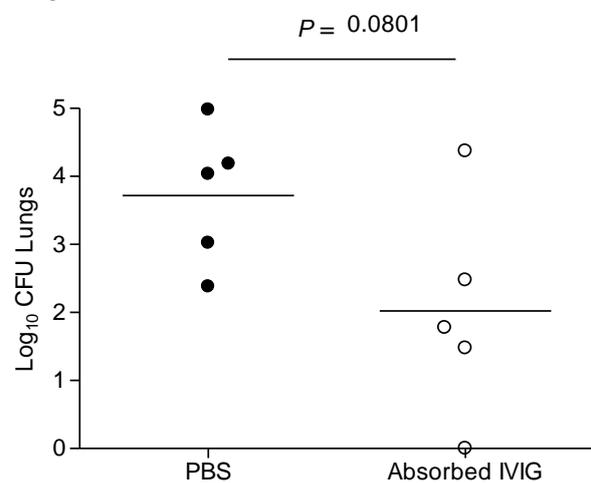
**Figure 5.7: IVIG depleted of anti-CPS IgG *in vivo*.**

(A)Total cell counts in BALF 24hrs following IN challenge with 1x10$^7$ CFU *S. pneumoniae* TIGR4 in mice treated with PBS or *S.mitis*T4*cps* absorbed IVIG. (B) CFU in blood24hrs following IN infection with 1x10$^7$ CFU *S. pneumoniae* TIGR4 in mice treated with PBS or absorbed IVIG. CFU in lungs 24hrs following IN infection with 1x10$^7$ CFU *S. pneumoniae* TIGR4 in mice treated with PBS or absorbed IVIG. (*P* values represent Student's unpaired T-test, error bars represent SD). Results are from one experiment.

### 5.1.7 Chapter summary

Passive transfer of IVIG into mice allowed assessment of the protective effect of naturally-acquired human IgG against *S. pneumoniae* infection *in vivo*. In this model human IgG was not detectable in the alveolar space without infection. There may therefore be an additional role for pre-existing IgG in the alveolar space in the initial clearance of *S. pneumoniae* from the lung. IVIG treatment partially protected mice from *S. pneumoniae* lung infection and very strongly against bacteraemia, lung protection required neutrophils and blood protection macrophages. Since protection in this model appeared to be dependent upon phagocytes these data indicate that *in vitro* assays of phagocytosis (used in the previous chapter) are relevant for assessing the protective effects of IVIG.

Importantly passive administration of IVIG depleted of IgG to the type-4 polysaccharide was still protective against *S. pneumoniae* TIGR4 infection, suggesting the protective effects of naturally-acquired IgG may target non-capsular antigen. This is in agreement with data presented in chapter 4, demonstrating IVIG depleted of type-4 capsular polysaccharide still bound to the surface of *S. pneumoniae* TIGR4. Although these data do not confirm that the non-capsular antigens targeted by IVIG *in vivo* are pneumococcal surface proteins, the *in vitro* data presented in chapter 3 and 4 strongly support that they are important targets for naturally-acquired anti-*S. pneumoniae* IgG.

# 6 Results (4): Acquired immunity to non-bacteraemic pneumonia

Passive transfer of IVIG to mice pre-challenge with *S. pneumoniae* TIGR4 demonstrated that IgG has a role to play in protection from experimental lung infection. Previous data using murine models has established that antibody is the dominant mechanism preventing systemic infection after prior colonisation. However humoral immunity represents only one mechanism of naturally-acquired immunity that may be responsible for protection within the lung. An aim was therefore to develop a model of murine colonisation followed by challenge using a 19F strain of *S. pneumoniae* (EF3030) that causes lung infection without causing bacteraemia (Briles et al., 2003). This would allow interrogation of the immune mechanisms induced by colonisation that may be protective against subsequent lung infection independently of the mechanisms required for prevention of bacteraemia.

### 6.1.1 Assessment of EF3030 as model of non-bacteraemic pneumonia

To assess if IN infection with *S. pneumoniae* EF3030 was an appropriate model of non-invasive *S. pneumoniae* pneumonia, mice were challenged IN with EF3030 or PBS in a volume of 50µl. Mice challenged with EF3030 had neutrophil infiltration into the alveolar space on histology (**Figure 6.1**), and a large increase in neutrophil numbers in BALF 24hrs post-infection (**Figure 6.2**). CFU counts confirmed that EF3030 was detectable in lung tissue and BALF samples (**Figure 6.2**), but importantly no bacteria were detectable in the blood 24hrs after challenge.  These data indicate that challenge with *S. pneumoniae* EF3030 recapitulates the import features of non-bacteraemic pneumonia, including cellular infiltration into the lungs and the absence of bacteria from the blood.

**A**
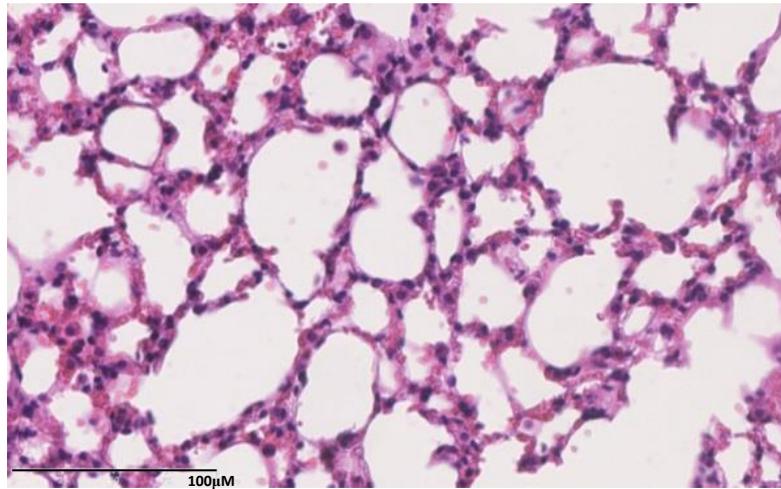


**B**
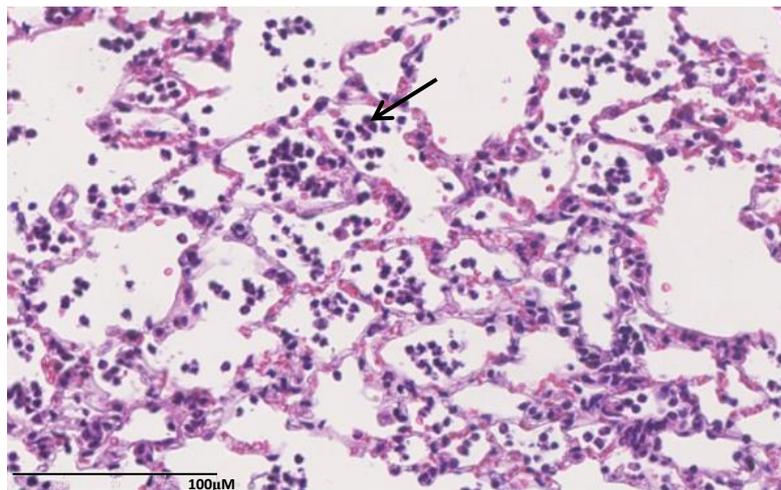


**Figure 6.1: Lung histology following EF3030 challenge**.

Histological lung sections stained with Haematoxylin and Eosin, from mice 24hrs following IN challenge with (A) PBS or (B) $2x10^7$ CFU of *S. pneumoniae* EF3030. (Arrow indicates cellular infiltration in to the alveolar space). Images are representative of the lungs from two uninfected mice (Figure A) and four mice infected with EF3030 (Figure B).
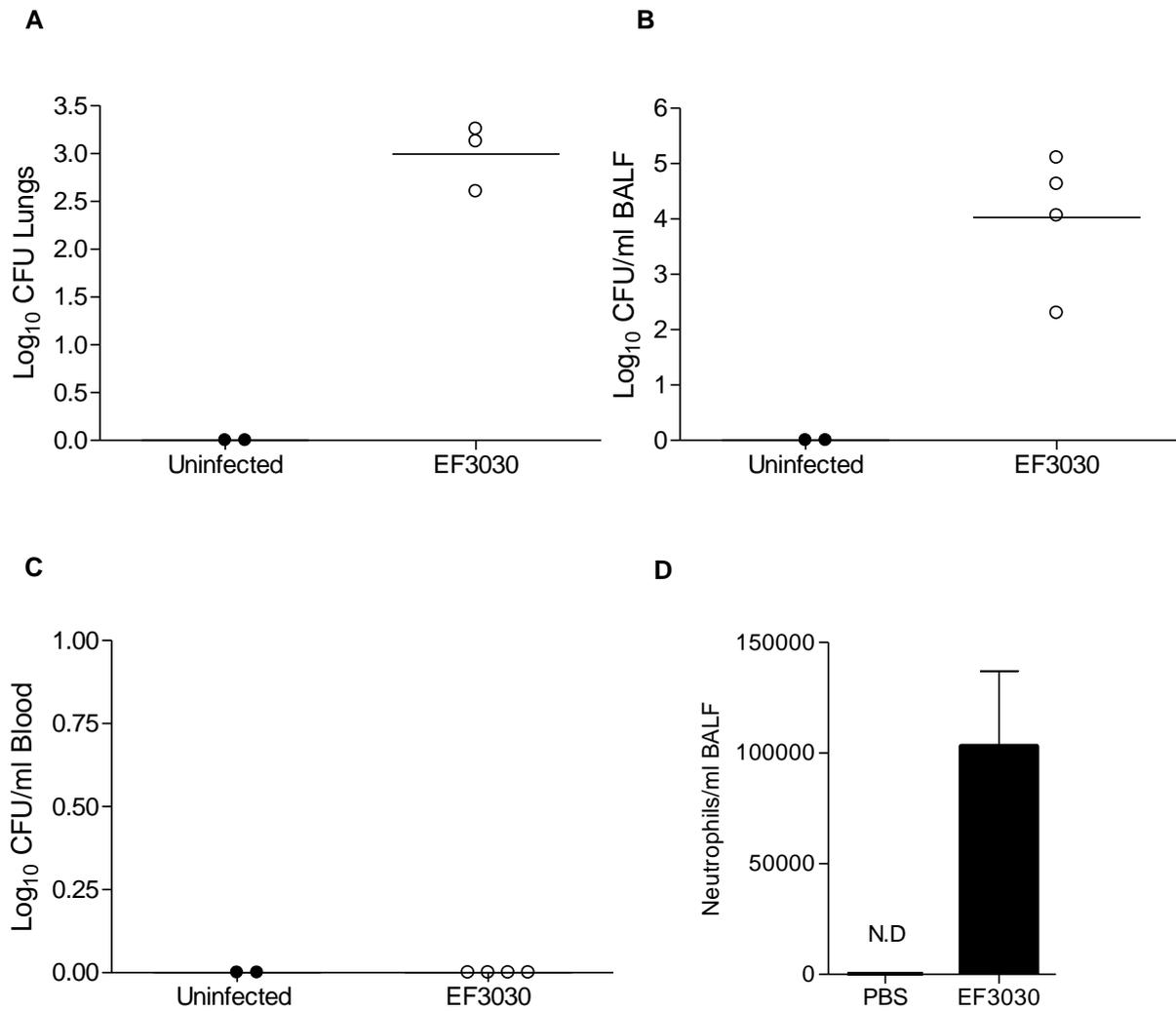
**Figure 6.2: Non-invasive lung infection with *S. pneumoniae* EF3030.**

(A) CFU in lungs of C57/BL6 mice 24hrs following IN challenge with $2\times10^7$ CFU of *S. pneumoniae* EF3030 or PBS. (B) CFU in BALF of mice 24hrs following challenge with *S. pneumoniae* EF3030 or PBS. (C) CFU in blood of mice 24hrs following challenge with *S. pneumoniae* EF3030 or PBS. (D) Neutrophil numbers in BALF of mice 24hrs following IN challenge with *S. pneumoniae* EF3030 or PBS. (Lines represent mean). Results are from one experiment.

### 6.1.2 Nasopharyngeal colonisation with EF3030

To assess if *S. pneumoniae* EF3030 could colonise the nasopharynx, C57/BL6 mice were experimentally colonised by IN inoculation of $1 \times 10^7$ CFU in 10µl of PBS, and culled at days 5, 13 and 30 post-infection. EF3030 was detectable in nasal washes at 5 days and 13 days post-inoculation (**Figure 6.3**) and had been cleared by 30 days, demonstrating that EF3030 can colonise the nares of C57/BL6 mice for up to 13 days. Other models of murine colonisation with *S. pneumoniae* that induce an adaptive immune response have demonstrated colonisation for similar lengths of time (Cohen et al., 2011), suggesting that this model of colonisation with *S. pneumoniae* EF3030 is appropriate for assessing potentially protective immune responses.
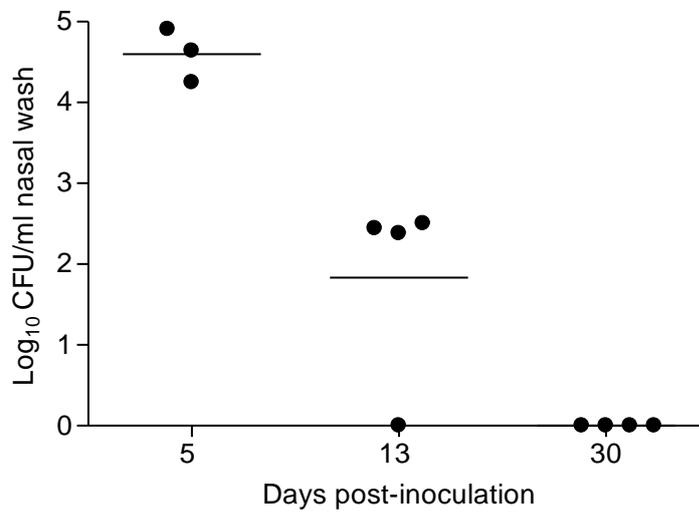
**Figure 6.3: *S. pneumoniae* in nares of mice post-colonisation.**

CFU in nasal washes of C57/BL6 mice at 5, 13, and 30 days following IN inoculation with $1x10^7$ CFU *S. pneumoniae* EF3030 in $10\mu l$ PBS. (Lines represent mean). Results are from one experiment at each time-point.

### 6.1.3    Protective effect of colonisation

To assess if colonisation with *S. pneumoniae* EF3030 is protective against subsequent lung infection, mice were either experimentally colonised with EF3030 or sham colonised with PBS. 30 days following colonisation mice were challenged IN with EF3030 and culled at 4, 24 and 72hrs post-infection. CFU were enumerated in the lungs and BALF of mice at each time point post-infection. 4hrs following colonisation there was no difference in bacterial numbers in colonised or control mice (**Figure 6.4**). At 24hrs post-infection there was a significant reduction in bacterial numbers in the BALF and lungs of previously colonised mice (**Figure 6.4**). At 72hrs following *S. pneumoniae* challenge a significant reduction in bacterial burden remained in the lungs, but not BALF of colonised mice (**Figure 6.4**). Together these data demonstrate that colonisation with EF3030 is partially protective against subsequent *S. pneumoniae* lung infection, significantly reducing bacterial burden in BALF and lungs at 24hrs and lungs 72hrs post-infection.

**Figure 6.4: CFU following lung infection in colonised mice.**

(A,B) CFU in BALF and lungs of mice colonised with EF3030 or PBS, 4hrs following challenge with $2x10^7$ CFU *S. pneumoniae* EF3030. (C,D) CFU in BALF and lungs of mice colonised with EF3030 or PBS, 24hrs following challenge with $2x10^7$ CFU *S. pneumoniae*. (E,F) ) CFU in BALF and lungs of mice colonised with EF3030 or PBS, 72hrs following challenge with *S. pneumoniae*. (*P* values represent Student's unpaired T-test, lines represent mean). Results are from one experiment at each time-point, except 24hrs which was repeated twice.

### 6.1.4    Antibody responses to colonisation

In previous models of highly invasive lung-infection the development of antibodies following colonisation was essential for protection. To explore the potential role for antibodies in the protection seen in this model, anti-pneumococcal antibody responses to colonisation were assessed. There was a significant increase in the binding titre of anti-EF3030 IgG in the sera of mice following colonisation (**Figure 6.5**) as assessed by whole cell ELISA. In colonised mice there was not a significant rise in IgG specific for the type-19F (EF3030) capsular polysaccharide as measured by ELISA (**Figure 6.5**). The concentration of IgM in sera recognising EF3030 by whole cell ELISA was also unchanged following colonisation (**Figure 6.5**). In these assays anti-EF3030 IgA was not detected in the sera or BALF of colonised or control mice by whole cell ELISA.
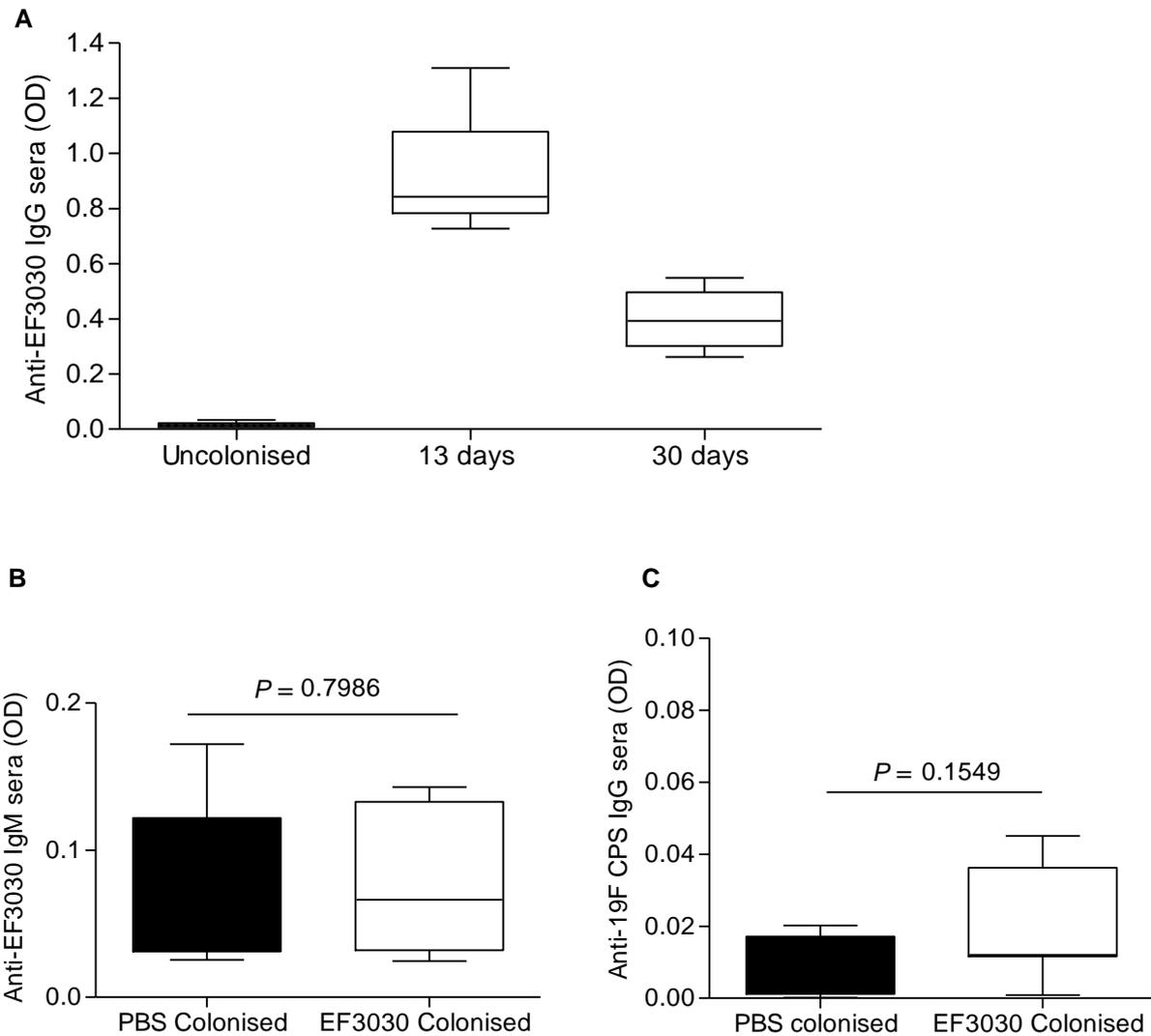
**Figure 6.5: Serological responses to EF3030 colonisation.**

(A) Anti-EF3030 IgG in the sera (diluted 1:100) of mice 13 and 30 days post-colonisation with $1x10^7$ CFU *S. pneumoniae* EF3030 by whole cell ELISA compared to uncolonised controls, represented as $OD_{405-630}$. (B) Anti-19F polysaccharide IgG in the sera (diluted 1:100) of mice 30 days post-colonisation with *S. pneumoniae* EF3030 or PBS by ELISA represented as $OD_{405-630}$. (C) Anti-EF3030 IgM in the sera (diluted 1:150) of mice 30 days post-colonisation with *S. pneumoniae* EF3030 or PBS by whole cell ELISA represented as $OD_{405-630}$. (*P* values represent Student's unpaired T-test). Results are from one experiment at each time-point, ELISAs were repeated twice.

To assess if IgG responses could be responsible for the protection against infection within the lung, EF3030 specific and total murine IgG was measured in BALF following challenge. In the absence of infection EF3030 colonised mice had significantly greater EF3030-specific IgG detected in BALF (**Figure 6.6**). 4hrs post-challenge the concentration of EF3030-specific IgG remained significantly higher in colonised mice compared to non-colonised controls (**Figure 6.6**), and was significantly higher than in colonised mice pre-challenge (**Figure 6.6**). From 4 to 24hrs following infection the concentration of EF3030-specific IgG in the BALF fell (**Figure 6.6**). The concentration total murine IgG in the BALF was also measured by ELISA. In colonised mice there was an early increase in total IgG at 4hrs that was not seen in uncolonised controls (**Figure 6.6**). By 24hrs post infection the total IgG concentration in the BALF of both groups was similar (**Figure 6.6**). Measurement of total IgG indicates that the reduction in pathogen specific IgG observed between 4 and 24hrs was not driven by global reduction in IgG concentration and may therefore be due to the adsorption of specific IgG onto *S. pneumoniae*. The increase in total IgG at 4hrs post-challenge may be responsible for the observed increase in EF3030-specific IgG in colonised mice at this time point. To assess if this increase in total IgG may be related to alveolar epithelial permeability albumin levels in the BALF were measured post-infection by ELISA. There was a strong correlation between the concentration of total IgG and albumin concentration in the BALF of colonised mice (**Figure 6.6**), indicating that IgG may accumulate in the BALF of mice in line with increased alveolar permeability following challenge.
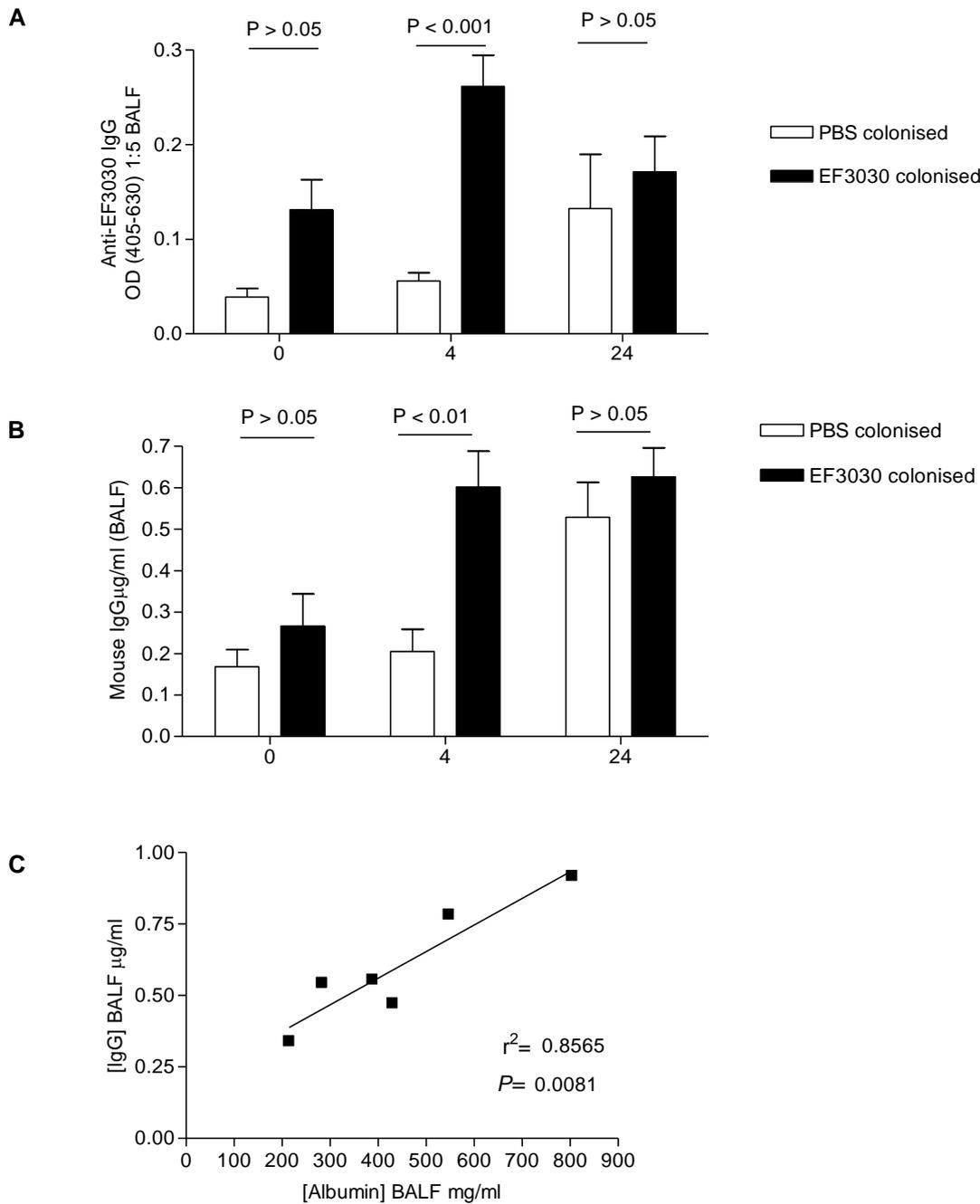
**Figure 6.6: Antibody dynamics in bronchoalveolar lavage fluid following challenge.**

(A) Anti-EF3030 IgG in BALF of mice colonised with EF3030 or PBS 0, 4 and 24hrs post- IN challenge with $2x10^7$ CFU *S. pneumoniae* EF3030, by whole cell ELISA. (B) Total murine IgG in BALF of mice colonised with EF3030 or PBS 0, 4 and 24hrs post- IN challenge with $2x10^7$ CFU *S. pneumoniae* EF3030, by whole cell ELISA. (C) Linear regression of total IgG concentration compared to albumin concentration in the BALF of EF3030 colonised mice 4hrs following EF3030 challenge**.** (*P* values represent one-way ANOVAs and Tukey's post-test to compare columns, error bars represent SD). Results are from one experiment at each time-point, ELISAs were repeated twice.

### 6.1.5 Identification of *S. pneumoniae* target antigens

Increased IgG levels in the BALF of colonised mice following challenge suggest a potential role for IgG in mediating the protection from lung infection observed in previously colonised mice. To investigate the potential targets of EF3030 specific IgG in colonised mice responses to purified pneumococcal polysaccharide (type 19F) and pneumococcal proteins were assessed. Very low levels of IgG to type 19F CPS were detectable in the sera of sham colonised mice (**Figure 6.5**). Though there was a trend towards increased anti-CPS-specific IgG in the sera of EF3030 colonised mice, this was not significant, and levels of anti-CPS IgG remained low following colonisation (**Figure 6.5**). Responses to pneumococcal proteins following colonisation were initially assessed by Western blot. Probing lysates of EF3030 with sera from sham colonised or EF3030 colonised mice indicated the development of IgG to specific *S. pneumoniae* protein targets following colonisation with EF3030 (**Figure 6.7**). Sera from sham colonised mice reacted with a single band at approximately 65KDa, whereas sera from different individual mice colonised with EF3030 gave a number of bands including at approximately 70, 55, 40, 35 and 25KDa (**Figure 6.7**). The pattern of responses in sera from colonised mice displayed some differences, with certain bands weaker or absent for sera from individual mice but present in others (**Figure 6.7**). To quantify antibody responses to different proteins and to identify some of the specific protein targets for post-colonisation IgG, the Luminex assay was used. Both sera and BALF from sham colonised mice displayed minimal IgG binding to all proteins (**Figure 6.7**). Sera and BALF from EF3030 colonised mice had significant IgG responses to the pneumococcal proteins PhtD, PsaA and PpmA (**Figure 6.7**).

These data demonstrate that following colonisation with EF3030 mice develop IgG responses to a limited number of protein antigens, including the surface proteins PhtD, PsaA and PpmA. Western blotting indicated IgG responses to more than 3 proteins following EF3030 colonisation (**Figure 6.7**). These responses may be to proteins not included on the Luminex assay or to proteins that show

allelic variation between EF3030 and the strains from which the Luminex proteins were purified

from (**Table 2.5**).
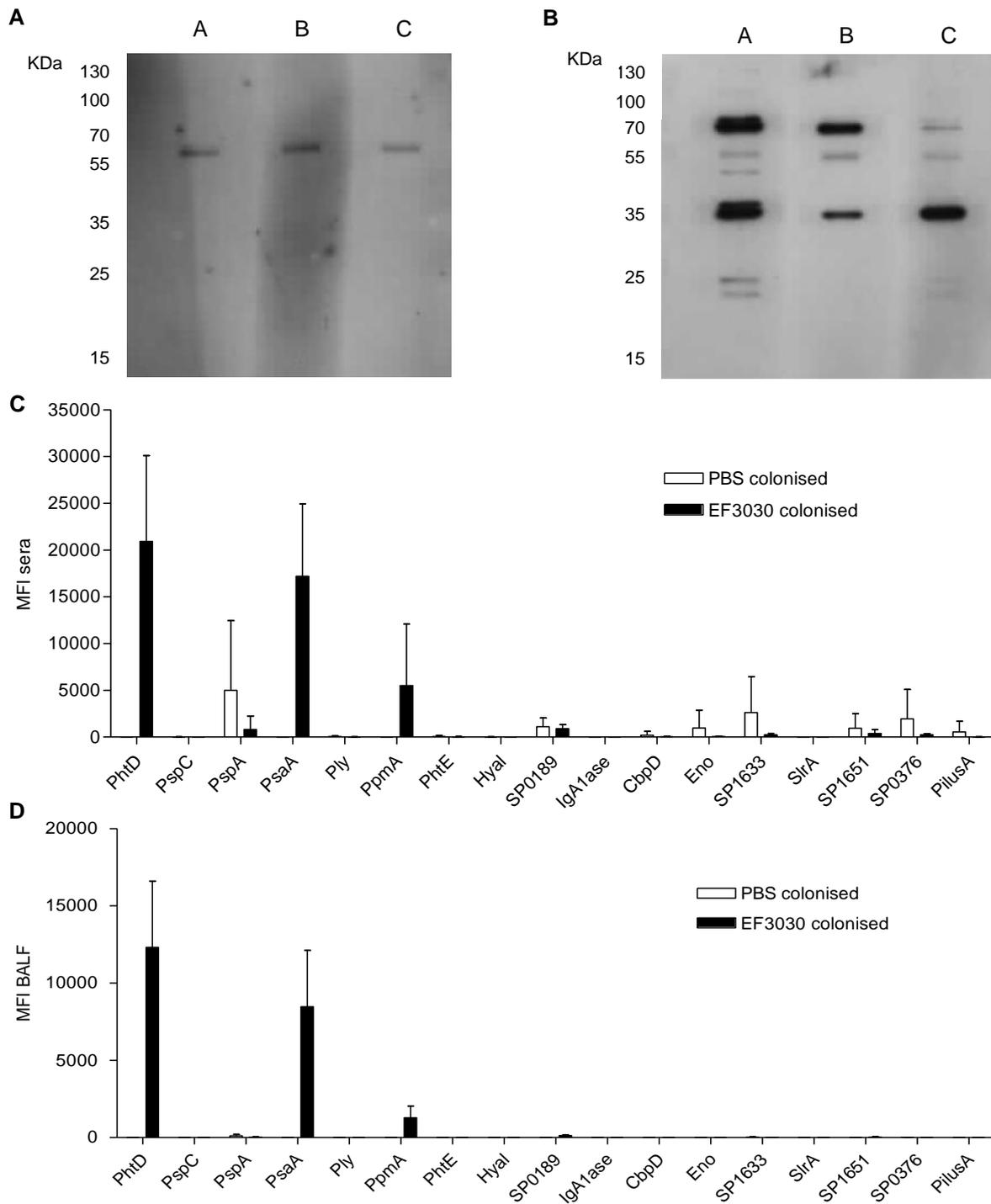
**Figure 6.7:** *S. pneumoniae* **protein targets of IgG in colonised mice.**

(A,B) Western blots of IgG binding to whole cell lysates of *S. pneumoniae* EF3030 probed with sera from individual mice colonised with PBS or EF3030 pre-infection. (C,D) Luminex assay of IgG binding to recombinant pneumococcal proteins in the sera (1:100) and BALF (1:1) of different individual mice colonised with PBS or EF3030 pre-infection, MFI represents binding of anti-mouse IgG-PE secondary antibody. (Columns represent mean of 5 mice per group, error bars represent SD). Results are from one experiment, Luminex assays were performed in duplicate.

### 6.1.6 Effect of colonisation on cellular responses in lungs of challenged mice

To assess if colonisation also affected cellular responses to subsequent lung infection cell numbers in BALF were quantified by differential cell counting, under light microscopy. Colonisation had no effect on baseline numbers of neutrophils or macrophages in BALF prior to challenge (**Figure 6.8**). At 4hrs following lung challenge the numbers of neutrophils in the BALF of mice previously colonised with EF3030 was significantly higher than PBS colonised controls (**Figure 6.8**). By 24hrs post-challenge neutrophil numbers in colonised and control mice were similar and both significantly enhanced compared to 4hrs post-challenge (**Figure 6.8**). There were no significant differences in macrophage numbers at any time point post-challenge in previously colonised or control mice (**Figure 6.8**). These changes in cell numbers indicate that colonisation may affect the neutrophil response to subsequent challenge, with more neutrophils appearing in the alveolar space early (4hrs) post-challenge in colonised mice.

**A**



**B**
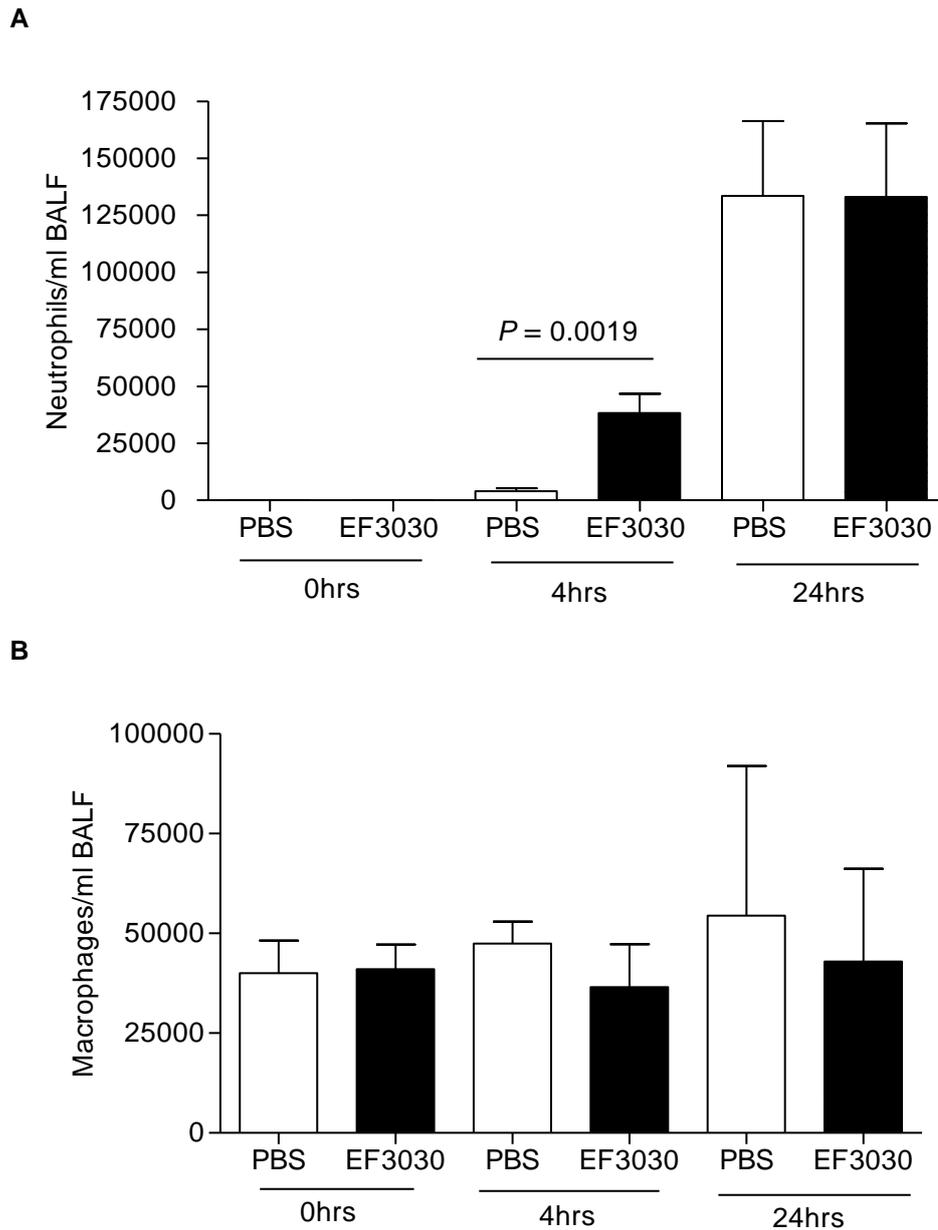


**Figure 6.8: Cell numbers in bronchoalveolar lavage fluid of colonised mice following challenge.**

(A) Neutrophil numbers in BALF of mice colonised with PBS or EF3030 0, 4 and 24hrs following IN infection with $2x10^7$ CFU *S. pneumoniae* EF3030. (B) Macrophage numbers in BALF of mice colonised with PBS or EF3030 0, 4 and 24hrs following IN infection with $2x10^7$ CFU *S. pneumoniae* EF3030. (*P* value represent Student's unpaired T-test, error bars represent SD). Results are from one experiment at each time point, except 24hrs which was repeated twice.

## 6.1.7   Effect of colonisation on cytokine responses in the lung

A number of different cytokines may have a role to play in immunity to pneumococcal lung infection, and may therefore mediate the improved protection from lung infection demonstrated following nasopharyngeal colonisation of mice. As previously colonised mice displayed an enhanced neutrophil response to challenge, the concentration of the neutrophil chemoattractant KC, (the murine functional homologue of IL-8) was measured in lung tissue homogenates. 4hrs post-challenge there were significantly higher levels of KC in the lungs of EF3030 colonised mice compared to controls (**Figure 6.9**). By 24hrs following challenge the concentration of KC in lung tissue was lower than at 4hrs for both groups and there was no significant difference in KC concentration between EF3030 colonised mice and controls (**Figure 6.9**). The pro-inflammatory cytokine TNF-α has an important role in the initial immune response *to S. pneumoniae* lung infection and may affect the release of chemokines including KC from epithelial cells (Sun et al., 2007). Colonised mice demonstrated significantly higher levels of TNF-α in lung homogenates both at 4 and 24hrs following challenge with *S. pneumoniae* EF3030 compared to controls (**Figure 6.9**).

Th17 responses and IL-17 in-particular have been demonstrated to be important for acquired immunity to *S. pneumoniae* within the nasopharynx (Zhang et al., 2009). IL-17 was therefore measured in the lungs of colonised and control mice following challenge with *S. pneumoniae* EF3030. 4hrs post-infection IL-17 was detectable in lung homogenates of colonised mice but not in controls (**Figure 6.10**). Similarly IL-17 was only detectable in the lungs of colonised mice at 24hrs post-infection (**Figure 6.10**). 3 days following challenge IL-17 was measureable in sham colonised mice, but remained significantly increased in EF3030 colonised mice (**Figure 6.10**). The cytokine IL-23 has a role in Th17 cell differentiation and initiation of IL-17 production (Ma et al., 2010). Levels of IL-23 in lung homogenates 4hrs following *S. pneumoniae* challenge were significantly increased in previously colonised mice, by 24hrs post-infection IL-23 concentrations in the lungs of both groups were similar (**Figure 6.10**).
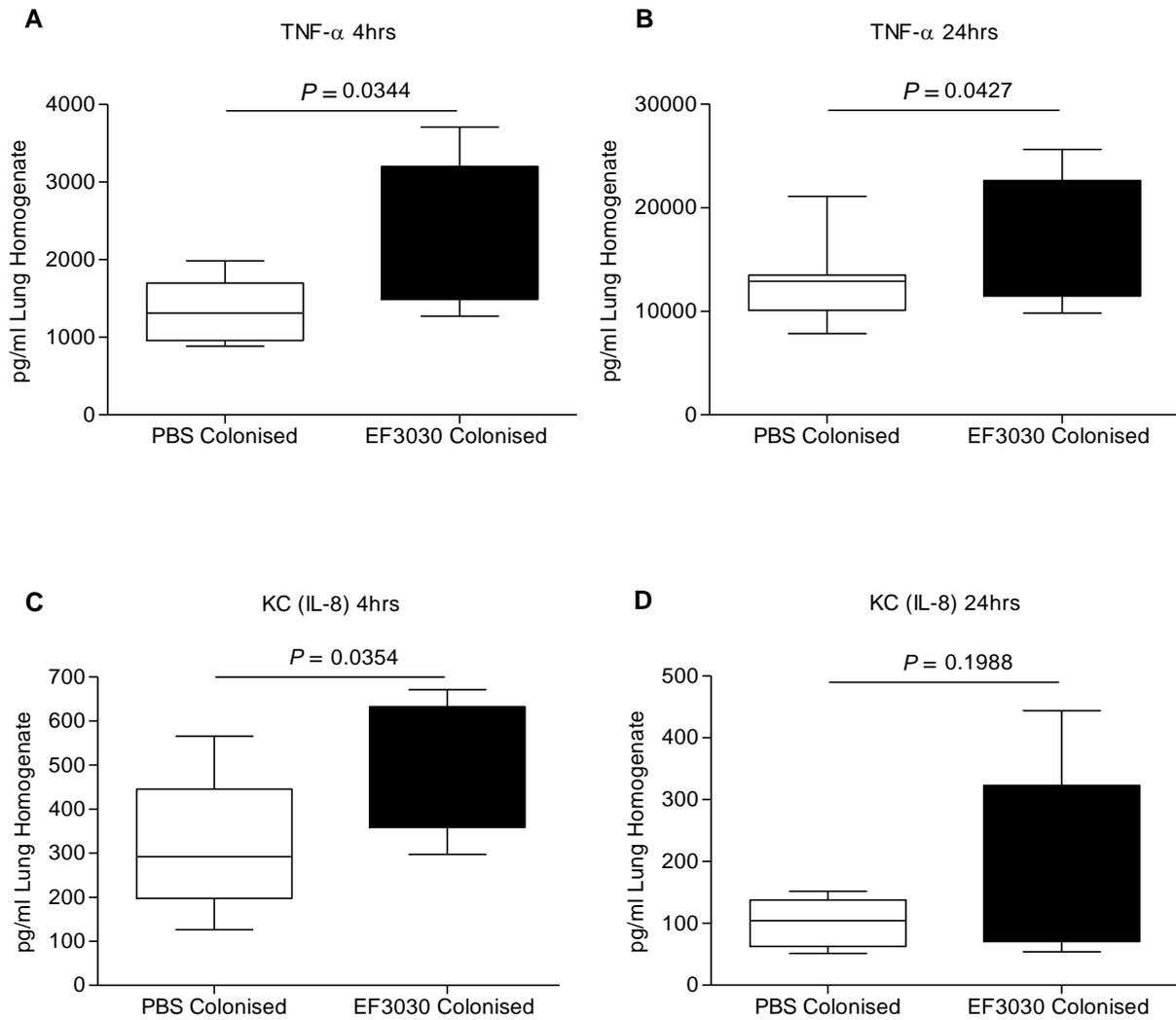
**Figure 6.9: KC and TNF-α responses in colonised mice following challenge.**

(A,B) Concentration (pg/ml) of TNF-α in the lung homogenates of mice colonised with EF3030 or PBS 4 and 24hrs following challenge with 2x10$^7$ CFU *S. pneumoniae* EF3030, measured by ELISA. (C,D) Concentration (pg/ml) of KC in the lung homogenates of mice colonised with EF3030 or PBS, 4 and 24hrs following challenge with 2x10$^7$ CFU *S. pneumoniae* EF3030, measured by ELISA. (*P* values represent Student's unpaired T-test). ELISA titres are representative of two independent experiments on the same samples.
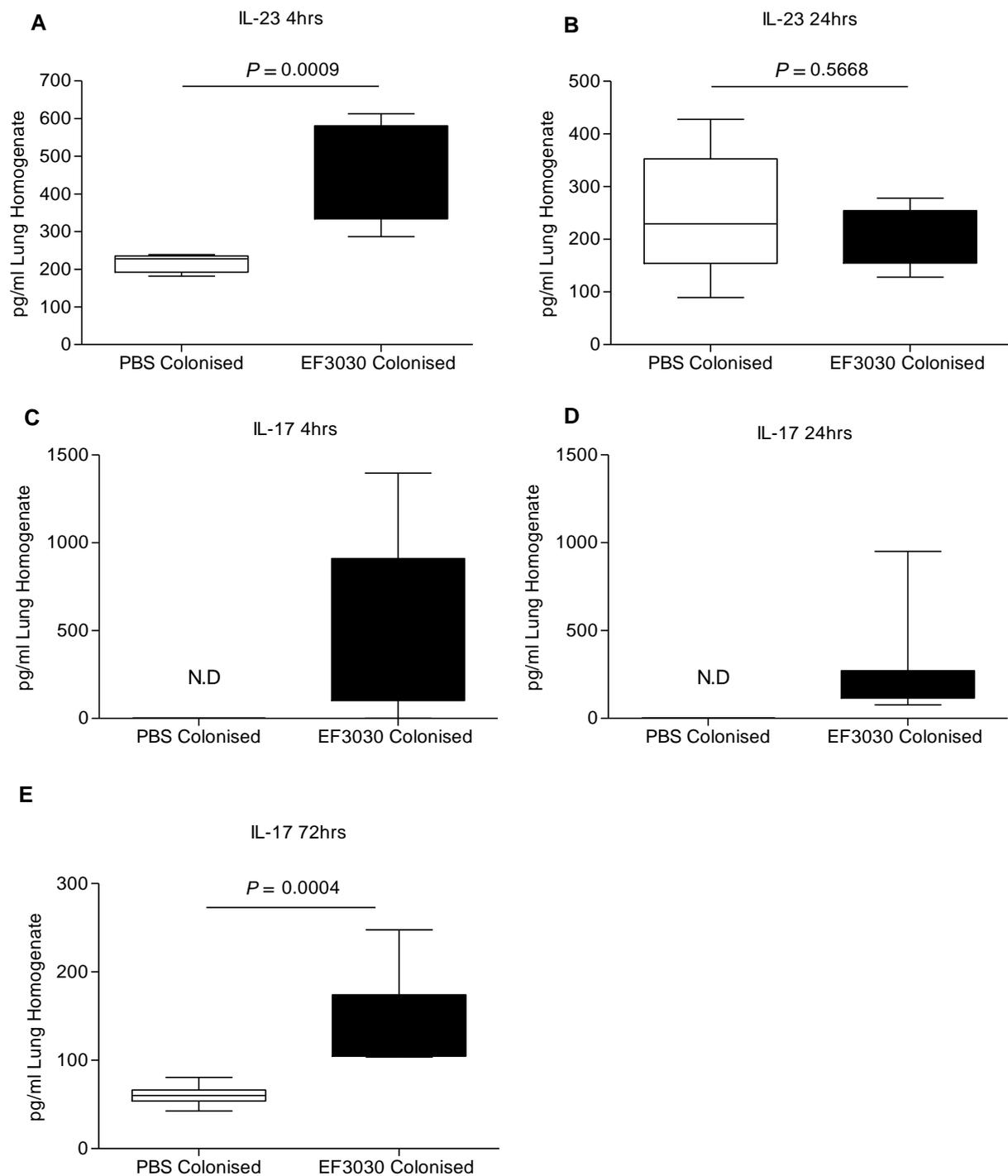
**Figure 6.10: IL-23 and IL-17 responses in colonised mice following challenge.**

(A,B) Concentration (pg/ml) of IL-23 in the lung homogenates of mice colonised with EF3030 or PBS, 4 and 24hrs following challenge with $2x10^7$ CFU *S. pneumoniae* EF3030, by ELISA. (C-E) Concentration (pg/ml) of IL-17 in the lung homogenates of mice colonised with EF3030 or PBS, 4, 24 and 72hrs following challenge with $2x10^7$ CFU *S. pneumoniae* EF3030, measured by ELISA. (*P* values represent Student's unpaired T-test). ELISA titres are representative of two independent experiments.

Differences in the regulatory cytokine IL-10 have previously been demonstrated as important in mediating susceptibility to experimental *S. pneumoniae* infection in mice (Neill et al., 2012). Therefore IL-10 concentration in lung homogenates at different time points post EF3030 infection was assessed. 4hrs post-infection there were significant increases in IL-10 in the lungs of mice previously colonised with EF3030, compared to PBS controls (**Figure 6.11**). However, at 24hrs post-infection there was no difference in lung IL-10 concentrations between EF3030 colonised or control mice. In colonised mice IL-10 levels remained significantly higher 3 days post *S. pneumoniae* infection, compared to controls (**Figure 6.11**). These data indicate that EF3030 colonisation appears to facilitate an early IL-10 response 4hrs following challenge, prior colonisation may also allow mice to maintain increased IL-10 responses for up to 72hrs post-challenge. These responses could be significant for the contribution to protection from *S. pneumoniae* EF3030 pneumonia, afforded by prior colonisation.
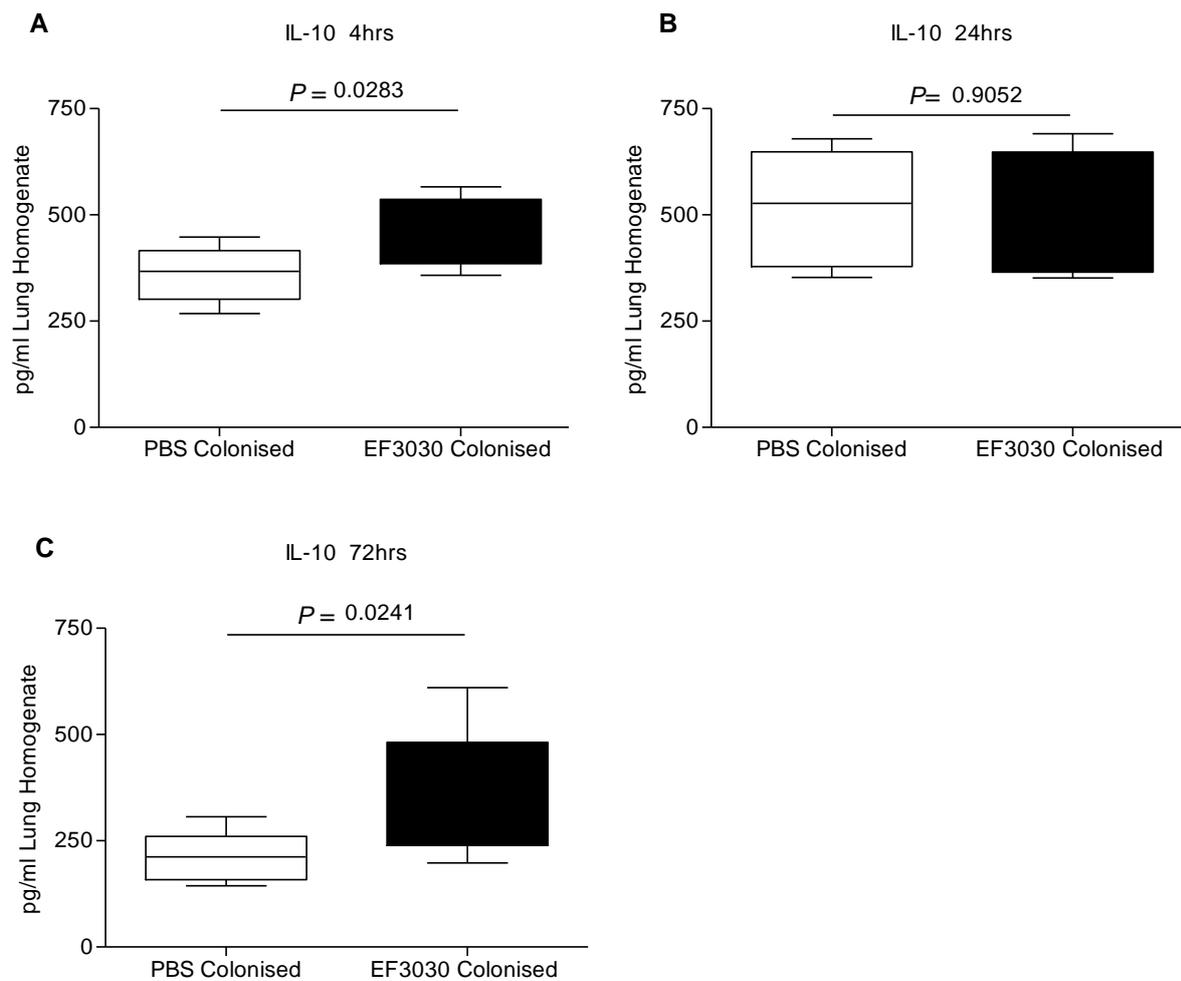
**Figure 6.11: IL-10 responses in colonised mice following challenge.**

(A-C) Concentration of IL-10 in the lung homogenates of mice colonised with EF3030 or PBS, 4  24hrs and 72hrs following challenge with 2x10$^7$ CFU *S. pneumoniae* EF3030, measured by ELISA. (*P* values represent Student's unpaired T-test). ELISA titres are representative of two independent experiments on the same samples.

Prior colonisation appears to have an effect on a number of different cytokine responses during subsequent challenge. Responses in previously colonised mice compared to uncolonised controls are summarised in **Table 6.1**, and include increases in TNF-α, KC, IL-23, IL-17 and IL-10. Enhanced in Th17 type immune responses (IL-17 and IL-23) in the lung are of particular interest as they have been shown to be protective against *S. pneumoniae* in the nasopharynx following experimental colonisation. IL-17 was not detected in the lungs of colonised or control mice prior to EF3030 lung infection, suggesting that the observed responses are to subsequent infection rather than increased baseline cytokine levels following colonisation.

| Cytokine | 4hrs | 24hrs | 72hrs |
|----------|------|-------|-------|
| KC | ↑ | → | ND |
| TNF-α | ↑ | ↑ | ND |
| IL-23 | ↑ | | ND |
| IL-17 | ↑ | ↑ | ↑ |
| IL-10 | ↑ | → | ↑ |

**Table 6.1:** Summary of cytokine responses in lung tissue of previously colonised mice at different time points post IN infection with $2x10^7$ CFU *S. pneumoniae* EF3030, relative to uncolonised controls (↑= increase,→ = no change, ND= not done).

### 6.1.8  Relevance of antibody, neutrophils, CD4 cells and IL-17 for protection

A number of arms of the protective immune response to lung infection are enhanced by prior colonisation with *S. pneumoniae* EF3030. It is not clear thus far which of these may be important in mediating the protective effect of colonisation against subsequent lung infection. EF3030 colonisation and challenge experiments were therefore repeated in mice that had undergone cellular or cytokine depletion strategies prior to challenge, or in μMT (B-cell deficient) mice.

### 6.1.8.1  Absence of antibody

Colonisation with *S. pneumoniae* EF3030 resulted in the development of a specific IgG response.  To assess if this response was required for protection from lung infection, μMT (B-cell deficient) mice were colonised with EF3030. Whole cell ELISAs on sera from colonised mice demonstrated the absence of a specific IgG response to colonisation in these mice (**Figure 6.12**). 24hrs following EF3030 challenge there was no difference in bacterial numbers in the BALF or lungs of previously colonised or sham colonised μMT mice (**Figure 6.12**). This suggests that anti-pneumococcal IgG responses are required for protection from lung infection with *S. pneumoniae* EF3030, following colonisation.
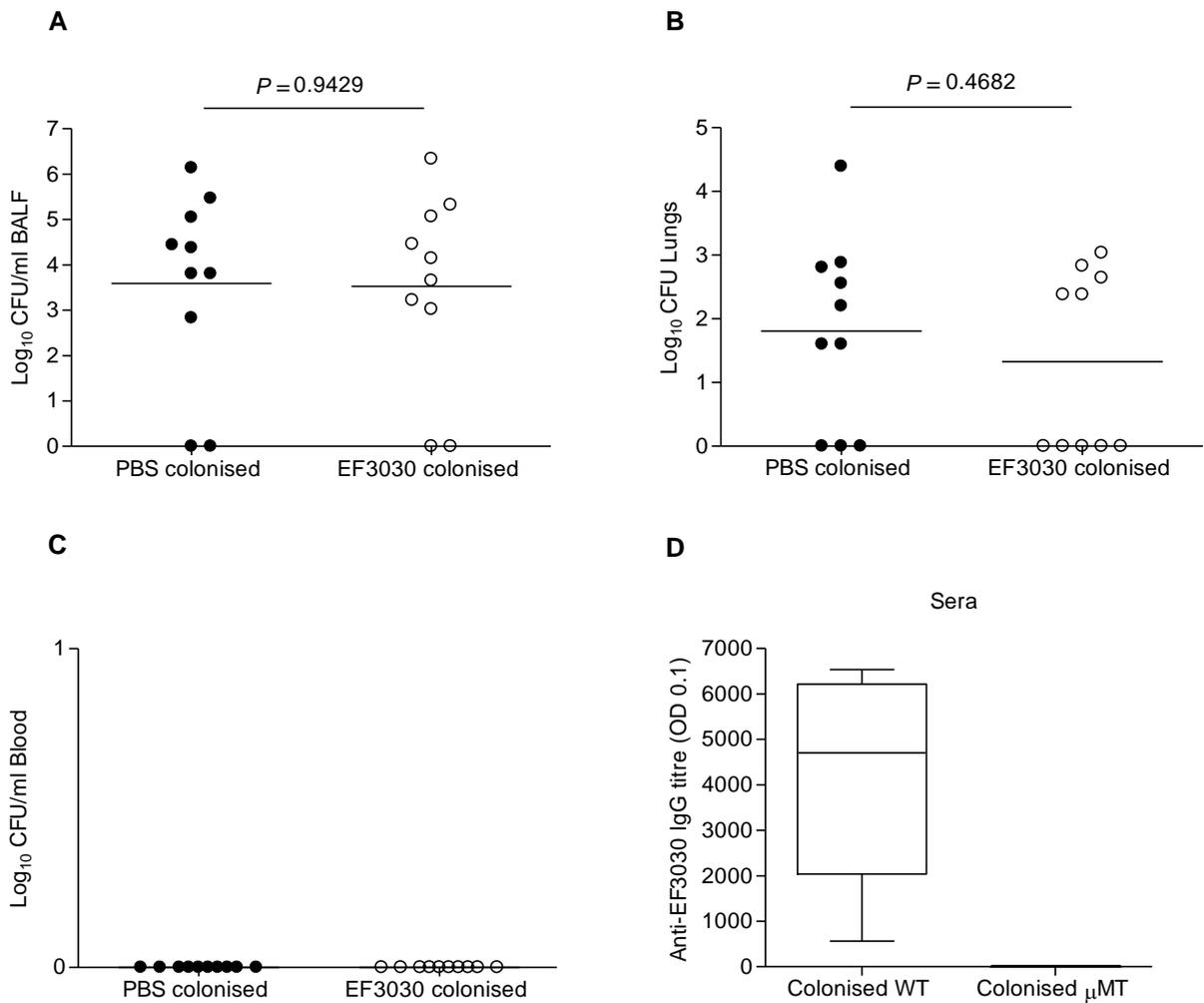
**Figure 6.12: CFU in antibody deficient mice following challenge.**

(A,B,C) CFU in BALF, lungs and blood of µMT (B-cell deficient) mice colonised with EF3030 or PBS 24hrs following challenge with 2x10$^7$ CFU *S. pneumoniae*. (D) Concentration of anti-EF3030 IgG in the sera of WT or µMT (B-cell deficient) mice colonised with 1x10$^7$ CFU EF3030 measured by whole cell ELISA. (*P* values represent Student's unpaired T-test). Results are from one experiment.

### 6.1.8.2 Absence of neutrophils

Neutrophils were the most abundant cell type in the BALF following EF3030 challenge. Colonised mice also displayed significantly greater neutrophil numbers in the BALF at 4hrs post-infection. To assess if neutrophils were important in the observed protection from lung infection mice previously colonised with EF3030 or PBS were depleted of neutrophils prior to challenge. Administration of anti-Ly6G monoclonal antibody via IP inoculation 24hrs prior to challenge led to an 8-fold reduction in neutrophils in the BALF of mice 24hrs post *S. pneumoniae* infection, as assessed by differential cell counting under light microscopy (**Figure 6.13**). Neutrophil depletion abrogated the protective effect of colonisation, with no significant difference in bacterial numbers in the BALF and lungs of colonised compared to control mice (**Figure 6.13**). A few of these neutrophil depleted mice had *S. pneumoniae* EF3030 in the blood; however the vast majority remained free of detectable bacteraemia.
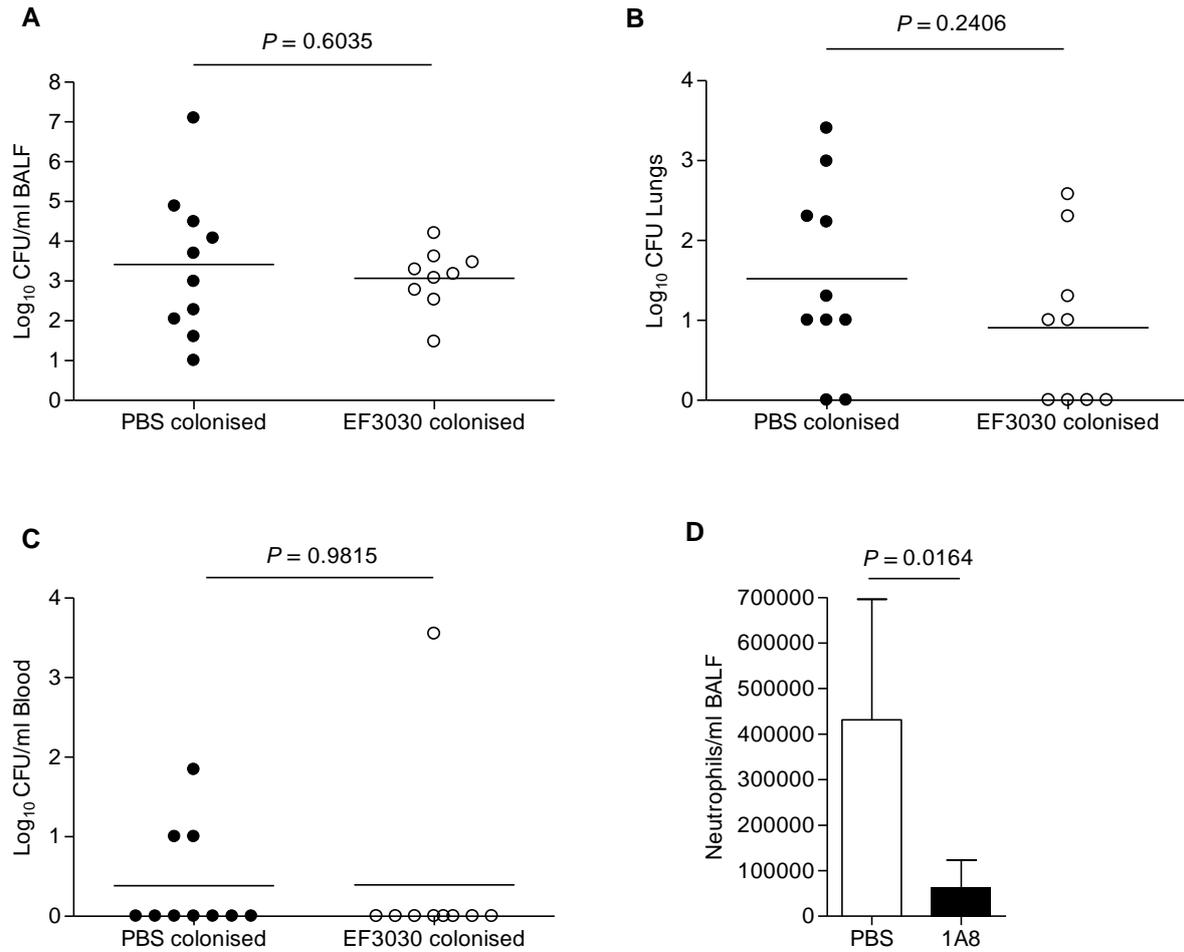
**Figure 6.13: CFU in neutrophil depleted mice following challenge**.

(A-C) CFU in BALF, lungs and blood of colonised or control mice treated with neutrophil-depleting antibody (1A8, anti-Ly6G) 24hrs following challenge with $2x10^7$ CFU *S. pneumoniae.* (D) Neutrophils in the BALF 24hrs following challenge with $2x10^7$ CFU *S. pneumoniae* EF3030 in EF3030 colonised mice treated with 1A8 (anti-Ly6G) neutrophil depleting antibody or PBS 24hrs prior to infection. (*P* values represent Student's unpaired T-test, lines represent mean, error bars represent SD (D)). Results are from one experiment.

### 6.1.8.3 Absence of CD4+ cells

As previously discussed Th17 cells have been implicated in immunity to secondary pneumococcal colonisation. To assess if CD4+ T-cells were important in this model of protection from lung infection these cells were depleted prior to EF3030 challenge by administration of an anti-CD4 monoclonal antibody (GK 1.5), given as two does 48 and 24hrs prior to challenge. The efficacy of CD4+ T-cell depletion was assessed by flow-cytometry analysis of lung homogenates post-infection. Antibody depletion led to a 6 fold reduction in the number of CD4+ cells in the lung, compared to undepleted controls (**Figure 6.14**). This reduction could reflect antibody masking of CD4 cells rather than reduction in numbers of CD4+ cells, so depletion of CD4+ cells was confirmed by flow-cytometry to show a reduction in CD3+ CD8- cells (**Figure 6.14**).

*S. pneumoniae* EF3030 CFU in the BALF and lungs of mice 24hrs following IN challenge were not significantly different in previously colonised or control mice following CD4+ T-cell depletion (**Figure 6.15**), indicating that CD4+ cells are important in mediating protection from lung infection following EF3030 colonisation. CD4+ T-cell depletion also abrogated the IL-17 response to challenge 24hrs following challenge in colonised mice, indicating that CD4+ T-cells are likely the cellular source of IL-17 in the lungs of colonised mice, at least at this time point following EF3030 challenge (**Figure 6.15**). The effects of CD4+ T-cell depletion on cytokine or cellular responses 4hrs following *S. pneumoniae* infection were not assessed.
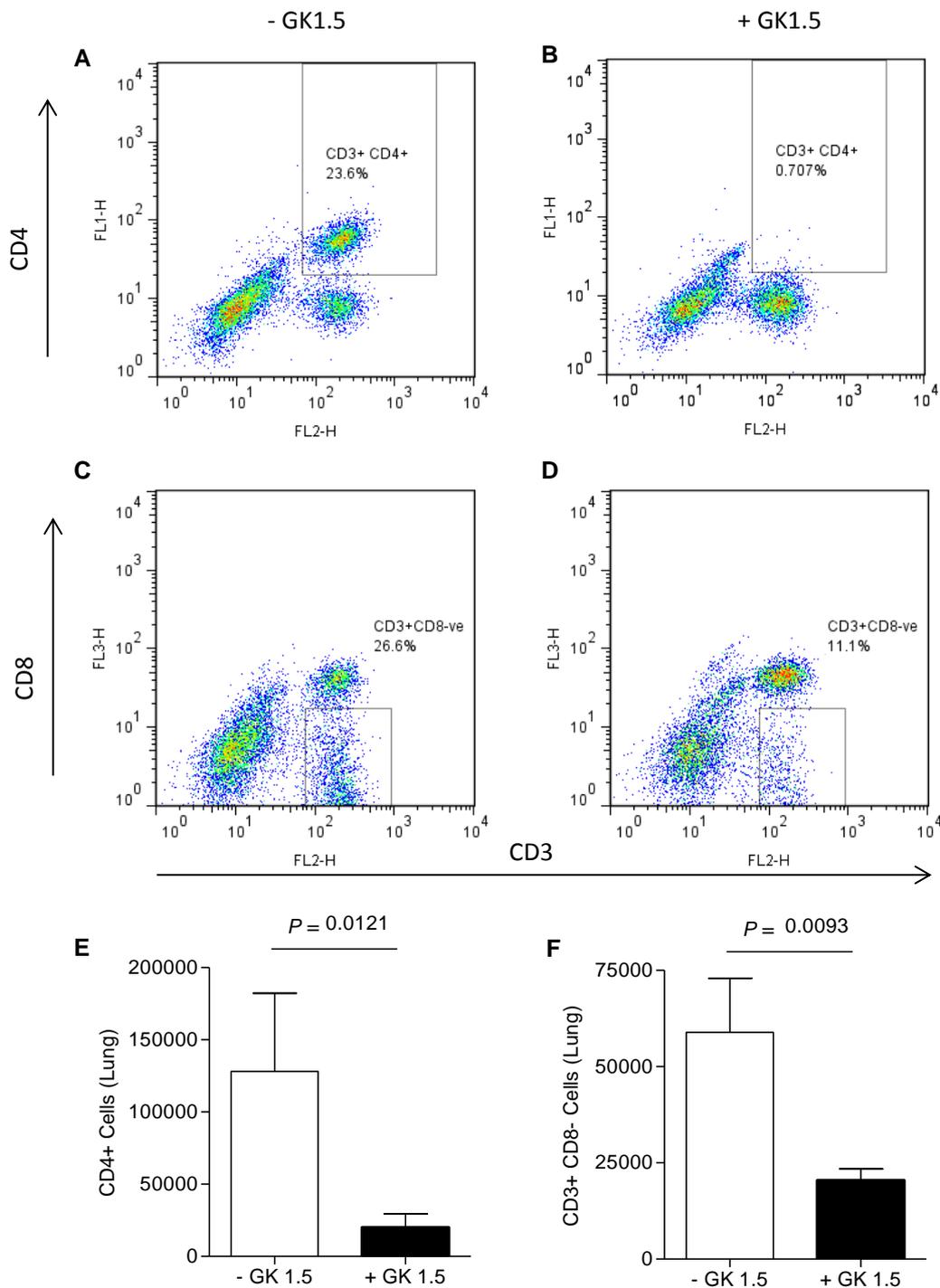
**Figure 6.14: CD4+ T-cells following depletion.**

(A,B) Representative flow-cytometry dot-plots of splenocytes showing gating on CD3+ CD4+ splenocytes from mice treated with anti-CD4 depleting antibody (GK1.5) or PBS. (C,D) Representative flow-cytometry dot-plots of splenocytes showing gating on CD3+ CD8- splenocytes from mice treated with anti-CD4 depleting antibody (GK1.5) or PBS. (E) Total CD4+ cells in the lungs of mice treated with GK1.5 (anti-CD4) antibody. (F) Total CD3+ CD8- cells in the lungs of mice treated with GK1.5 (anti-CD4 antibody). (*P* values represent Student's unpaired T-test, error bars represent SD). Results are from one experiment.
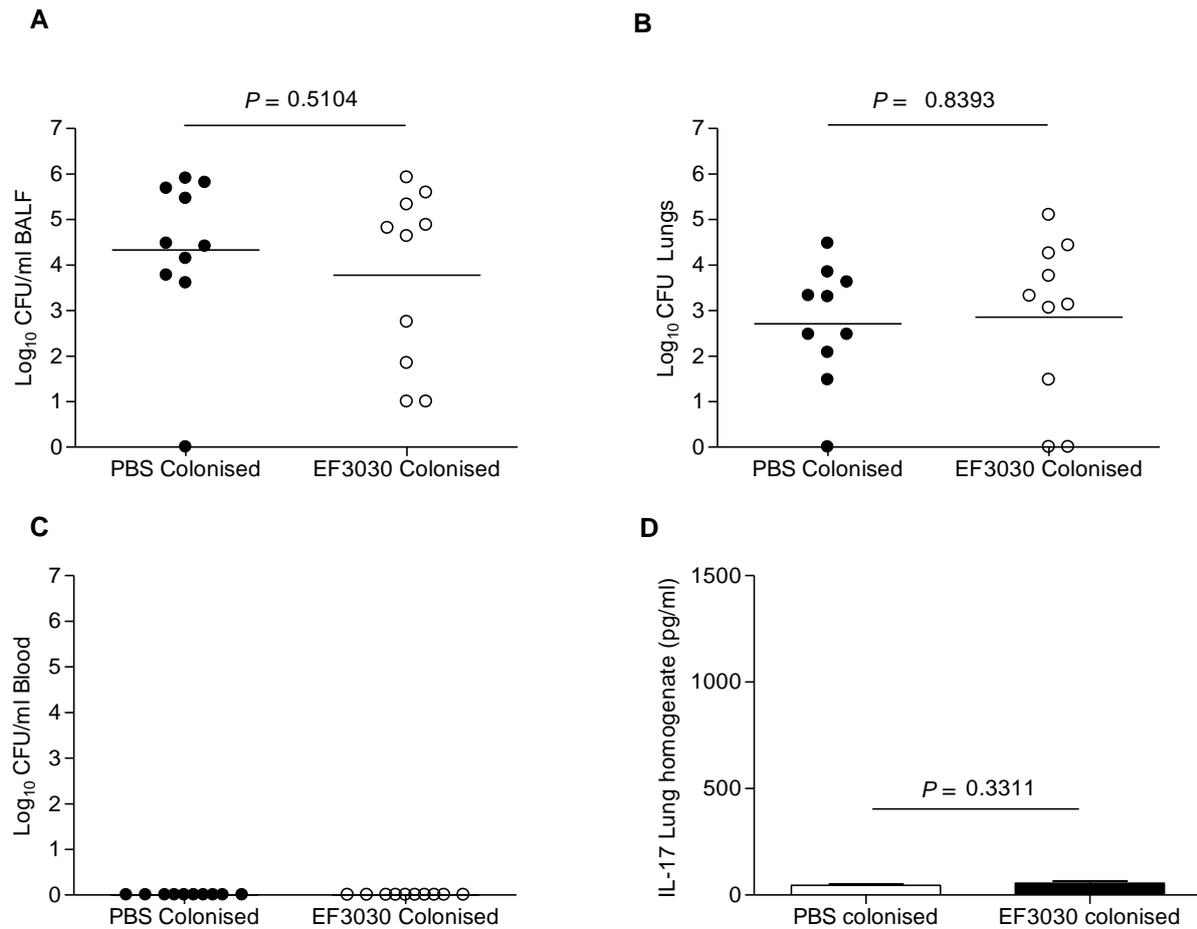
**Figure 6.15: CFU in CD4+ T-cell depleted mice following challenge.**

(A-C) CFU 24hrs following challenge with $2x10^7$ CFU *S. pneumoniae* EF3030 in BALF, lungs and blood of mice colonised with EF3030 or PBS treated IP with anti-CD4 antibody GK1.5 24 and 48hrs prior to infection. (D) Concentration of IL-17 in the lung homogenates of mice colonised with EF3030 or PBS, 24hrs following challenge with $2x10^7$ CFU EF3030, in mice treated IP with GK1.5 (CD4 depleting antibody) 24 and 48hrs prior to infection. (*P* values represent Student's unpaired T-test, lines represent mean). Results are from one experiment.

### 6.1.8.4    Absence of IL-17

The concentration of IL-17 was markedly reduced following antibody depletion of CD4+ cells, and depletion of CD4+ T-cells cells abrogated the protective effect of prior colonisation. To confirm If IL-17 itself had a role in colonisation-induced protection from infection, mice were treated with an IL-17 neutralising antibody (anti-IL-17A). Again there was no difference in bacterial numbers 24hrs post-infection in the BALF or lungs of EF3030 or sham colonised mice, following IL-17 neutralisation (**Figure 6.16**). This suggests that IL-17 is a key cytokine required for protection from EF3030 lung infection induced by prior colonisation in this murine model of non-invasive disease.
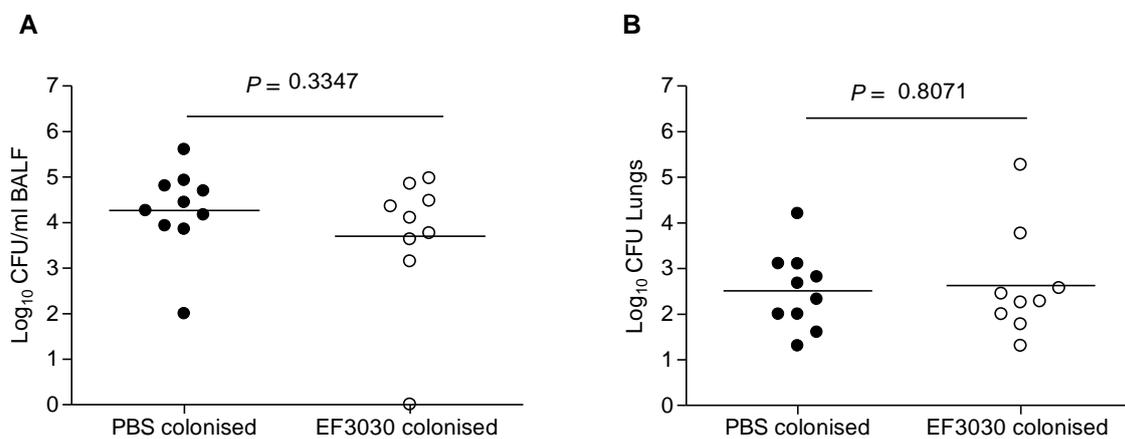


**Figure 6.16: CFU in IL-17 depleted mice following challenge.**

(A,B) CFU 24hrs following challenge with $2\times10^7$ CFU *S. pneumoniae* EF3030 in BALF and lungs of mice colonised with EF3030 or PBS treated IP with IL-17 neutralising antibody (anti-mouse IL-17A) 24hrs prior to infection. (*P* values represent Student's unpaired T-test, lines represent mean). Results are from one experiment.

### 6.1.9    Chapter summary

*S. pneumoniae* EF3030 is able to colonise the nares of C57/BL6 mice. Colonised mice developed a specific antibody response, associated with the development of IgG to protein antigens of *S. pneumoniae*. Additionally colonised mice displayed altered cellular responses to *S. pneumoniae* lung infection, including an early influx of neutrophils into the alveolar space and enhanced cytokine responses. Challenge experiments indicated that these immune responses to colonisation protected against lung infection. Repeat challenge in B-cell deficient and neutrophil depleted mice indicated that the protective effect of colonisation required both antibodies and neutrophils, suggesting that enhanced phagocytosis of bacteria opsonised with specific antibody is the main mechanism by which colonisation protects against subsequent lung infection. However, enhanced IL-17 and IL-23 responses in the lungs of colonised mice suggested a potential additional role for Th17 cell-mediated immunity in protection from lung infection following colonisation. Depletion of CD4+ cells confirmed that these cells were the likely source of IL-17 and important for the protective effect of colonisation against subsequent pneumonia. Furthermore, neutralisation of IL-17 confirmed that this cytokine was required for the protective effect of colonisation. These data suggest early T-cell responses in colonised mice may facilitate the early influx of neutrophils into the lung that can clear opsonised bacteria.

Combined these data indicate that both humoral and cellular immune responses to nasal colonisation are required for subsequent protection in a mouse model of non-invasive pneumonia. Whilst these data confirm the importance of IL-17 and CD4+ cells for protection, they do not necessarily rule out important roles for other cytokines including IL-10 and TNF-$\alpha$ that were both elevated in the lungs of previously colonised mice after challenge with *S. pneumoniae* EF3030.

# Summary of findings

o The *S. pneumoniae* polysaccharide capsule is not a major target of naturally-acquired IgG binding to the surface of *S. pneumoniae*.

o Naturally-acquired IgG targets proteins on the surface of *S. pneumoniae*.

o The major *S. pneumoniae* protein targets appear conserved in pooled IgG from different populations, and between different *S. pneumoniae* strains.

o IgG to non-capsular antigens facilitates agglutination, phagocytosis and killing of *S. pneumoniae*.

o Naturally-acquired human IgG mediates protection from lung infection and bacteraemia *in vivo*. This requires neutrophils and macrophages respectively.

o Experimental *S. pneumoniae* colonisation in mice protects against subsequent pneumonia, using a non-invasive model of infection with *S. pneumoniae* EF3030 (19F).

o *S. pneumoniae* EF3030 colonisation enhances cytokine (KC, TNF-α, IL-10, IL-23, IL-17), cellular, and antibody (IgG) responses in the lung to subsequent pneumonia challenge.

o Protection from lung infection with EF3030 following colonisation requires both humoral (IgG) and cellular (neutrophils, CD4+ T-cells) immune responses.

# 7 Discussion

## 7.1 *S. pneumoniae* targets of naturally-acquired IgG

### 7.1.1 Anti-polysaccharide IgG

Vaccine induced antibodies against the pneumococcal polysaccharide capsule clearly protect against *S. pneumoniae*, and can rapidly clear pneumococci from the blood (Alonso De Velasco et al., 1995). However, epidemiological evidence suggests anti-capsular antibodies may be redundant for the natural development of immunity to *S. pneumoniae* (Lipsitch et al., 2005), and the antigen targets for naturally-acquired IgG are not well understood. This thesis has investigated the naturally-acquired antibody response to *S. pneumoniae*.

Experimental colonisation of mice with *S. pneumoniae* EF3030 led to a rise in *S. pneumoniae*-specific IgG, but no detectable rise in IgG targeting the type 19F capsular polysaccharide, suggesting that following exposure in the nasopharynx the *S. pneumoniae* capsule is not a major target for the development of protective IgG. In other models of murine colonisation with *S. pneumoniae* D39 (serotype 2), there was also no detectable rise in IgG targeting the type-2 polysaccharide in the sera of colonised mice (Cohen et al., 2012). Furthermore, colonisation of mice with unencapsulated mutants induced protective IgG responses to *S. pneumoniae* (Malley et al., 2001, Cohen et al., 2012). In contrast anti-capsular IgG (against a number of different *S. pneumoniae* serotypes including 19F) was detectable in both human sera and pooled human IVIG products. The differences in mouse and human antibody responses may reflect recurrent exposure of humans to *S. pneumoniae* compared to a single experimental colonisation event in mice. The duration of colonisation events in humans is also longer than the two week colonisation period in mouse models (Turner et al., 2012). However, whilst human sera do contain IgG to the polysaccharide capsule, it is not necessarily the major *S. pneumoniae* antigen target of naturally-acquired IgG in humans. Multiple data obtained using IVIG suggest that capsular antigen does not mediate acquired immunity. Purified capsular polysaccharide was unable to inhibit IVIG or pooled human sera binding to *S. pneumoniae* TIGR4 by whole cell ELISA and binding of IVIG to the surface of a number of different *S. pneumoniae* strains was increased, not

reduced by removal of the polysaccharide capsule. Additionally, depletion of IgG targeting the type-4 polysaccharide capsule had no effect on IVIG binding to the surface of *S. pneumoniae* TIGR4 by flow-cytometry, and mice treated with depleted IVIG were still protected against experimental *S. pneumoniae* infection. Human sera also contained detectable IgG to *S. pneumoniae* CWPS. However, levels of CWPS-specific IgG in individual sera did not correlate with binding to *S. pneumoniae*, and purified CWPS was not able to inhibit IVIG binding to *S. pneumoniae* by whole cell ELISA, indicating it is not a major target of naturally-acquired IgG. These data are supported by previous investigations that demonstrated antibodies raised against the pneumococcal CWPS antigen did not protect mice from lethal *S. pneumoniae* challenge (Szu et al., 1986).

These data appear to contradict previous studies suggesting that opsonisation of *S. pneumoniae* may be exclusively dependent upon anti-polysaccharide IgG (Vitharsson et al., 1994, Vernacchio et al., 2000). These conclusions were made from data comparing opsonic activity of sera pre and post-polysaccharide vaccination, and not from assessing the naturally-acquired IgG targets in unvaccinated sera. Data presented here from both experimental colonisation and human sera/IVIG indicate that anti-capsular antibody may be dispensable for the protective effect of naturally-acquired IgG. With this in mind, assessing titres of anti-capsular antibodies in unvaccinated individuals may not be a good correlate of protection. In patients receiving IVIG therapy increased serotype-specific anti-capsular antibody levels did not provide additional benefit (reduced incidence of respiratory infection), compared to increasing the total IgG concentration alone (Chua et al., 2011).

Specific antibody deficiency (SAD) is a clinical condition described in both adults and children associated with impaired antibody responses to polysaccharide vaccination, and increased incidence of respiratory tract infection (Boyle et al., 2006). The assumption in these patients is that the increased incidence of recurrent respiratory tract infections is due to the lack of development of protective anti-capsular IgG. Perhaps this assumption requires re-examination in the context of data

presented here which indicate that anti-capsular IgG may not be required for the development of protective humoral immunity to *S. pneumoniae*. In support of this notion it has recently been suggested that in patients with SAD, the concentration of antibodies to purified polysaccharide by ELISA does not necessarily correlate with the functional (opsonophagocytic) activity of patient sera (Gelfand et al., 2013). The concentration of antibodies to pneumococcal proteins in this patient population has not been assessed. SAD may be a marker for more general immune defects and these rather than anti-CPS antibody could underpin the susceptibility to respiratory tract infection. For example, in a recent controlled study of SAD it has been demonstrated that many children diagnosed with SAD may have other underlying immunological defects including phagocytic disorders, (Ruuskanen et al., 2013).

### 7.1.2 Anti-protein IgG

Previous studies have demonstrated the acquisition of IgG to surface proteins following naturally-acquired and experimental *S. pneumoniae* carriage (Lebon et al., 2011, Ferreira et al., 2013). Experimental colonisation of humans with *S. pneumoniae* 6B increased serum IgG to a number of pneumococcal proteins including PspC, PspA, PcsB and PiuA, that was detectable 2 weeks following inoculation (Ferreira et al., 2013). Hence colonisation results in anti-protein antibody responses, and this is likely to be why Western blotting and Luminex assays indicated that adult sera and IVIG contain IgG to a range of pneumococcal surface proteins. Furthermore, data presented here demonstrate that colonisation of mice with *S. pneumoniae* EF3030 led to an increase in IgG against a limited number of specific *S. pneumoniae* protein antigens, including PhtD, PsaA and PpmA. Previous models of colonisation in mice with *S. pneumoniae* D39 also demonstrated increases in IgG to the pneumococcal proteins PspA, PsaA and PpmA but not to PhtD post-colonisation (Cohen et al., 2012). In comparison to individual human sera, mice colonised with *S. pneumoniae* EF3030 demonstrated antibody responses to relatively few protein targets. This perhaps reflects that humans are likely to have been colonised multiple times by different *S. pneumoniae* serotypes and therefore acquire significant antibody responses to a wider range of pneumococcal proteins. The effect on antibody responses of multiple colonisation events with different *S. pneumoniae* strains in mice has not been investigated.

The relative functional importance of pneumococcal proteins as targets for naturally-acquired IgG to *S. pneumoniae* has previously been unclear and was investigated in this thesis. With the exception of serotype 1 *S. pneumoniae* whole cell ELISA titres in different sera correlated with the concentration of IgG to different protein targets, and not to anti-capsular IgG. The structure of the type-1 polysaccharide is distinct from other serotypes in that it is zwitterionic and can be presented on MHC class II (Mertens et al., 2009). Therefore the development of IgG to the type-1 capsule may be T-cell dependent. Protease treatment of pneumococcal lysates impaired their ability to compete out

IgG in pooled human sera binding to *S. pneumoniae* TIGR4, indicating that the whole cell ELISA measured IgG binding to protease-sensitive proteins of *S. pneumoniae*. Trypsin treatment of lysates did not fully restore whole cell ELISA titres of IgG binding to *S. pneumoniae*, suggesting either that not all antigen targets for IgG are proteins or that trypsin treatment only partially denatured pneumococcal proteins, allowing some inhibition of IgG binding to be maintained. A limitation of whole cell ELISAs is that they are biased towards detecting antibodies to proteins as these are exposed by bacterial lysis (Cohen et al., 2013). However, flow-cytometry analysis to whole *S. pneumoniae* also indicated that IVIG binding to the surface of intact encapsulated *S. pneumoniae* was reduced by pre-incubation of *S. pneumoniae* with a protease (pronase). These data together therefore indicate that surface proteins are important pneumococcal antigen targets for naturally-acquired IgG present in IVIG preparations

Assessment of the potential target proteins for IgG by Luminex revealed well described vaccine candidates including PhtD, PspC and PspA as IgG targets in different IVIG preparations and adult sera. Multiplex assays do not provide a comprehensive assessment of antibody responses against all proteins of *S. pneumoniae.* Instead they allow the strength of antibody responses to a pre-selected panel of antigens to be determined semi-quantitatively. This allows comparison of the pattern of responses to previously characterised important surface-protein antigens in different sources of sera. This approach does mean responses to potentially-important antigens not included on the panel are missed. Targets of naturally-acquired IgG identified in this thesis have previously been identified as strong IgG targets in human sera by screening an expression library of pneumococcal proteins (Giefing et al., 2008), generated by randomly fragmenting the genome and expressing proteins on the surface of *E. coli*. This screen also identified proteins absent from the Luminex panel (for example PcsB) as dominant targets of IgG binding in human sera. The Luminex assay should therefore be considered as providing a 'snapshot' of responses to a range of pneumococcal protein targets. Screening of expression libraries although not biased by pre-selection of protein antigens

also has limitations and may be biased by the ability of the expression system to display different proteins.

It is possible that the Luminex assay does not provide an accurate quantification of antibody levels to each of the proteins included on the panel. However the ability of the Luminex assay to measure responses to PspC and PhtE was confirmed by ELISA, and the proteins PspA, PspC, and PhtD to which strong responses were detected by Luminex could also be detected as absent bands in Western blots of knockout strains probed with pooled IgG. Western blots of pneumococcal whole cell lysates in contrast to the multiplex assay allow a comprehensive assessment of antibody targets, but not necessarily the identification of antigens, except those for which mutant strains are available. 1-dimensional (1-D) Western blotting also suffers from the problem of poor resolution, and proteins of a similar molecular weight may not always be distinguishable from each other as separate bands. Due to these limitations the assessment of antibody responses to *S. pneumoniae* lysates separated by 2-dimensional (2-D) electrophoresis may be warranted. This involves the separation of proteins first by their isoelectric point and then by size (Choi et al., 2012). The better separation achieved by 2-D electrophoresis should allow responses to pneumococcal antigens in different sera and to different *S. pneumoniae* strains to be more completely compared. 2-D separation also facilitates the identification of antibody targets by mass-spectrometry. This would allow a comprehensive non-biased identification of the range of immunogenic protein antigen targets for IgG in different sera.

### 7.1.3 Surface-accessibility of *S. pneumoniae* protein antigens

Multiplexed assays and immunoblotting demonstrated that *S. pneumoniae* proteins are targets of naturally-acquired IgG in IVIG preparations and adult sera. For IgG to be relevant for protective immunity it will have to bind the bacterial surface to facilitate functional effects including opsonophagocytosis and bacterial agglutination. Flow-cytometry based binding assays allow assessment of IgG binding to the surface of whole bacteria (Cohen et al., 2013). Binding of IVIG to unencapsulated mutants of *S. pneumoniae* demonstrated that removal of the polysaccharide

capsule enhanced IgG deposition for most strains, further indicating the target for the binding of naturally-acquired IgG to the bacterial surface are sub-capsular antigens such as proteins. The major targets of IgG binding to unencapsulated mutant of *S. pneumoniae* may well be different to the WT parent strain, as the presence of a polysaccharide capsule can mask immunogenic surface proteins (Abeyta et al., 2003, Gor et al., 2005). To overcome this problem the surface binding assay was repeated using protease treated encapsulated *S. pneumoniae* TIGR4. Protease treatment reduced the binding of IVIG to the bacterial surface, indicating that protease-sensitive proteins are targets for naturally-acquired IgG even in the presence of the type-4 capsule. My data suggests that whilst the capsule may reduce IgG binding to some surface proteins, they are still potential targets for opsonisation with IgG. Antibodies raised against a number of purified pneumococcal surface proteins, including PspA and PspC, were still able to bind to the surface of WT (encapsulated) *S. pneumoniae* (Ren et al., 2003, Ricci et al., 2011) supporting this observation. The presence of a capsule does not therefore necessarily prevent antibody binding to proteins that appear to be major targets of naturally-acquired IgG, and antibodies to *S. pneumoniae* surface proteins could thus be functional for protection.

Protease treatment of *S. pneumoniae* as well as other Gram-positive bacteria including *Streptococcus pyogenes* has been used to screen for surface proteins that could be potential vaccine candidates (Olaya-Abril et al., 2012, Rodriguez-Ortega et al., 2006). In these studies the 'shaved' proteins have been separated by 2D gel electrophoresis and identified by mass spectrometry. This approach could be used to identify the surface proteins removed from *S. pneumoniae* by protease (pronase) treatment and allow a specific assessment of the targets of naturally-acquired IgG that are recognised on the surface of intact *S. pneumoniae*, and therefore are likely to be protective. Incubation with whole bacteria has also been used to deplete immune sera of antibodies to surface proteins, so allowing proteomic identification of antibody targets accessible on the surface of *S. pneumoniae* (Zhang et al., 2011). Hence incubation of *S. pneumoniae* in IVIG could be used to deplete antibodies to pneumococcal surface antigens, in order to investigate their function or

facilitate the comprehensive identification of surface-accessible protein targets of naturally-acquired IgG.

### 7.1.4    Consistency of IgG responses to *S. pneumoniae* in different sera/IVIG

Luminex data indicated that the protein targets of IgG binding in human sera and pooled immunoglobulin preparations appear conserved between populations. These assays demonstrated consistently high levels of anti- PhtD, PspA, PspC PsaA and Ply in geographically distinct sources of immunoglobulin, with weaker responses to PpmA, PhtE, Hyal, Sp0189, IgA1ase, and absent responses to several antigens including Eno, SlrA and NanA. Similarly, immunoblots demonstrated a similar pattern of bands with lysates of TIGR4 probed with pooled IgG from Europe, USA or Malawi. There is a surprising amount of genetic diversity between strains of *S. pneumoniae*, occurring on a number of levels. Firstly there is variation in gene content between different pneumococcal serotypes, and it has been suggested that as little as 50% of the pneumococcal genome may be 'core' (conserved across all strains) (Hiller et al., 2007). Hence the accessory genome is very large and could readily contain important antigens that are specific only for a subset of strains such as PsrP and the pilus proteins (Munoz-Almagro et al., 2010, Basset et al., 2007b). Secondly, numerous single-nucleotide polymorphisms have been detected amongst even closely related strains (Pandya et al., 2011, Croucher et al., 2011). Finally, allelic variants of a number of surface proteins have been well described especially the choline binding proteins like PspA and PspC (Iannelli et al., 2002, Hollingshead et al., 2000). Because of the uneven global distribution of *S. pneumoniae* serotypes (Gordon et al., 2003, Hausdorff et al., 2000b) different populations will have been colonised with different strains, which are likely to express different allelic variants of the variable surface proteins and have novel antigens encoded by the accessory genome. Despite this, our data indicate that immunoglobulin preparations and sera pooled from geographically distinct populations contain IgG that recognised a remarkably consistent pattern of protein antigens. Interestingly, the pooled immunoglobulin and sera tested all contained high levels of IgG that recognised PspA and PspC

isolated from a serotype 4 (TIGR4) strain of *S. pneumoniae* (as measured by Luminex). Therefore despite allelic variation, IgG pooled from different populations is able to recognise PspA and PspC from one particular genetic background. Studies by other investigators have indicated that children develop antibody specific to the PspA type they have been exposed to (Melin et al., 2008). In the same study adult sera contained significant levels of antibody to both PspA families. It could therefore be the case that as an individual's cumulative exposure to different strains of *S. pneumoniae* increases, so does their level of cross-reactive antibody to structurally-variant proteins such as PspA. This may be down to allelic variants sharing similar immunogenic epitopes that can induce cross-reactive antibodies, as has been demonstrated for PspA (Darrieux et al., 2008). Alternatively in a population different individuals are likely to be colonised with strains expressing different allelic variants and therefore pooled sera and IVIG preparations will contain IgG that react to most of the common allelic variants.

Pooled serum is by its nature not representative of every individual within a population. Instead it gives an 'average' response of those individuals that make up the pool. This is supported by the pattern of antibody targets in sera from different individuals to (as measured by Luminex) broadly reflecting those of pooled sera, with dominant responses to the same sub-set of pneumococcal antigens (PhtD, PspA, PspC and PsaA). Despite this, the relative response of each individual to a particular protein varied, with some individuals having lower responses to antigens to which most other sera had strong IgG responses. This variation is reflected both in Luminex assays of individual sera and Western blots against whole cell lysates probed with different individual sera, where for some individuals seemingly immunodominant bands were absent. Variation in responses to specific antigens in individual sera was seen both in volunteers from Malawi and the UK. This variation in individual responses to particular pneumococcal protein antigens may occur due to genetic variation of the host or due to differences in the strains that have colonised each individual (as discussed above). Different HLA alleles have been implicated in variant responses to protein antigens (Kruskall et al., 1992), and HLA variation may result in some individuals failing to mount antibody responses to

certain pneumococcal proteins. Host responses to pneumococcal proteins may also be affected by restriction in the range of peptides that can be presented by host MHC II (Brodsky et al., 1996), a requirement for the development of T-dependent IgG responses. Certain haplotypes of IL-4 and IL-4Rα have been associated with lower antibody responses following pneumococcal polysaccharide vaccination (Wiertsema et al., 2007), and these polymorphisms may also affect individual's antibody levels to specific pneumococcal proteins. Interestingly Western blotting against whole cell lysates indicated that sera from inbred mice all colonised with *S. pneumoniae* EF3030 displayed some variation in the intensity of IgG responses, acquired following colonisation. The reasons for this are unclear but could for example be as a result of varied expression of pneumococcal proteins; the potential impact of bacterial protein expression on the development of protective immunity is discussed later. On-going experimental colonisation models in mice and humans may help elucidate the important factors influencing an individual's immune response to different protein antigens following exposure to *S. pneumoniae*.

**7.1.5    Consistency of IgG responses to different *S. pneumoniae* strains**

Individual sera demonstrated whole cell ELISA IgG titres against several *S. pneumoniae* strains. The similar pattern of immunogenic bands on immunoblots against the different *S. pneumoniae* serotypes indicated that the protein antigens recognised by IgG are generally conserved across different *S. pneumoniae* serotypes. This suggests that genetic variation of *S. pneumoniae* does not necessarily prevent the natural development of antibodies to common protein targets, and furthermore also suggests that perhaps with some exceptions dominant antigens are not usually part of the accessory genome. There was for example no appreciable antibody response observed in any of the sera or pooled IgG preparations tested to one of the pilus proteins (PilusA),  a protein present in only a minority of pneumococcal strains  (Basset et al., 2007b). Whilst this may reflect poor immunogenicity of this particular protein it may also indicate a relative lack of exposure to the human immune system (on a population level) compared to proteins common to all strains of *S. pneumoniae.*

**7.1.6    Antibody Isotypes and IgG sub-classes**

This thesis focussed on the role of naturally-acquired IgG in the context of protection from lung infection, based on the observation that purified IgG protects against *S. pneumoniae* when passively transferred to patients with immunoglobulin deficiency (Quinti et al., 2011).  IgG has previously been shown to be the main sub-class induced following experimental *S. pneumoniae* colonisation in mice (Cohen et al., 2011). Other antibody isotypes may possibly have a role to play in naturally-acquired immunity to pneumococcal lung infection, however in the murine model of colonisation there was no significant rise in anti-*S. pneumoniae* IgA or IgM detected by whole cell ELISA.  Small rises in anti-*S. pneumoniae* IgA have previously been demonstrated in the lavage fluid of colonised mice (Cohen et al., 2011). These studies assessed IgA concentration in neat lavage fluid; whereas a dilution of 1:5 was used here (due to limitations in quantity). Therefore small increases in IgA post-colonisation

may have been missed. Other murine models have also demonstrated a modest rise in anti-*S. pneumoniae* IgM in the sera of mice post-colonisation (Cohen et al., 2011) and a rise in specific anti-capsular IgM (Cohen et al., 2011). However, no significant rise in anti-*S. pneumoniae* IgM post-colonisation by whole cell ELISA was detected in this thesis.

Each of the subclasses of human IgG (IgG 1-4) exhibit different effector functions. For example IgG1 and IgG4 can activate the complement protein C1 more than IgG2 (Jefferis and Kumararatne, 1990). Different antigens may also preferentially stimulate production of certain IgG sub-classes (Jefferis and Kumararatne, 1990). Studies investigating subclass responses to vaccination have indicated that the concentration of IgG1 following vaccination most closely correlated with enhanced opsonic activity of human sera following immunisation with the 23-valent polysaccharide vaccine (Bardardottir et al., 1990). This thesis has not specifically examined the natural acquisition of anti-pneumococcal IgG of different sub-classes, instead focussing on the functional targets of total IgG. The proportion of anti-pneumococcal IgG of different subclasses in IVIG represent those in human sera, acquired through natural exposure to *S. pneumoniae*. Therefore using IVIG as a surrogate of naturally-acquired IgG is not biased by over or under-representation of certain subclasses. Further understanding of the specific IgG sub-class responses required for naturally-acquired immunity to *S. pneumoniae* may however be important for the development of prospective vaccines.

### 7.1.7 Immunogenicity of *S. pneumoniae* proteins

The dominant protein targets of IgG in humans appear consistent irrespective of location and population. The proteins identified as being major targets of naturally-acquired IgG fall into a number of different classes. They include choline binding proteins (PspA, PspC), lipoproteins (PsaA), and non-classical surface proteins (PhtD). The reason for the immunogenicity of certain proteins over others in the development of an antibody response is not clear. It could be related to their abundance on the bacterial cell surface, an inherent ability to activate innate immune receptors, or preferential trafficking to local lymph nodes. The site of development of immune response to

pneumococcal proteins is also not well understood. Adenoidal mononuclear cells appear to be capable of producing IgG to protein antigens upon stimulation *in vitro* (Zhang et al., 2006b). Capsular polysaccharide antigen is deposited in the nasal associated lymphoid tissue following colonisation, in a process that requires neutrophils and the virulence factor pneumolysin (Zhang et al., 2006b). T-cell help is required for the development of IgG to pneumococcal proteins and IgG levels to a number of pneumococcal proteins including PspA and PspC are reduced in the sera of children with HIV infection (Ditse et al., 2013). It is possible that protein presentation to T-cells on MHC II may determine the immunogenicity of certain pneumococcal proteins. Following antigen exposure B-cells differentiate into either memory plasma cells that secrete immunoglobulin, or into memory B-cells which respond to subsequent antigenic stimulation (Yoshida et al., 2010). It is not entirely clear which population of cells is most important for the long term maintenance of serum antibody levels to pathogenic proteins (Amanna et al., 2007). Further understanding of the B-cell response to *S. pneumoniae*, including its site of development and the way in which B-cell memory persists may help to determine why antibodies to certain protein targets are maintained in adult sera.

### 7.1.8 *S. pneumoniae* gene expression and IgG responses

A number of studies have investigated the up-regulation of pneumococcal virulence genes during invasive disease (Mahdi et al., 2008, Orihuela et al., 2004). More recently the relative expression of genes between pneumococci isolated from the nasopharynx and lungs of challenged mice has been compared, and there is varied expression of certain pneumococcal proteins between these body compartments which may affect virulence (Ogunniyi et al., 2012). For example, maltose/maltodextrin-binding protein (MalX) was up-regulated in the lungs compared to nasopharynx. It could be that genes up-regulated specifically during bacterial invasion into the lungs (or blood) may be important functionally as targets for protective IgG responses. Furthermore, antibodies induced following colonisation to proteins highly expressed in the nasopharynx may not be protective against lung infection if there is reduced expression of these proteins when the

bacteria enter the lungs. However, naturally-acquired IgG (acquired through colonisation) was protective against subsequent pneumococcal lung infection, both in a mouse model of colonisation or by passive transfer of human IVIG in to mice. Therefore despite variation in bacterial gene expression between these body compartments, antibodies induced following colonisation are still protective in the lung. Additionally, cell-mediated immunity acquired during colonisation still appears to be effective against subsequent pneumonia challenge, despite differences in expression of potentially immunogenic proteins of *S. pneumoniae*.

As well as pneumococcal gene expression potentially affecting the development of antibody responses, IgG responses to specific protein antigens may affect the molecular ecology of *S. pneumoniae*. Sequencing of the PMEN1 isolate of *S. pneumoniae* over time has identified the genes encoding the surface proteins PspA and PspC as 'recombination hotspots', where horizontal gene transfer events occur at higher frequency (Croucher et al., 2011). These gene transfer events may alter antigen expression and suggest that these surface proteins are under selective pressure. This selective pressure may be due to the development of naturally-acquired IgG to these proteins, high levels of IgG to PspC and PspA were detected in all sera samples assessed in this thesis.

## 7.2 Functional effects of naturally-acquired IgG

### 7.2.1 Protective effects of IgG

Passive transfer of IVIG protected mice from lung infection with *S. pneumoniae* TIGR4, reducing CFU in the lungs and clearing *S. pneumoniae* from the blood 24hrs post-challenge. Following EF3030 colonisation mice were protected from re-challenge and had reduced CFU in the lungs and BALF 24hrs post-challenge. This protective effect was lost in μMT (antibody deficient) mice. Together these data indicated an important role for IgG in naturally-acquired immunity to *S. pneumoniae*. *In vitro* assays and cellular depletion experiment *in vivo* were used to assess the mechanisms by which antibodies could be protective against *S. pneumoniae* infection.

### 7.2.2 IgG mediated bacterial agglutination

Bacterial agglutination has been described as a mechanism by which antibodies may mediate protection. In 1915 it was noted that pneumococcal antisera when injected into rabbits with pneumococcal bacteraemia, induced clumping of *S. pneumoniae in vivo* and facilitated the clearance of bacteria from the blood (Bull, 1915b). More recently it has been demonstrated that anti-capsular antibody can mediate bacterial agglutination, which can promote complement dependent killing of *S. pneumoniae* (Dalia and Weiser, 2011). The targets of naturally-acquired IgG that may facilitate *S. pneumoniae* agglutination have not previously been investigated. Data in this thesis demonstrated that incubation in IVIG results in agglutination of both encapsulated and unencapsulated *S. pneumoniae* TIGR4. This indicates that naturally-acquired IgG can target non-capsular antigens to mediate bacterial agglutination. Addition of 10% IVIG to the culture medium inhibited growth of a number of *S. pneumoniae* strains and their unencapsulated derivatives, as assessed by a change in OD. As well as agglutination, naturally-acquired IgG may have additional effects on *in vitro* growth of *S. pneumoniae*, for example binding to and inhibiting the function of pneumococcal proteins involved in nutrient acquisition.

### 7.2.3 IgG mediated phagocytosis and killing

Ingestion and killing of opsonised bacteria by phagocytes including macrophages and neutrophils is essential for host defence against *S. pneumoniae* (Standish and Weiser, 2009, Marriott and Dockrell, 2007). Serotype-specific IgG enhances phagocytosis of *S. pneumoniae* (Burton and Nahm, 2006) and improved opsonophagocytosis is a major readout for the effectiveness of current polysaccharide-based vaccines (Song et al., 2013). Antibodies to *S. pneumoniae* surface proteins can also facilitate enhanced phagocytosis (Jomaa et al., 2005, Arulanandam et al., 2001). In this thesis opsonisation with IVIG increased the association of *S. pneumoniae* with both neutrophils and macrophages. The opsonising effect of IVIG was enhanced upon removal of the *S. pneumoniae* capsule, indicating that naturally-acquired IgG in IVIG may facilitate phagocytosis, targeting non-capsular antigens. Opsonisation in IVIG also improved the killing of both unencapsulated and WT *S. pneumoniae* by human neutrophils, further demonstrating that naturally-acquired IgG against non-capsular targets is functional. Phagocytic assays were performed in the absence of complement, therefore enhanced uptake of unencapsulated *S. pneumoniae* was independent of any effects of complement deposition, and directly due to opsonisation of IgG. Cellular depletion studies demonstrated that both neutrophils and macrophages were important for the protective effect of IgG *in vivo*, indicating *in vitro* assays of phagocyte function are relevant for assessing the potential protective effect of naturally-acquired IgG.

### 7.2.4 IgG accumulation in the lungs post-challenge

The low level of IgG in airway lining fluid prior to infection indicates that prevention of infection by IVIG is unlikely. Indeed in the model of passive IgG transfer and lung infection presented in this thesis, adoptive transfer of naturally-acquired human IgG failed to protect against early lung infection or colonisation. IgG may accumulate in the lungs by active transport (Kim et al., 2004), local production in response to antigen (Bice and Muggenburg, 1996), or passive leak (Renegar et al., 2004). In mice treated with IVIG the accumulation of human IgG correlated with albumin

concentration in the BALF. IgG accumulation post-infection in the BALF of EF3030 colonised mice also correlated with albumin concentration, and enhanced accumulation of total IgG in the lavage fluid of EF3030 colonised mice was temporally associated with a neutrophil influx. Combined these data indicate that much of the IgG in the lung post-infection may be due to passive leak as a consequence of inflammation.

The IVIG challenge model used in this thesis involved adoptive transfer of human IVIG into mice. Unlike when transferring murine IgG, human IgG was undetectable in the alveolar space in the absence of infection, demonstrating IgG accessibility to the lung in the absence of inflammation may be species dependent. Therefore mouse models cannot be used to fully evaluate the role for human IgG in lung immunity to *S. pneumoniae*. For example, this model does not properly assess the relevance of IgG for bacterial interactions with alveolar macrophages prior to established lung infection, which may be important for early clearance of *S. pneumoniae* (Gordon et al., 2000). Pre-existing IgG in the lungs of EF3030 colonised mice led to reduced CFU in the BALF 24hrs post-challenge. In contrast, mice treated with IVIG had no human IgG detectable in the BALF prior to challenge and no reduction in CFU in BALF 24hrs post-challenge with *S. pneumoniae* TIGR4. The potential role of pre-existing IgG in protection from pneumococcal lung infection in humans is highlighted by the detection of anti-*S. pneumoniae* IgG in the lavage fluid of healthy volunteers. Though compared to sera low levels of IgG were detected in human BALF samples, obtaining these samples involves significant dilution of the epithelial lining fluid, and hence IgG concentration in neat epithelial lining fluid is likely substantial. Despite limitations with this model, passive transfer of human IgG to mice provided an important tool to study the mechanisms of protection post-infection with *S. pneumoniae* by naturally-acquired human IgG *in vivo*, using cellular depletion strategies that clearly could not be performed in humans.

### 7.2.5 Effect of IVIG on inflammatory responses to *S. pneumoniae* infection

IVIG may have immunomodulatory effects and has been used in the treatment of a number of autoimmune and inflammatory conditions (Schwab and Nimmerjahn, 2013). In a caecal-ligation and puncture (CLP) model of sepsis high dose IVIG (1000mg/kg) delivered intravenously to rats improved survival, and this was associated with reduced TNF-α levels in the sera 3hrs post-CLP and reduced IL-6 6hrs post-CLP (Hagiwara et al., 2008). IVIG treatment also reduces inflammatory cell infiltration into the lungs 12hrs following CLP (Hagiwara et al., 2008). The effect of this IVIG therapy on bacterial numbers in the blood post-CLP was not assessed, and the effect on inflammation may have been related to IgG mediated clearance of bacteria following IVIG treatment. Cytokine concentrations in the sera of mice challenged with *S. pneumoniae* TIGR4 were not measured, however both TNF-α concentration in lung homogenates and neutrophil infiltration into the alveolar space were unaffected by IVIG treatment 24hrs post-infection. This indicates that changes in the inflammatory response were not necessarily responsible for the observed protective effect of adoptively transferred IVIG 24hrs post-lung infection. In this IVIG doses of approximately 500mg/kg were administered and delivered via the IP route, therefore the concentration of IgG achieved in the sera was approximately 1.5mg/ml. Normal doses for humans on IVIG replacement therapy are between 400-600mg/kg IgG administered monthly (Gelfand et al., 2013).

## 7.3 Cell-mediated immunity

### 7.3.1 Cellular effectors mediating naturally-acquired immunity

The importance of neutrophils in host defence against bacterial respiratory infection is highlighted by the higher incidence of pneumonia in patients with neutropenia (Lanoix et al., 2012). Neutrophils were required for the protective effect of prior colonisation against EF3030 pneumonia. Previously colonised mice demonstrated a heightened early influx of neutrophils (4hrs post-challenge) compared to controls. Although differences in bacterial numbers in the lungs of mice were only observed by 24hrs post-infection, it is likely that this early neutrophil response contributed to the control of *S. pneumoniae* infection in colonised mice. In the nasopharynx, immunity to re-colonisation has been demonstrated to depend on neutrophil influx into the nasal cavity following challenge (Zhang et al., 2009). Significant increases in KC (CXCL1-the murine functional homologue of human IL-8) were observed 4hrs following challenge in the lungs of colonised compared to control mice. KC has been well described as a neutrophil chemoattractant and it is possible that this increase in KC contributes to the early influx of neutrophils into the alveolar space of colonised mice. CXCL1(-/-) mice infected with *Klebsiella pneumoniae* demonstrate a reduced neutrophil influx into the lungs, as well as reduced production of the inflammatory mediator leukotriene B4 (LTB4). In these studies exogenous LTB4 treatment restored neutrophil migration to the lungs (Batra et al., 2012). The potential role of LTB4 in the enhanced neutrophil responses to lung infection following experimental colonisation has not been assessed in this thesis.

In addition to neutrophils the protective effect of prior colonisation on lung infection also required antibodies. It is therefore likely that neutrophils phagocytose EF3030 opsonised by specific antibody induced following colonisation. It has been demonstrated in the nasopharynx that neutrophils are important for the clearance of secondary but not primary *S. pneumoniae* colonisation, and neutrophils may be functional against secondary challenge due to the presence of opsonising antibody (Zhang et al., 2009). In other models of murine colonisation with *S. pneumoniae* D39

opsonisation in sera from colonised mice improved *S. pneumoniae* uptake by human neutrophils *in vitro*, compared to sera from uncolonised controls (Cohen et al., 2011).

Enhancement of neutrophil mediated phagocytosis is one mechanism by which naturally-acquired human IgG can prevent *S. pneumoniae* lung infection. Other mechanisms of neutrophil mediated protection against extracellular bacteria have also been described. Neutrophils may contribute to the killing of extracellular pathogens including *S. pneumoniae* by the formation of neutrophil extracellular traps (NETs) (Brinkmann et al., 2004, Yamada et al., 2011). These NETs consist of released chromatin and anti-microbial granule proteins. NET formation has been associated with IFN-γ production by neutrophils in response to *S. pneumoniae* infection (Yamada et al., 2011). However, *S. pneumoniae* expresses an endonuclease (endA) that may allow it to escape from NETs (Beiter et al., 2006). The contribution of NET formation to *S. pneumoniae* killing *in vivo*, and the potential relevance of antibody in this process remains unclear.

Although neutrophils were required for the protective effect of naturally-acquired IgG, the data suggest they may also contribute to the pathogenesis of disease. Neutrophil depletion significantly reduced the number of bacteria in the blood of untreated mice following *S. pneumoniae* lung challenge, indicating that neutrophils may contribute to the development of bacteraemia. In other models of pulmonary *S. pneumoniae* infection mice depleted of neutrophils are protected from bacteraemia, and demonstrate enhanced survival following challenge with $5 \times 10^3$ CFU serotype 8 *S. pneumoniae* (Marks et al., 2007). Results presented here demonstrate that neutrophil depletion also significantly increases *S. pneumoniae* TIGR4 CFU in BALF 24hrs post-challenge. Therefore although neutrophil influx may facilitate bacterial invasion into the blood, neutrophils are required for the control of infection within the lung. Hence a regulated early influx of neutrophils may achieve clearance of *S. pneumoniae* from the lungs without contributing to bacteraemia. The enhanced susceptibility of male mice to pneumococcal disease may be in part due to neutrophil kinetics (Kadioglu et al., 2011a). Female mice demonstrate an earlier neutrophil influx post-infection, but

significantly reduced neutrophil numbers compared to male mice 24hrs following infection. Data in this thesis indicate the increased development of sepsis in male mice may be in part due to this enhanced late neutrophil influx and associated inflammation, perhaps by increasing pulmonary epithelial barrier breakdown.

Macrophages appear to be important for the increased clearance of *S. pneumoniae* from the blood mediated by human IgG. Systemic clodronate treatment of mice was associated with depletion of splenic macrophages and impaired clearance of *S. pneumoniae* from the blood following IVIG treatment.  IVIG treatment may also reduce (though not completely clear) *S. pneumoniae* in the blood of splenectomised mice, via phagocytosis in the liver (Nakamura et al., 2013). The effect of clodronate treatment on cells of the liver was not assessed in this thesis, though Kupffer cells of the liver can be depleted by IV administration of liposomal clodronate (Meijer et al., 2000). However, the importance of the spleen in the clearance of blood-borne *S. pneumoniae* can be seen in the higher incidence of *S. pneumoniae* bacteraemia in patients with asplenia (Schutze et al., 2002).  In a model of sepsis with group B streptococci specific antibodies required the complement protein C3 to effectively clear *S. pneumoniae* from the blood (Wessels et al., 1995). It is likely that complement, in addition to macrophages is required for the protective effect of naturally-acquired IgG against sepsis, and IgG in human sera can facilitate C3b deposition on encapsulated and unencapsulated strains of *S. pneumoniae* (Hyams et al., 2010a). IgG could mediate protection from sepsis following lung infection by preventing invasion of bacteria into the blood from the lungs, rather than facilitating clearance of *S. pneumoniae* from the blood. However,  in  a study of IVIG treatment alone and in combination with ampicillin Intranasal administration of IVIG failed to prevent sepsis following lung infection with *S. pneumoniae* pn4241, whereas intravenous administration did (De Hennezel et al., 2001). Additionally, in data presented here human IgG treatment led to direct clearance from the blood following IV challenge with *S. pneumoniae.*

### 7.3.2  Cytokine responses to colonisation

A number of cytokines were increased in the lungs of EF3030 colonised mice following challenge. Of particular interest was an increase in Th17-type cytokines (IL-23, IL-17). Th17-type responses are important for acquired immunity to *S. pneumoniae* colonisation in mouse models (Zhang et al., 2009). In particular IL-17A responses in the nasopharynx are important for protection from re-colonisation and IL-17A depletion abrogates the protective effect of prior colonisation (Zhang et al., 2009). In models of invasive D39 pneumonia, prior colonisation lead to enhanced IL-17 and IL-22 responses in the lavage fluid 18hrs post-challenge. However these responses appeared dispensable for protection against lung challenge with this highly invasive strain, in which lethality is largely due to the development of septicaemia.

Protective immunity to non-invasive pneumonia challenge with EF3030 developed as a result of previous colonisation was abrogated by IL-17A depletion. IL-17A has a role in the recruitment of neutrophils and can induce the release of the neutrophil chemoattractant KC from other cell types including epithelial and endothelial cells (Swaidani et al., 2009). An early increase in IL-17 concentration following EF3030 challenge was temporally associated with increased KC concentration in colonised mice. This suggests that IL-17 release post-infection may facilitate KC production, though it was not assessed if IL-17 was directly responsible for the enhanced KC response 4hrs post-challenge in the lungs of colonised mice. IL-23 contributes to the differentiation of naive CD4+ T-cells into Th17 cells (Silverpil et al., 2013). IL-23 is released from alveolar macrophages, mice lacking the IL-23 subunit alpha gene (IL-23p19) have impaired lung defence against the fungal pathogen *Pneumocystis carinii*, associated with reduced IL-17 and CD4+ T-cell responses to infection. IL-23 was increased in the lungs of EF3030 colonised mice at 4 but not 24hrs following *S. pneumoniae* challenge. This early increase in IL-23 perhaps reflects its role in the initiation of IL-17 responses that were maintained for up to 72hrs post-challenge in the lungs of colonised mice.

As well as Th17-type cytokine responses, previous EF3030 colonisation led to an increase in levels of TNF-$\alpha$ and IL-10 in the lungs of mice following infection. TNF-$\alpha$ was also elevated 4hrs post-challenge in models of *S. pneumoniae* D39 colonisation and challenge (Cohen et al., 2011). TNF-$\alpha$ is important for innate immunity to *S. pneumoniae* infection and TNF-$\alpha$ can stimulate the production of the neutrophil chemoattractant KC from murine lung cells (Sun et al., 2007). IL-10 is a regulatory cytokine produced by regulatory T-cells (Tregs). IL-10 production occurs in human adenoidal mononuclear cell in response to *S. pneumoniae* stimulation (Zhang et al., 2006a). IL-10 producing Tregs have been implicated in protection from primary *S. pneumoniae* lung infection, in particular protection from bacteraemia following challenge with *S. pneumoniae* D39 (Neill et al., 2012). This protection has been associated with reduced inflammation, preventing translocation of bacteria from the lungs to the blood. On the contrary other investigators have advocated that IL-10 may be negative for the outcome of primary pneumonia challenge in mice. Following lung-infection with *S. pneumoniae* ST3 IL-10 depletion reduced bacterial burden in the lungs of mice, which was associated with increased TNF-$\alpha$ production (van der Poll et al., 1996). The role of IL-10 may depend upon the pneumonia model used, including the invasiveness of the strain and the host genetic background. For example, CBA/Ca mice have a heightened susceptibility to infection due to reduced regulatory responses compared to BALB/c mice (Neill et al., 2012). The significance of IL-10 in protection from lung infection induced by colonisation in this model is not clear; however the presence of IL-10 did not prevent a rise in TNF-$\alpha$, or clearance of lung infection in colonised mice. Depletion of IL-10 in colonised mice would help to determine its functional relevance for protection in this model. Other cytokines that could have a role in protection from pneumococcal lung infection including IFN-$\gamma$ were not measured in colonised mice following challenge. Assessing a wider range of cytokines (perhaps utilising a multiplex platform) would provide a more complete picture of the different responses to lung infection following prior colonisation.

### 7.3.3 T-cell responses acquired following colonisation

In the model of non-bacteraemic pneumonia presented here CD4+ T-cells were required for the protective effect of prior colonisation. The absence of IL-17A in the lungs of challenged mice depleted of CD4+ cells indicates that these cells are the cellular source of the enhanced IL-17 detected in the lungs of colonised mice. Previous studies of sub-lethal infection with *S. pneumoniae* ST1, followed 7 days later by challenge, have demonstrated (by intracellular cytokine staining) that CD4+ T-cells from the lungs of protected mice produce high levels of IL-17A (Marques et al., 2012).

γδ T-cells may be an important T-cell type required for IL-17 production in response to primary infection of the lungs (Ma et al., 2010). TCRδ(-/-) mice display significantly reduced IL-17 production 4hrs post-challenge with *S. pneumoniae* ST3, and reduced clearance of *S. pneumoniae* ST3 from the lungs 24hrs post-challenge (Ma et al., 2010). However, most gamma-delta T-cells lack CD4 expression (Lockhart et al., 2006), and thus would not have been depleted by anti-CD4 antibody treatment. Therefore the cells responsible for IL-17 production in this model are either a subset of γδT-cells expressing CD4 or αβ T-cells. A process of antigen presentation by γδT-cells, to CD4+ αβT-cells has been described (Collins et al., 1998). It is therefore possible that via this mechanism both cell types are required for the enhanced IL-17 production in response to lung infection in mice previously colonised with EF3030.

In models of *S. pneumoniae* colonisation, protection from re-colonisation mediated by CD4+ IL-17+ cells occurs over the course of 5 days (Zhang et al., 2009). In contrast protection from re-infection in this model of pneumonia is seen as early as 24hrs post-challenge, but still required CD4+ T-cell responses. Although 24hrs seems very quick for the development of a protective T-cell response, T-cells isolated from mice colonised with *S. pneumoniae* have demonstrated high levels of intracellular IL-17 after only 6hrs of stimulation with heat killed *S. pneumoniae ex vivo* (Zhang et al., 2009).

Experimental pneumococcal carriage in humans can lead to an increase the percentage of CD4+ cells expressing IL-17 in BALF samples, following stimulation with *S. pneumoniae ex vivo* (Wright et al., 2013). Data from EF3030 colonised mice indicate that these Th17-type responses in humans may be relevant for protection from subsequent pneumonia. The higher incidence of pneumococcal disease in patients with HIV and associated reduction in CD4+ cell numbers further suggests an important role for T-cells in immunity to *S. pneumoniae* in humans (Hyams et al., 2010b, Dworkin et al., 2001). Impaired Th17 cell differentiation occurs in individuals with hyper-IgE syndrome, which is associated with an increased risk of bacterial lung infection (Milner et al., 2008). Patients with T-cell immunodeficiency may be at a higher risk of pneumonia due to a lack of naturally-acquired Th17-type immunity to *S. pneumoniae*.

Some *S. pneumoniae* antigens that can elicit of Th17-type immunity have been identified. Proteins of *S. pneumoniae* have been screened based on their ability to stimulate IL-17 secretion from the splenocytes of mice immunised intranasaly with a killed whole cell pneumococcal vaccine. Proteins identified in this manner include a ribosomal protein SP0862 and SP1534 a pyrophosphatase. Intranasal immunisation with these proteins is protective against nasal colonisation (Moffitt et al., 2012). Separately, human CD4+ T-cells co-cultured with dendritic cells may produce IL-17 in response to stimulation with pneumococcal peptidoglycan, following secondary exposure *in vitro* (Olliver et al., 2013).

## 7.4 Schematic of naturally-acquired immunity to *S. pneumoniae* lung infection

Data presented in this thesis demonstrate that exposure to *S. pneumoniae* in the nasopharynx results in the development of immunity that is protective against future lung infection. In-particular data implicate an important role for both Th17 and humoral immunity. However, as discussed, additional arms of the immune response that are potentially protective against lung infection are enhanced following nasopharyngeal colonisation with *S. pneumoniae*. **Figure 7.1** summarises responses to colonisation that enhance or may enhance immunity to subsequent *S. pneumoniae* infection within the lung, and the potential interactions between each.
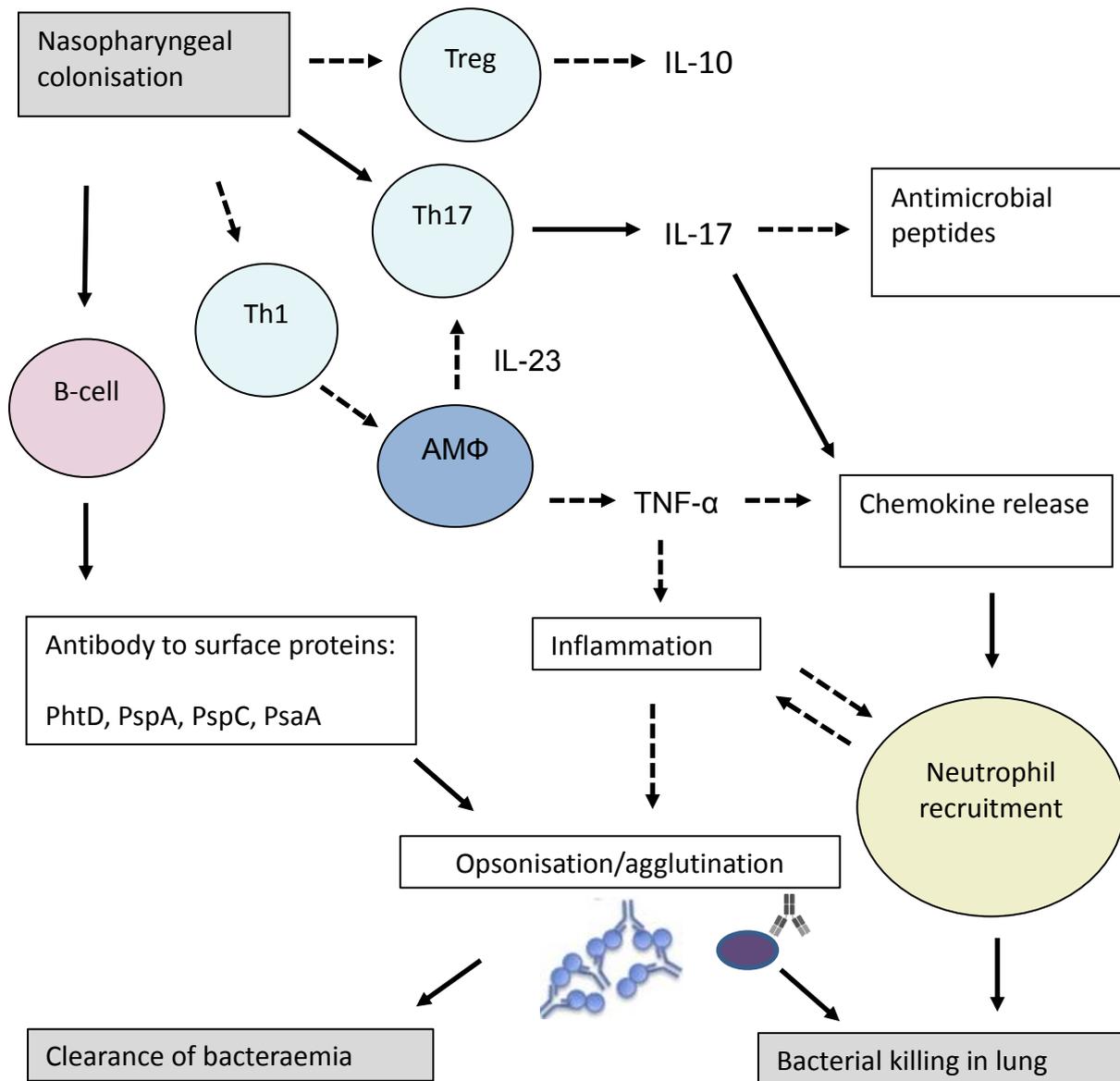
**Figure 7.1: Mechanisms of acquired immunity to *S. pneumoniae* lung infection.**

Schematic demonstrating the potential effects of nasopharyngeal colonisation on immunity to subsequent lung infection with *S. pneumoniae*. Solid arrows indicate mechanisms of acquired immunity directly supported by data in this thesis, dashed arrows represent other potential mechanisms of acquired immunity and their possible interactions.

## 7.5    Relevance for vaccine development

Polysaccharide-based vaccines for *S. pneumoniae* suffer from a number of limitations including poor coverage and serotype replacement. Current purified polysaccharide vaccines used in adults are poorly protective against pneumonia (Dear et al., 2003), though the use of conjugate vaccines in adults may provide improved protection within the lung (Paradiso, 2012). Any new pneumococcal vaccine*s* should have protection against pneumonia as an aim, since it represents the highest burden of disease caused by *S. pneumoniae* (O'Brien et al., 2009).

Several vaccines based on conserved *S. pneumoniae* protein antigens are in development (Darrieux et al., 2013). These vaccines aim to induce protective antibodies to surface proteins of *S. pneumoniae*.  A number of protein based vaccines have demonstrated protection in animal models of disease, and  some including PhtD and PspA are immunogenic in adult humans (Darrieux et al., 2013).  Based on data demonstrating that a Th17 based immunity is required for immunity to colonisation, vaccines aimed at stimulating cell-mediated immunity to *S. pneumoniae* are also in development (Moffitt et al., 2011).

Data presented in this thesis demonstrate the need for both humoral and cell-mediated immunity in full protection from non-invasive pneumonia following colonisation. Any new vaccine that aims to provide protection from infection within lung may need to recapitulate both arms of this protective immune response. The proteins that induce protective antibody responses following colonisation in mice or humans include (but are not limited to) PhtD, PspA, PspC and PsaA. These appear distinct from the pneumococcal protein antigens that may stimulate cell-mediated immunity to *S. pneumoniae* (Moffitt et al., 2012). Vaccines against pneumonia may therefore be most effective if they contain combinations of antigens that stimulate both cell-mediated and humoral immunity. This could be either as a whole cell vaccine or as combination protein based vaccines.

A number of protein antigens that are in development as pneumococcal vaccines are also targets of naturally-acquired IgG in adult human sera. This raises the question of how effective vaccines inducing antibodies against these proteins may be, if such antibodies are already present and functional in normal sera. It may be that boosting antibodies to antigens that are also targets of naturally-acquired IgG may only be effective in subjects with reduced levels of naturally-acquired IgG to those antigens. Interestingly IgG to *S. pneumoniae* protein antigens including PspC, PhtD and PspA are reduced in sera from older individuals (>65) compared to younger controls (Simell et al., 2008); potentially this may be one reason why the elderly are at more risk of *S. pneumoniae* infection. It is therefore possible that boosting antibodies to these proteins may help reduce the increased risk of *S. pneumoniae* pneumonia in this population.

The phagocytic function of human sera has been correlated with polysaccharide-specific IgG responses post-vaccination (Bardardottir et al., 1990). Current vaccination boosts phagocytosis by inducing capsule-specific IgG. If vaccines against common protein antigens of *S. pneumoniae* are to be successful they would have to boost the function of sera at least as much as current polysaccharide vaccines, but on the background of non-capsular (protein) antigens already being mediators of naturally-acquired protection. There is therefore the potential for the failure of functional improvement in antibody responses following vaccination with protein antigens, due to the presence of pre-existing antibody acquired through colonisation.

Both data from EF3030 colonised mice and human sera indicate that colonisation has an immunising effect that protects against subsequent lung infection. One key aim of pneumococcal vaccination could be the elimination of carriage (Bogaert et al., 2004), so removing the reservoir for transmission of *S. pneumoniae* for the development of invasive disease (Bogaert et al., 2004). However, removal of carriage is likely to reduce the opportunity for the acquisition of natural immunity to *S. pneumoniae*, which can protect against invasive disease.  A recent rise in the adult incidence of whooping cough has been attributed to a lack of natural exposure to *Bordatella pertussis* during

childhood, as a consequence of vaccination (Lavine et al., 2011). It is possible that a similar increase in pneumococcal pneumonia could occur following a vaccine-induced reduction in pneumococcal carriage.

## 7.6　Future directions

These data demonstrate the function for naturally-acquired IgG to pneumococcal protein antigens in human sera. However, a number of different factors are likely to influence an individual's risk of pneumococcal pneumonia. Therefore in order to assess if levels of naturally-acquired IgG may be clinically relevant, a more thorough assessment of antibody levels to *S. pneumoniae* proteins in patient groups at risk of pneumonia is warranted. This should include detailed assessment of serological responses in the elderly. Any serological assessment of antibody responses in these patient groups should include assays of surface binding and phagocytosis to ensure potential differences can be correlated to function. Assessment of cellular immunity in patients at risk of *S. pneumoniae* infection may also we warranted. As discussed, a more comprehensive assessment of the targets of naturally-acquired IgG would be useful to fully understand the antibody targets that may be required for protection. This could involve assessment of antibody responses by 2D gel electrophoresis. Full understanding of the reasons for the immunogenicity of certain proteins following carriage also remains elusive, and understanding this would aid the development of new vaccines.

In mice it appears that Th17-type responses are required for protection from *S. pneumoniae* pneumonia following colonisation. Identification and phenotyping of an IL-17 producing population of cells in the lungs of colonised mice (by flow-cytometry), may be important for understanding the precise cellular responses required for protection. The mechanism of enhanced neutrophil recruitment to the lungs of colonised mice, and the precise contribution of Th17 responses and KC require additional clarification. This could involve cellular depletion at early time points post-infection. A more comprehensive assessment of cytokine responses to lung infection could be achieved using a multiplexed assay. Additionally, the role of other cytokines including IL-10 on the colonisation induced protection from non-invasive lung infection seen in this model requires further

investigation. Depletion of these cytokines in colonised mice prior to challenge could help elucidate their function.

The need for both cell-mediated and humoral immunity for colonisation-induced protection from lung infection requires further assessment in the context of vaccine development. Protection following experimental vaccination of mice with antigens that elicit either cell-mediated or humoral immunity, alone and in combination would be informative. This vaccination approach in different models of lung infection would allow the rational design of vaccines aimed at protecting against different forms of pneumococcal disease.

### 7.6.1 Recommendations for future experiments

1. Assessment of antibody levels to *S. pneumoniae* proteins in patient groups at risk of pneumonia.

2. A comprehensive assessment of the S*. pneumoniae* protein targets of naturally-acquired human IgG by 2D gel electrophoresis and mass-spectrometry.

3. Phenotyping of the IL-17 producing population of cells in the lungs of previously colonised mice by flow-cytometry.

4. A more comprehensive assessment of cytokine responses enhanced by previous colonisation, using a multiplexed platform.

5. Assessment of immune responses following CD4+ T-cell depletion at early time points (4hrs) post-challenge, in previously colonised mice.

6. Investigation of the protective effect of experimental vaccination with antigens that elicit either cell-mediated or humoral immunity, in different murine models of *S. pneumoniae* infection.

# References

Abeyta, M., Hardy, G. G. & Yother, J. 2003. Genetic alteration of capsule type but not PspA type affects accessibility of surface-bound complement and surface antigens of *Streptococcus pneumoniae*. *Infection and immunity,* 71**,** 218-25.

Adamou, J. E., Heinrichs, J. H., Erwin, A. L., Walsh, W., Gayle, T., Dormitzer, M., Dagan, R., Brewah, Y. A., Barren, P., Lathigra, R., Langermann, S., Koenig, S. & Johnson, S. 2001. Identification and characterization of a novel family of pneumococcal proteins that are protective against sepsis. *Infection and immunity,* 69**,** 949-58.

Aichele, P., Zinke, J., Grode, L., Schwendener, R. A., Kaufmann, S. H. & Seiler, P. 2003. Macrophages of the splenic marginal zone are essential for trapping of blood-borne particulate antigen but dispensable for induction of specific T cell responses. *J Immunol,* 171**,** 1148-55.

Alonso De Velasco, E., Verheul, A. F., Verhoef, J. & Snippe, H. 1995. *Streptococcus pneumoniae*: virulence factors, pathogenesis, and vaccines. *Microbiological reviews,* 59**,** 591-603.

Alugupalli, K. R. & Gerstein, R. M. 2005. Divide and conquer: division of labor by B-1 B cells. *Immunity,* 23**,** 1-2.

Amanna, I. J., Carlson, N. E. & Slifka, M. K. 2007. Duration of humoral immunity to common viral and vaccine antigens. *The New England journal of medicine,* 357**,** 1903-15.

Ansaldi, F., De Florentiis, D., Canepa, P., Zancolli, M., Martini, M., Orsi, A., Durando, P. & Icardi, G. 2012. Carriage of *Streptoccoccus pneumoniae* 7 years after implementation of vaccination program in a population with very high and long-lasting coverage, Italy. *Vaccine,* 30**,** 2288-94.

Armstrong, R. R. & Johnson, R. S. 1932. Treatment of lobar pneumonia by anti-pneumococcal serum. *Br Med J,* 1932**,** 662-665.

Arulanandam, B. P., Lynch, J. M., Briles, D. E., Hollingshead, S. & Metzger, D. W. 2001. Intranasal vaccination with pneumococcal surface protein A and interleukin-12 augments antibody-mediated opsonization and protective immunity against *Streptococcus pneumoniae* infection. *Infection and immunity,* 69**,** 6718-24.

Austrian, R. 1977. Prevention of Pneumococcal Infection by Immunization with Capsular Polysaccharides of *Streptococcus pneumonia*e - Current Status of Polyvalent Vaccines. *Journal of Infectious Diseases,* 136**,** S38-S42.

Austrian, R. & Gold, J. 1964. Pneumococcal Bacteremia with Especial Reference to Bacteremic Pneumococcal Pneumonia. *Ann Intern Med,* 60**,** 759-76.

Baggiolini, M., Dewald, B. & Moser, B. 1994. Interleukin-8 and related chemotactic cytokines--CXC and CC chemokines. *Adv Immunol,* 55**,** 97-179.

Bardardottir, E., Jonsson, S., Jonsdottir, I., Sigfusson, A. & Valdimarsson, H. 1990. IgG subclass response and opsonization of *Streptococcus pneumoniae* after vaccination of healthy adults. *J Infect Dis,* 162**,** 482-8.

Barocchi, M. A., Censini, S. & Rappuoli, R. 2007. Vaccines in the era of genomics: the pneumococcal challenge. *Vaccine,* 25**,** 2963-73.

Barocchi, M. A., Ries, J., Zogaj, X., Hemsley, C., Albiger, B., Kanth, A., Dahlberg, S., Fernebro, J., Moschioni, M., Masignani, V., Hultenby, K., Taddei, A. R., Beiter, K., Wartha, F., Von Euler, A., Covacci, A., Holden, D. W., Normark, S., Rappuoli, R. & Henriques-Normark, B. 2006. A pneumococcal pilus influences virulence and host inflammatory responses. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 2857-62.

Barrett, D. J. 1985. Human immune responses to polysaccharide antigens: an analysis of bacterial polysaccharide vaccines in infants. *Adv Pediatr,* 32**,** 139-58.

Basset, A., Thompson, C. M., Hollingshead, S. K., Briles, D. E., Ades, E. W., Lipsitch, M. & Malley, R. 2007a. Antibody-independent, CD4+ T-cell-dependent protection against pneumococcal colonization elicited by intranasal immunization with purified pneumococcal proteins. *Infect Immun,* 75**,** 5460-4.

Basset, A., Trzcinski, K., Hermos, C., O'brien, K. L., Reid, R., Santosham, M., Mcadam, A. J., Lipsitch, M. & Malley, R. 2007b. Association of the pneumococcal pilus with certain capsular serotypes but not with increased virulence. *Journal of clinical microbiology,* 45**,** 1684-9.

Batra, S., Cai, S., Balamayooran, G. & Jeyaseelan, S. 2012. Intrapulmonary administration of leukotriene B(4) augments neutrophil accumulation and responses in the lung to Klebsiella infection in CXCL1 knockout mice. *J Immunol,* 188**,** 3458-68.

Baumgarth, N. 2011. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol,* 11**,** 34-46.

Baxendale, H. E., Johnson, M., Stephens, R. C., Yuste, J., Klein, N., Brown, J. S. & Goldblatt, D. 2008. Natural human antibodies to pneumococcus have distinctive molecular characteristics and protect against pneumococcal disease. *Clin Exp Immunol,* 151**,** 51-60.

Beiter, K., Wartha, F., Albiger, B., Normark, S., Zychlinsky, A. & Henriques-Normark, B. 2006. An endonuclease allows *Streptococcus pneumoniae* to escape from neutrophil extracellular traps. *Curr Biol,* 16**,** 401-7.

Bentley, S. D., Aanensen, D. M., Mavroidi, A., Saunders, D., Rabbinowitsch, E., Collins, M., Donohoe, K., Harris, D., Murphy, L., Quail, M. A., Samuel, G., Skovsted, I. C., Kaltoft, M. S., Barrell, B., Reeves, P. R., Parkhill, J. & Spratt, B. G. 2006. Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. *PLoS genetics,* 2**,** e31.

Bergmann, S. & Hammerschmidt, S. 2006. Versatility of pneumococcal surface proteins. *Microbiology,* 152**,** 295-303.

Bewick, T., Sheppard, C., Greenwood, S., Slack, M., Trotter, C., George, R. & Lim, W. S. 2012. Serotype prevalence in adults hospitalised with pneumococcal non-invasive community-acquired pneumonia. *Thorax,* 67**,** 540-545.

Bice, D. E. & Muggenburg, B. A. 1996. Pulmonary immune memory: localized production of antibody in the lung after antigen challenge. *Immunology,* 88**,** 191-7.

Bogaert, D., De Groot, R. & Hermans, P. W. 2004. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. *Lancet Infect Dis,* 4**,** 144-54.

Boyle, R. J., Le, C., Balloch, A. & Tang, M. L. 2006. The clinical syndrome of specific antibody deficiency in children. *Clin Exp Immunol,* 146**,** 486-92.

Briles, D. E., Forman, C., Horowitz, J. C., Volanakis, J. E., Benjamin, W. H., Jr., Mcdaniel, L. S., Eldridge, J. & Brooks, J. 1989. Antipneumococcal effects of C-reactive protein and monoclonal antibodies to pneumococcal cell wall and capsular antigens. *Infect Immun,* 57**,** 1457-64.

Briles, D. E., Hollingshead, S. K., Paton, J. C., Ades, E. W., Novak, L., Van Ginkel, F. W. & Benjamin, W. H. 2003. Immunizations with pneumococcal surface protein A and pneumolysin are protective against pneumonia in a murine model of pulmonary infection with *Streptococcus pneumoniae*. *Journal of Infectious Diseases,* 188**,** 339-348.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y. & Zychlinsky, A. 2004. Neutrophil extracellular traps kill bacteria. *Science,* 303**,** 1532-5.

Brodsky, F. M., Lem, L. & Bresnahan, P. A. 1996. Antigen processing and presentation. *Tissue antigens,* 47**,** 464-71.

Brown, E. J., Hosea, S. W. & Frank, M. M. 1983. The role of antibody and complement in the reticuloendothelial clearance of pneumococci from the bloodstream. *Rev Infect Dis,* 5 Suppl 4**,** S797-805.

Brown, E. J., Hosea, S. W., Hammer, C. H., Burch, C. G. & Frank, M. M. 1982. A quantitative analysis of the interactions of antipneumococcal antibody and complement in experimental pneumococcal bacteremia. *J Clin Invest,* 69**,** 85-98.

Brown, J. S., Gilliland, S. M. & Holden, D. W. 2001. A *Streptococcus pneumoniae* pathogenicity island encoding an ABC transporter involved in iron uptake and virulence. *Molecular microbiology,* 40**,** 572-85.

Brown, J. S., Gilliland, S. M., Ruiz-Albert, J. & Holden, D. W. 2002a. Characterization of pit, a *Streptococcus pneumoniae* iron uptake ABC transporter. *Infect Immun,* 70**,** 4389-98.

Brown, J. S., Hussell, T., Gilliland, S. M., Holden, D. W., Paton, J. C., Ehrenstein, M. R., Walport, M. J. & Botto, M. 2002b. The classical pathway is the dominant complement pathway required for innate immunity to *Streptococcus pneumoniae* infection in mice. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 16969-74.

Brueggemann, A. B., Peto, T. E., Crook, D. W., Butler, J. C., Kristinsson, K. G. & Spratt, B. G. 2004. Temporal and geographic stability of the serogroup-specific invasive disease potential of *Streptococcus pneumoniae* in children. *The Journal of infectious diseases,* 190**,** 1203-11.

Bui, N. K., Eberhardt, A., Vollmer, D., Kern, T., Bougault, C., Tomasz, A., Simorre, J. P. & Vollmer, W. 2012. Isolation and analysis of cell wall components from *Streptococcus pneumoniae*. *Anal Biochem,* 421**,** 657-66.

Bull, C. G. 1915a. The Agglutination of Bacteria in Vivo. *J Exp Med,* 22**,** 484-91.

Bull, C. G. 1915b. The Mechanism of the Curative Action of Antipneumococcus Serum. *J Exp Med,* 22**,** 457-64.

Bull, C. G. 1915c. A Method of Serum Treatment of Pneumococcic Septicemia in Rabbits. *J Exp Med,* 22**,** 466-74.

Bullowa, J. G. & Wilcox, C. 1936. Therapeutic Serum for Pneumococcus Type V (Cooper) Pneumonia. *J Clin Invest,* 15**,** 711-4.

Burton, R. L. & Nahm, M. H. 2006. Development and validation of a fourfold multiplexed opsonization assay (MOPA4) for pneumococcal antibodies. *Clin Vaccine Immunol,* 13**,** 1004-9.

Calbo, E. & Garau, J. 2010. Of mice and men: innate immunity in pneumococcal pneumonia. *Int J Antimicrob Agents,* 35**,** 107-13.

Casadevall, A., Dadachova, E. & Pirofski, L. A. 2004. Passive antibody therapy for infectious diseases. *Nature reviews. Microbiology,* 2**,** 695-703.

Casadevall, A. & Scharff, M. D. 1994. Serum therapy revisited: animal models of infection and development of passive antibody therapy. *Antimicrob Agents Chemother,* 38**,** 1695-702.

Casadevall, A. & Scharff, M. D. 1995. Return to the past: the case for antibody-based therapies in infectious diseases. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America,* 21**,** 150-61.

Casal, J. & Tarrago, D. 2003. Immunity to *Streptococcus pneumoniae*: Factors affecting production and efficacy. *Current opinion in infectious diseases,* 16**,** 219-24.

Chen, J., Deng, Y., Zhao, J., Luo, Z., Peng, W., Yang, J., Ren, L., Wang, L., Fu, Z., Yang, X. & Liu, E. 2010. The polymorphism of IL-17 G-152A was associated with childhood asthma and bacterial colonization of the hypopharynx in bronchiolitis. *J Clin Immunol,* 30**,** 539-45.

Chiavolini, D., Pozzi, G. & Ricci, S. 2008. Animal models of *Streptococcus pneumoniae* disease. *Clin Microbiol Rev,* 21**,** 666-85.

Chidiac, C. 2012. Pneumococcal infections and adult with risk factors. *Med Mal Infect,* 42**,** 517-24.

Chimalapati, S., Cohen, J., Camberlein, E., Durmort, C., Baxendale, H., De Vogel, C., Van Belkum, A. & Brown, J. S. 2011. Infection with conditionally virulent *Streptococcus pneumoniae* Deltapab strains induces antibody to conserved protein antigens but does not protect against systemic infection with heterologous strains. *Infect Immun,* 79**,** 4965-76.

Choi, C. W., Lee, Y. G., Kwon, S. O., Kim, H. Y., Lee, J. C., Chung, Y. H., Yun, C. Y. & Kim, S. I. 2012. Analysis of *Streptococcus pneumoniae* secreted antigens by immuno-proteomic approach. *Diagn Microbiol Infect Dis,* 72**,** 318-27.

Chua, I., Lagos, M., Charalambous, B. M., Workman, S., Chee, R. & Grimbacher, B. 2011. Pathogen-specific IgG antibody levels in immunodeficient patients receiving immunoglobulin replacement do not provide additional benefit to therapeutic management over total serum IgG. *J Allergy Clin Immunol,* 127**,** 1410-1.

Coffey, T. J., Enright, M. C., Daniels, M., Morona, J. K., Morona, R., Hryniewicz, W., Paton, J. C. & Spratt, B. G. 1998. Recombinational exchanges at the capsular polysaccharide biosynthetic locus lead to frequent serotype changes among natural isolates of *Streptococcus pneumoniae*. *Mol Microbiol,* 27**,** 73-83.

Cohen, J. M., Chimalapati, S., De Vogel, C., Van Belkum, A., Baxendale, H. E. & Brown, J. S. 2012. Contributions of capsule, lipoproteins and duration of colonisation towards the protective immunity of prior *Streptococcus pneumoniae* nasopharyngeal colonisation. *Vaccine*.

Cohen, J. M., Khandavilli, S., Camberlein, E., Hyams, C., Baxendale, H. E. & Brown, J. S. 2011. Protective Contributions against Invasive *Streptococcus pneumoniae* Pneumonia of Antibody and Th17-Cell Responses to Nasopharyngeal Colonisation. *PloS one,* 6**,** e25558.

Cohen, J. M., Wilson, R., Shah, P., Baxendale, H. E. & Brown, J. S. 2013. Lack of cross-protection against invasive pneumonia caused by heterologous strains following murine *Streptococcus pneumoniae* nasopharyngeal colonisation despite whole cell ELISAs showing significant cross-reactive IgG. *Vaccine,* 31**,** 2328-32.

Collins, R. A., Werling, D., Duggan, S. E., Bland, A. P., Parsons, K. R. & Howard, C. J. 1998. Gammadelta T cells present antigen to CD4+ alphabeta T cells. *J Leukoc Biol,* 63**,** 707-14.

Coonrod, J. D. & Yoneda, K. 1981. Complement and opsonins in alveolar secretions and serum of rats with pneumonia due to *Streptococcus pneumoniae*. *Reviews of infectious diseases,* 3**,** 310-22.

Cornu, C., Yzebe, D., Leophonte, P., Gaillat, J., Boissel, J. P. & Cucherat, M. 2001. Efficacy of pneumococcal polysaccharide vaccine in immunocompetent adults: a meta-analysis of randomized trials. *Vaccine,* 19**,** 4780-90.

Craig, A., Mai, J., Cai, S. & Jeyaseelan, S. 2009. Neutrophil recruitment to the lungs during bacterial pneumonia. *Infect Immun,* 77**,** 568-75.

Croucher, N. J., Harris, S. R., Fraser, C., Quail, M. A., Burton, J., Van Der Linden, M., Mcgee, L., Von Gottberg, A., Song, J. H., Ko, K. S., Pichon, B., Baker, S., Parry, C. M., Lambertsen, L. M., Shahinas, D., Pillai, D. R., Mitchell, T. J., Dougan, G., Tomasz, A., Klugman, K. P., Parkhill, J., Hanage, W. P. & Bentley, S. D. 2011. Rapid pneumococcal evolution in response to clinical interventions. *Science,* 331**,** 430-4.

Dalia, A. B. & Weiser, J. N. 2011. Minimization of bacterial size allows for complement evasion and is overcome by the agglutinating effect of antibody. *Cell host & microbe,* 10**,** 486-96.

Dallaire, F., Ouellet, N., Bergeron, Y., Turmel, V., Gauthier, M. C., Simard, M. & Bergeron, M. G. 2001. Microbiological and inflammatory factors associated with the development of pneumococcal pneumonia. *J Infect Dis,* 184**,** 292-300.

Darrieux, M., Goulart, C., Briles, D. & Leite, L. C. 2013. Current status and perspectives on protein-based pneumococcal vaccines. *Crit Rev Microbiol*.

Darrieux, M., Moreno, A. T., Ferreira, D. M., Pimenta, F. C., De Andrade, A. L., Lopes, A. P., Leite, L. C. & Miyaji, E. N. 2008. Recognition of pneumococcal isolates by antisera raised against PspA fragments from different clades. *Journal of medical microbiology,* 57**,** 273-8.

Davidson, R. N. & Wall, R. A. 2001. Prevention and management of infections in patients without a spleen. *Clin Microbiol Infect,* 7**,** 657-60.

De Gracia, J., Vendrell, M., Alvarez, A., Pallisa, E., Rodrigo, M. J., De La Rosa, D., Mata, F., Andreu, J. & Morell, F. 2004. Immunoglobulin therapy to control lung damage in patients with common variable immunodeficiency. *International Immunopharmacology,* 4**,** 745-753.

De Hennezel, L., Ramisse, F., Binder, P., Marchal, G. & Alonso, J. M. 2001. Effective combination therapy for invasive pneumococcal pneumonia with ampicillin and intravenous immunoglobulins in a mouse model. *Antimicrobial agents and chemotherapy,* 45**,** 316-8.

Dear, K., Holden, J., Andrews, R. & Tatham, D. 2003. Vaccines for preventing pneumococcal infection in adults. *Cochrane Database Syst Rev*, CD000422.

Defrance, T., Taillardet, M. & Genestier, L. 2011. T cell-independent B cell memory. *Current Opinion in Immunology,* 23**,** 330-336.

Dillard, J. P. & Yother, J. 1994. Genetic and molecular characterization of capsular polysaccharide biosynthesis in *Streptococcus pneumoniae* type 3. *Mol Microbiol,* 12**,** 959-72.

Dintilhac, A., Alloing, G., Granadel, C. & Claverys, J. P. 1997. Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. *Molecular microbiology,* 25**,** 727-39.

Ditse, Z., Adrian, P. V., Kuwanda, L. & Madhi, S. A. 2013. Association of *Streptococcus pneumoniae* common protein antigen (CPA) antibodies and pneumococcal nasopharyngeal colonization in HIV-infected and HIV-uninfected African children. *Vaccine*.

Dochez, A. R. & Avery, O. T. 1917. The elaboration of specific soluble substance by pneumococcus during growth. *Journal of Experimental Medicine,* 26**,** 477-493.

Dockrell, D. H., Whyte, M. K. B. & Mitchell, T. J. 2012. Pneumococcal Pneumonia Mechanisms of Infection and Resolution. *Chest,* 142**,** 482-491.

Dominguez, A., Ciruela, P., Garcia-Garcia, J. J., Moraga, F., Sevilla, M. F., Selva, L., Coll, F., Munoz-Almagro, C., Planes, A. M., Codina, G., Jordan, I., Esteva, C., Hernandez, S., Soldevila, N., Cardenosa, N., Batalla, J. & Salleras, L. 2011. Effectiveness of 7-valent pneumococcal conjugate vaccine in the prevention of invasive pneumococcal disease in children aged 7-59 months. A matched case-control study. *Vaccine*.

Donkor, E. S., Adegbola, R. A., Wren, B. W. & Antonio, M. 2013. Population Biology of *Streptococcus pneumoniae* in West Africa: Multilocus Sequence Typing of Serotypes That Exhibit Different Predisposition to Invasive Disease and Carriage. *PLoS One,* 8**,** e53925.

Dorrington, M. G., Roche, A. M., Chauvin, S. E., Tu, Z., Mossman, K. L., Weiser, J. N. & Bowdish, D. M. 2013. MARCO is required for TLR2- and Nod2-mediated responses to *Streptococcus pneumoniae* and clearance of pneumococcal colonization in the murine nasopharynx. *J Immunol,* 190**,** 250-8.

Dworkin, M. S., Ward, J. W., Hanson, D. L., Jones, J. L., Kaplan, J. E. & Dis, A. a. S. H. 2001. Pneumococcal disease among human immunodeficiency virus-infected persons: Incidence, risk factors, and impact of vaccination. *Clinical Infectious Diseases,* 32**,** 794-800.

Echenique, J., Kadioglu, A., Romao, S., Andrew, P. W. & Trombe, M. C. 2004. Protein serine/threonine kinase StkP positively controls virulence and competence in *Streptococcus pneumoniae*. *Infection and immunity,* 72**,** 2434-7.

Eisen, D. P. 2010. Mannose-binding lectin deficiency and respiratory tract infection. *J Innate Immun,* 2**,** 114-22.

Feldman, C. & Anderson, R. 2011. Bacteraemic pneumococcal pneumonia: current therapeutic options. *Drugs,* 71**,** 131-53.

Felton, L. D., Jordan, C. F., Hesbacher, E. N. & Vaubel, E. K. 1941. Studies on Immunizing Substances in Pneumococci Xii. Comparison of the Effect of Whole-Cell Vaccine and of Polysaccharide Antigen in Human Beings. *Public Health Reports,* 56**,** 1041-1054.

Ferreira, D. M., Jambo, K. C. & Gordon, S. B. 2011. Experimental human pneumococcal carriage models for vaccine research. *Trends Microbiol*.

Ferreira, D. M., Neill, D. R., Bangert, M., Gritzfeld, J. F., Green, N., Wright, A. K., Pennington, S. H., Moreno, L. B., Moreno, A. T., Miyaji, E. N., Wright, A. D., Collins, A. M., Goldblatt, D., Kadioglu, A. & Gordon, S. B. 2013. Controlled Human Infection and Re-Challenge with *Streptococcus pneumoniae* Reveals the Protective Efficacy of Carriage in Healthy Adults. *Am J Respir Crit Care Med*.

Finland, M. & Ruegsegger, J. M. 1935. Immunization of human subjects with the specific carbohydrates of Type III and the related Type VIII pneumococcus. *Journal of Clinical Investigation,* 14**,** 829-832.

Fischetti, V. A., Pancholi, V. & Schneewind, O. 1990. Conservation of a hexapeptide sequence in the anchor region of surface proteins from gram-positive cocci. *Molecular microbiology,* 4**,** 1603-5.

Fitzwater, S. P., Chandran, A., Santosham, M. & Johnson, H. L. 2012. The worldwide impact of the seven-valent pneumococcal conjugate vaccine. *Pediatr Infect Dis J,* 31**,** 501-8.

Flasche, S., Van Hoek, A. J., Sheasby, E., Waight, P., Andrews, N., Sheppard, C., George, R. & Miller, E. 2011. Effect of pneumococcal conjugate vaccination on serotype-specific carriage and invasive disease in England: a cross-sectional study. *PLoS medicine,* 8**,** e1001017.

Frisch, A. W. 1939. Clumping of extracellular encapsulated pneumococci in sputum for control of serum therapy. *Proceedings of the Society for Experimental Biology and Medicine,* 40**,** 495-496.

Fukuyama, Y., King, J. D., Kataoka, K., Kobayashi, R., Gilbert, R. S., Oishi, K., Hollingshead, S. K., Briles, D. E. & Fujihashi, K. 2010. Secretory-IgA antibodies play an important role in the immunity to *Streptococcus pneumoniae*. *J Immunol,* 185**,** 1755-62.

Garcia-Suarez Mdel, M., Vazquez, F. & Mendez, F. J. 2006. *Streptococcus pneumoniae* virulence factors and their clinical impact: An update. *Enfermedades infecciosas y microbiologia clinica,* 24**,** 512-7.

Gasc, A. M., Kauc, L., Barraille, P., Sicard, M. & Goodgal, S. 1991. Gene localization, size, and physical map of the chromosome of *Streptococcus pneumoniae*. *J Bacteriol,* 173**,** 7361-7.

Gauthier, J. F., Fortin, A., Bergeron, Y., Dumas, M. C., Champagne, M. E. & Bergeron, M. G. 2007. Differential contribution of bacterial N-formyl-methionyl-leucyl-phenylalanine and host-derived CXC chemokines to neutrophil infiltration into pulmonary alveoli during murine pneumococcal pneumonia. *Infection and Immunity,* 75**,** 5361-5367.

Gelfand, E. W., Ochs, H. D. & Shearer, W. T. 2013. Controversies in IgG replacement therapy in patients with antibody deficiency diseases. *J Allergy Clin Immunol,* 131**,** 1001-5.

Giefing, C., Meinke, A. L., Hanner, M., Henics, T., Bui, M. D., Gelbmann, D., Lundberg, U., Senn, B. M., Schunn, M., Habel, A., Henriques-Normark, B., Ortqvist, A., Kalin, M., Von Gabain, A. & Nagy, E. 2008. Discovery of a novel class of highly conserved vaccine antigens using genomic scale antigenic fingerprinting of pneumococcus with human antibodies. *J Exp Med,* 205**,** 117-31.

Gladstone, R. A., Jefferies, J. M., Faust, S. N. & Clarke, S. C. 2011. Continued control of pneumococcal disease in the UK - the impact of vaccination. *Journal of medical microbiology,* 60**,** 1-8.

Glennie, S. J., Sepako, E., Mzinza, D., Harawa, V., Miles, D. J., Jambo, K. C., Gordon, S. B., Williams, N. A. & Heyderman, R. S. 2011. Impaired CD4 T cell memory response to *Streptococcus pneumoniae* precedes CD4 T cell depletion in HIV-infected Malawian adults. *PLoS One,* 6**,** e25610.

Goldblatt, D., Hussain, M., Andrews, N., Ashton, L., Virta, C., Melegaro, A., Pebody, R., George, R., Soininen, A., Edmunds, J., Gay, N., Kayhty, H. & Miller, E. 2005. Antibody responses to nasopharyngeal carriage of *Streptococcus pneumoniae* in adults: a longitudinal household study. *J Infect Dis,* 192**,** 387-93.

Goodner, K. & Horsfall, F. L. 1937. Properties of the Type Specific Proteins of Antipneumococcus Sera : Ii. Immunological Fractionation of Type I Antipneumococcus Horse and Rabbit Sera. *J Exp Med,* 66**,** 425-35.

Gor, D. O., Ding, X., Briles, D. E., Jacobs, M. R. & Greenspan, N. S. 2005. Relationship between surface accessibility for PpmA, PsaA, and PspA and antibody-mediated immunity to systemic infection by *Streptococcus pneumoniae. Infect Immun,* 73**,** 1304-12.

Gordon, S. B., Irving, G. R., Lawson, R. A., Lee, M. E. & Read, R. C. 2000. Intracellular trafficking and killing of *Streptococcus pneumoniae* by human alveolar macrophages are influenced by opsonins. *Infect Immun,* 68**,** 2286-93.

Gordon, S. B., Kanyanda, S., Walsh, A. L., Goddard, K., Chaponda, M., Atkinson, V., Mulwafu, W., Molyneux, E. M., Zijlstra, E. E., Molyneux, M. E. & Graham, S. M. 2003. Poor potential coverage for 7-valent pneumococcal conjugate vaccine, Malawi. *Emerg Infect Dis,* 9**,** 747-9.

Gordon, S. B. & Read, R. C. 2002. Macrophage defences against respiratory tract infections. *British medical bulletin,* 61**,** 45-61.

Green, B. A., Zhang, Y., Masi, A. W., Barniak, V., Wetherell, M., Smith, R. P., Reddy, M. S. & Zhu, D. 2005. PppA, a surface-exposed protein of *Streptococcus pneumoniae*, elicits cross-reactive antibodies that reduce colonization in a murine intranasal immunization and challenge model. *Infect Immun,* 73**,** 981-9.

Guevara, M., Barricarte, A., Gil-Setas, A., Garcia-Irure, J. J., Beristain, X., Torroba, L., Petit, A., Polo Vigas, M. E., Aguinaga, A. & Castilla, J. 2009. Changing epidemiology of invasive pneumococcal disease following increased coverage with the heptavalent conjugate vaccine in Navarre, Spain. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases,* 15**,** 1013-9.

Hagiwara, S., Iwasaka, H., Hasegawa, A., Asai, N. & Noguchi, T. 2008. High-dose intravenous immunoglobulin G improves systemic inflammation in a rat model of CLP-induced sepsis. *Intensive Care Med,* 34**,** 1812-9.

Hammerschmidt, S., Talay, S. R., Brandtzaeg, P. & Chhatwal, G. S. 1997. SpsA, a novel pneumococcal surface protein with specific binding to secretory Immunoglobulin A and secretory component. *Molecular Microbiology,* 25**,** 1113-1124.

Hanage, W. P., Kaijalainen, T., Herva, E., Saukkoriipi, A., Syrjanen, R. & Spratt, B. G. 2005. Using multilocus sequence data to define the pneumococcus. *Journal of Bacteriology,* 187**,** 6223-6230.

Harfouche, C., Filippini, S., Gianfaldoni, C., Ruggiero, P., Moschioni, M., Maccari, S., Pancotto, L., Arcidiacono, L., Galletti, B., Censini, S., Mori, E., Giuliani, M., Facciotti, C., Cartocci, E., Savino, S., Doro, F., Pallaoro, M., Nocadello, S., Mancuso, G., Haston, M., Goldblatt, D., Barocchi, M. A., Pizza, M., Rappuoli, R. & Masignani, V. 2012. RrgB321, a fusion protein of the three variants of the pneumococcal pilus backbone RrgB, is protective in vivo and elicits opsonic antibodies. *Infect Immun,* 80**,** 451-60.

Hartzell, J. D., Oster, C. N. & Gaydos, J. C. 2003. How contagious are common respiratory tract infections? *New England Journal of Medicine,* 349**,** 95-95.

Harvey, R. M., Stroeher, U. H., Ogunniyi, A. D., Smith-Vaughan, H. C., Leach, A. J. & Paton, J. C. 2011. A Variable Region within the Genome of *Streptococcus pneumoniae* Contributes to Strain-Strain Variation in Virulence. *Plos One,* 6.

Hathaway, L. J., Brugger, S. D., Morand, B., Bangert, M., Rotzetter, J. U., Hauser, C., Graber, W. A., Gore, S., Kadioglu, A. & Muhlemann, K. 2012. Capsule type of *Streptococcus pneumoniae* determines growth phenotype. *PLoS Pathog,* 8**,** e1002574.

Hausdorff, W. P. 2007. The roles of pneumococcal serotypes 1 and 5 in paediatric invasive disease. *Vaccine,* 25**,** 2406-12.

Hausdorff, W. P., Bryant, J., Kloek, C., Paradiso, P. R. & Siber, G. R. 2000a. The contribution of specific pneumococcal serogroups to different disease manifestations: implications for conjugate vaccine formulation and use, part II. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America,* 30**,** 122-40.

Hausdorff, W. P., Bryant, J., Paradiso, P. R. & Siber, G. R. 2000b. Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America,* 30**,** 100-21.

Heidelberger, M. & Avery, O. T. 1923. The soluble specific substance of pneumococcus. *Journal of Experimental Medicine,* 38**,** 0073-0079.

Heidelberger, M., Goebel, W. F. & Avery, O. T. 1925. The Soluble Specific Substance of Pneumococcus. Third Paper. *Journal of Experimental Medicine,* 42**,** 727-745.

Hiller, N. L., Janto, B., Hogg, J. S., Boissy, R., Yu, S., Powell, E., Keefe, R., Ehrlich, N. E., Shen, K., Hayes, J., Barbadora, K., Klimke, W., Dernovoy, D., Tatusova, T., Parkhill, J., Bentley, S. D., Post, J. C., Ehrlich, G. D. & Hu, F. Z. 2007. Comparative genomic analyses of seventeen *Streptococcus pneumoniae* strains: insights into the pneumococcal supragenome. *Journal of bacteriology,* 189**,** 8186-95.

Hollingshead, S. K., Becker, R. & Briles, D. E. 2000. Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. *Infection and immunity,* 68**,** 5889-900.

Horowitz, J., Volanakis, J. E. & Briles, D. E. 1987. Blood clearance of *Streptococcus pneumoniae* by C-reactive protein. *J Immunol,* 138**,** 2598-603.

Hussain, M., Melegaro, A., Pebody, R. G., George, R., Edmunds, W. J., Talukdar, R., Martin, S. A., Efstratiou, A. & Miller, E. 2005. A longitudinal household study of *Streptococcus pneumoniae* nasopharyngeal carriage in a UK setting. *Epidemiology and infection,* 133**,** 891-8.

Hyams, C., Camberlein, E., Cohen, J. M., Bax, K. & Brown, J. S. 2010a. The *Streptococcus pneumoniae* capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. *Infect Immun,* 78**,** 704-15.

Hyams, C., Opel, S., Hanage, W., Yuste, J., Bax, K., Henriques-Normark, B., Spratt, B. G. & Brown, J. S. 2011. Effects of *Streptococcus pneumoniae* strain background on complement resistance. *PLoS One,* 6**,** e24581.

Hyams, C., Tam, J. C., Brown, J. S. & Gordon, S. B. 2010b. C3b/iC3b deposition on *Streptococcus pneumoniae* is not affected by HIV infection. *PLoS One,* 5**,** e8902.

Hyams, C., Trzcinski, K., Camberlein, E., Weinberger, D. M., Chimalapati, S., Noursadeghi, M., Lipsitch, M. & Brown, J. S. 2013. *Streptococcus pneumoniae* capsular serotype invasiveness correlates with the degree of factor H binding and opsonization with C3b/iC3b. *Infect Immun,* 81**,** 354-63.

Hyams, C., Yuste, J., Bax, K., Camberlein, E., Weiser, J. N. & Brown, J. S. 2010c. *Streptococcus pneumoniae* resistance to complement-mediated immunity is dependent on the capsular serotype. *Infection and immunity,* 78**,** 716-25.

Iannelli, F., Oggioni, M. R. & Pozzi, G. 2002. Allelic variation in the highly polymorphic locus pspC of *Streptococcus pneumoniae*. *Gene,* 284**,** 63-71.

Jackson, L. A., Neuzil, K. M., Yu, O. C., Benson, P., Barlow, W. E., Adams, A. L., Hanson, C. A., Mahoney, L. D., Shay, D. K., Thompson, W. W. & Datalink, V. S. 2003. Effectiveness of pneumococcal polysaccharide vaccine in older adults. *New England Journal of Medicine,* 348**,** 1747-1755.

Jacobs, M. R., Dagan, R., Appelbaum, P. C. & Burch, D. J. 1998. Prevalence of antimicrobial-resistant pathogens in middle ear fluid: multinational study of 917 children with acute otitis media. *Antimicrob Agents Chemother,* 42**,** 589-95.

Janoff, E. N., Breiman, R. F., Daley, C. L. & Hopewell, P. C. 1992. Pneumococcal Disease during Hiv-Infection - Epidemiologic, Clinical, and Immunological Perspectives. *Annals of Internal Medicine,* 117**,** 314-324.

Jedrzejas, M. J. 2004. Extracellular virulence factors of *Streptococcus pneumoniae*. *Front Biosci,* 9**,** 891-914.

Jefferis, R. & Kumararatne, D. S. 1990. Selective IgG subclass deficiency: quantification and clinical relevance. *Clin Exp Immunol,* 81**,** 357-67.

Jomaa, M., Yuste, J., Paton, J. C., Jones, C., Dougan, G. & Brown, J. S. 2005. Antibodies to the iron uptake ABC transporter lipoproteins PiaA and PiuA promote opsonophagocytosis of *Streptococcus pneumoniae*. *Infection and immunity,* 73**,** 6852-9.

Jonsson, G., Truedsson, L., Sturfelt, G., Oxelius, V. A., Braconier, J. H. & Sjoholm, A. G. 2005. Hereditary C2 deficiency in Sweden: frequent occurrence of invasive infection, atherosclerosis, and rheumatic disease. *Medicine (Baltimore),* 84**,** 23-34.

Jorgensen, G. H., Gardulf, A., Sigurdsson, M. I., Sigurdardottir, S. T., Thorsteinsdottir, I., Gudmundsson, S., Hammarstrom, L. & Ludviksson, B. R. 2013. Clinical symptoms in adults with selective IgA deficiency: a case-control study. *J Clin Immunol,* 33**,** 742-7.

Jounblat, R., Clark, H., Eggleton, P., Hawgood, S., Andrew, P. W. & Kadioglu, A. 2005. The role of surfactant protein D in the colonisation of the respiratory tract and onset of bacteraemia during pneumococcal pneumonia. *Respir Res,* 6**,** 126.

Kadioglu, A., Cuppone, A. M., Trappetti, C., List, T., Spreafico, A., Pozzi, G., Andrew, P. W. & Oggioni, M. R. 2011a. Sex-based differences in susceptibility to respiratory and systemic pneumococcal disease in mice. *J Infect Dis,* 204**,** 1971-9.

Kadioglu, A., De Filippo, K., Bangert, M., Fernandes, V. E., Richards, L., Jones, K., Andrew, P. W. & Hogg, N. 2011b. The Integrins Mac-1 and alpha(4)beta(1) Perform Crucial Roles in Neutrophil and T Cell Recruitment to Lungs during *Streptococcus pneumoniae* Infection. *Journal of Immunology,* 186**,** 5907-5915.

Kadioglu, A., Gingles, N. A., Grattan, K., Kerr, A., Mitchell, T. J. & Andrew, P. W. 2000. Host cellular immune response to pneumococcal lung infection in mice. *Infection and immunity,* 68**,** 492-501.

Kadioglu, A., Weiser, J. N., Paton, J. C. & Andrew, P. W. 2008. The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. *Nat Rev Microbiol,* 6**,** 288-301.

Kamtchoua, T., Bologa, M., Hopfer, R., Neveu, D., Hu, B., Sheng, X., Corde, N., Pouzet, C., Zimmerman, G. & Gurunathan, S. 2012. Safety and immunogenicity of the pneumococcal pneumolysin derivative PlyD1 in a single-antigen protein vaccine candidate in adults. *Vaccine*.

Kang, H. Y., Srinivasan, J. & Curtiss, R., 3rd 2002. Immune responses to recombinant pneumococcal PspA antigen delivered by live attenuated Salmonella enterica serovar typhimurium vaccine. *Infection and immunity,* 70**,** 1739-49.

Kang, Y. S., Kim, J. Y., Bruening, S. A., Pack, M., Charalambous, A., Pritsker, A., Moran, T. M., Loeffler, J. M., Steinman, R. M. & Park, C. G. 2004. The C-type lectin SIGN-R1 mediates uptake of the capsular polysaccharide of *Streptococcus pneumoniae* in the marginal zone of mouse spleen. *Proc Natl Acad Sci U S A,* 101**,** 215-20.

Kao, C. Y., Chen, Y., Thai, P., Wachi, S., Huang, F., Kim, C., Harper, R. W. & Wu, R. 2004. IL-17 markedly up-regulates beta-defensin-2 expression in human airway epithelium via JAK and NF-kappaB signaling pathways. *J Immunol,* 173**,** 3482-91.

Kaur, R., Casey, J. R. & Pichichero, M. E. 2011. Serum Antibody Response to Five *Streptococcus pneumoniae* Proteins During Acute Otitis Media in Otitis-prone and Non-otitis-prone Children. *Pediatr Infect Dis J*.

Kelly, T., Dillard, J. P. & Yother, J. 1994. Effect of genetic switching of capsular type on virulence of *Streptococcus pneumoniae. Infect Immun,* 62**,** 1813-9.

Khan, M. N. & Pichichero, M. E. 2012. Vaccine candidates PhtD and PhtE of *Streptococcus pneumoniae* are adhesins that elicit functional antibodies in humans. *Vaccine*.

Khan, M. N., Sharma, S. K., Filkins, L. M. & Pichichero, M. E. 2012. PcpA of *Streptococcus pneumoniae* mediates adherence to nasopharyngeal and lung epithelial cells and elicits functional antibodies in humans. *Microbes and Infection,* 14**,** 1102-1110.

Kim, B. J., Lee, S., Berg, R. E., Simecka, J. W. & Jones, H. P. 2013. Interleukin-23 (IL-23) deficiency disrupts Th17 and Th1-related defenses against *Streptococcus pneumoniae* infection. *Cytokine*.

Kim, J. O. & Weiser, J. N. 1998. Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of *Streptococcus pneumoniae*. *Journal of Infectious Diseases,* 177**,** 368-77.

Kim, K. J., Fandy, T. E., Lee, V. H., Ann, D. K., Borok, Z. & Crandall, E. D. 2004. Net absorption of IgG via FcRn-mediated transcytosis across rat alveolar epithelial cell monolayers. *American journal of physiology. Lung cellular and molecular physiology,* 287**,** L616-22.

Kirby, A. C., Newton, D. J., Carding, S. R. & Kaye, P. M. 2007. Evidence for the involvement of lung-specific gammadelta T cell subsets in local responses to *Streptococcus pneumoniae* infection. *Eur J Immunol,* 37**,** 3404-13.

Koppel, E. A., Wieland, C. W., Van Den Berg, V. C. M., Litjens, M., Florquin, S., Van Kooyk, Y., Van Der Poll, T. & Geijtenbeek, T. B. H. 2005. Specific ICAM-3 grabbing nonintegrin-related 1 (SIGNR1) expressed by marginal zone macrophages is essential for defense against pulmonary *Streptococcus pneumoniae* infection. *European Journal of Immunology,* 35**,** 2962-2969.

Kreuz, W., Erdos, M., Rossi, P., Bernatowska, E., Espanol, T. & Marodi, L. 2010. A multi-centre study of efficacy and safety of Intratect (R) , a novel intravenous immunoglobulin preparation. *Clinical and Experimental Immunology,* 161**,** 512-517.

Kruskall, M. S., Alper, C. A., Awdeh, Z., Yunis, E. J. & Marcus-Bagley, D. 1992. The immune response to hepatitis B vaccine in humans: inheritance patterns in families. *The Journal of experimental medicine,* 175**,** 495-502.

Kyes, P. 1918. The treatment of lobar pneumonia with an anti-pneumococcus serum. *Journal of Medical Research,* 38**,** 495-501.

Laan, M., Cui, Z. H., Hoshino, H., Lotvall, J., Sjostrand, M., Gruenert, D. C., Skoogh, B. E. & Linden, A. 1999. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J Immunol,* 162**,** 2347-52.

Lanoix, J. P., Schmit, J. L. & Douadi, Y. 2012. Bacterial lung sepsis in patients with febrile neutropenia. *Curr Opin Pulm Med,* 18**,** 175-80.

Lavine, J. S., King, A. A. & Bjornstad, O. N. 2011. Natural immune boosting in pertussis dynamics and the potential for long-term vaccine failure. *Proc Natl Acad Sci U S A,* 108**,** 7259-64.

Lebon, A., Verkaik, N. J., Labout, J. A., De Vogel, C. P., Hooijkaas, H., Verbrugh, H. A., Van Wamel, W. J., Jaddoe, V. W., Hofman, A., Hermans, P. W., Ma, J., Mitchell, T. J., Moll, H. A. & Van Belkum, A. 2011. Natural antibodies against several pneumococcal virulence proteins in children in the pre-pneumococcal vaccine-era: The Generation R Study. *Infect Immun*.

Lemessurier, K., Hacker, H., Tuomanen, E. & Redecke, V. 2010. Inhibition of T Cells Provides Protection against Early Invasive Pneumococcal Disease. *Infect Immun,* 78**,** 5287-94.

Lemessurier, K. S., Ogunniyi, A. D. & Paton, J. C. 2006. Differential expression of key pneumococcal virulence genes in vivo. *Microbiology,* 152**,** 305-11.

Leung, M. H., Oriyo, N. M., Gillespie, S. H. & Charalambous, B. M. 2011. The adaptive potential during nasopharyngeal colonisation of *Streptococcus pneumoniae*. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases*.

Levine, O. S., O'brien, K. L., Knoll, M., Adegbola, R. A., Black, S., Cherian, T., Dagan, R., Goldblatt, D., Grange, A., Greenwood, B., Hennessy, T., Klugman, K. P., Madhi, S. A., Mulholland, K., Nohynek, H., Santosham, M., Saha, S. K., Scott, J. A., Sow, S., Whitney, C. G. & Cutts, F. 2006. Pneumococcal vaccination in developing countries. *Lancet,* 367**,** 1880-2.

Li, W. J., Moltedo, B. & Moran, T. M. 2012. Type I Interferon Induction during Influenza Virus Infection Increases Susceptibility to Secondary *Streptococcus pneumoniae* Infection by Negative Regulation of gamma delta T Cells. *Journal of Virology,* 86**,** 12304-12312.

Lipsitch, M., Whitney, C. G., Zell, E., Kaijalainen, T., Dagan, R. & Malley, R. 2005. Are anticapsular antibodies the primary mechanism of protection against invasive pneumococcal disease? *PLoS Med,* 2**,** e15.

Lockhart, E., Green, A. M. & Flynn, J. L. 2006. IL-17 production is dominated by gamma delta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. *Journal of Immunology,* 177**,** 4662-4669.

Lofling, J., Vimberg, V., Battig, P. & Henriques-Normark, B. 2011. Cellular interactions by LPxTG-anchored pneumococcal adhesins and their streptococcal homologues. *Cell Microbiol,* 13**,** 186-97.

Lu, Y. J., Gross, J., Bogaert, D., Finn, A., Bagrade, L., Zhang, Q., Kolls, J. K., Srivastava, A., Lundgren, A., Forte, S., Thompson, C. M., Harney, K. F., Anderson, P. W., Lipsitch, M. & Malley, R. 2008. Interleukin-17A mediates acquired immunity to pneumococcal colonization. *PLoS pathogens,* 4**,** e1000159.

Lu, Y. J., Skovsted, I. C., Thompson, C. M., Anderson, P. W. & Malley, R. 2009. Mechanisms in the serotype-independent pneumococcal immunity induced in mice by intranasal vaccination with the cell wall polysaccharide. *Microb Pathog,* 47**,** 177-82.

Ludwig, E., Bonanni, P., Rohde, G., Sayiner, A. & Torres, A. 2012. The remaining challenges of pneumococcal disease in adults. *Eur Respir Rev,* 21**,** 57-65.

Lynch, J. P., 3rd & Zhanel, G. G. 2009. *Streptococcus pneumoniae*: does antimicrobial resistance matter? *Seminars in respiratory and critical care medicine,* 30**,** 210-38.

Lysenko, E. S., Ratner, A. J., Nelson, A. L. & Weiser, J. N. 2005. The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces. *PLoS Pathog,* 1**,** e1.

Ma, J., Wang, J., Wan, J., Charboneau, R., Chang, Y., Barke, R. A. & Roy, S. 2010. Morphine disrupts interleukin-23 (IL-23)/IL-17-mediated pulmonary mucosal host defense against *Streptococcus pneumoniae* infection. *Infect Immun,* 78**,** 830-7.

Mahdi, L. K., Ogunniyi, A. D., Lemessurier, K. S. & Paton, J. C. 2008. Pneumococcal virulence gene expression and host cytokine profiles during pathogenesis of invasive disease. *Infect Immun,* 76**,** 646-57.

Malley, R., Lipsitch, M., Stack, A., Saladino, R., Fleisher, G., Pelton, S., Thompson, C., Briles, D. & Anderson, P. 2001. Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci. *Infection and immunity,* 69**,** 4870-3.

Marks, M., Burns, T., Abadi, M., Seyoum, B., Thornton, J., Tuomanen, E. & Pirofski, L. A. 2007. Influence of neutropenia on the course of serotype 8 pneumococcal pneumonia in mice. *Infect Immun,* 75**,** 1586-97.

Marques, J. M., Rial, A., Munoz, N., Pellay, F. X., Van Maele, L., Leger, H., Camou, T., Sirard, J. C., Benecke, A. & Chabalgoity, J. A. 2012. Protection against *Streptococcus pneumoniae* serotype 1 acute infection shows a signature of Th17- and IFN-gamma-mediated immunity. *Immunobiology,* 217**,** 420-9.

Marriott, H. M. & Dockrell, D. H. 2007. The role of the macrophage in lung disease mediated by bacteria. *Experimental lung research,* 33**,** 493-505.

Mathews, C. E., Brown, E. L., Martinez, P. J., Bagaria, U., Nahm, M. H., Burton, R. L., Fisher-Hoch, S. P., Mccormick, J. B. & Mirza, S. 2012. Impaired function of antibodies to pneumococcal surface protein a but not to capsular polysaccharide in mexican american adults with type 2 diabetes mellitus. *Clin Vaccine Immunol,* 19**,** 1360-9.

Matthias, K. A., Roche, A. M., Standish, A. J., Shchepetov, M. & Weiser, J. N. 2008. Neutrophil-toxin interactions promote antigen delivery and mucosal clearance of *Streptococcus pneumoniae*. *Journal of immunology,* 180**,** 6246-54.

Mccool, T. L., Cate, T. R., Moy, G. & Weiser, J. N. 2002. The immune response to pneumococcal proteins during experimental human carriage. *J Exp Med,* 195**,** 359-65.

Mccool, T. L. & Weiser, J. N. 2004. Limited role of antibody in clearance of *Streptococcus pneumoniae* in a murine model of colonization. *Infect Immun,* 72**,** 5807-13.

Mcdaniel, L. S., Scott, G., Kearney, J. F. & Briles, D. E. 1984. Monoclonal antibodies against protease-sensitive pneumococcal antigens can protect mice from fatal infection with *Streptococcus pneumoniae*. *J Exp Med,* 160**,** 386-97.

Mcintosh, E. D. & Reinert, R. R. 2011. Global prevailing and emerging pediatric pneumococcal serotypes. *Expert Rev Vaccines,* 10**,** 109-29.

Mcneela, E. A., Burke, A., Neill, D. R., Baxter, C., Fernandes, V. E., Ferreira, D., Smeaton, S., El-Rachkidy, R., Mcloughlin, R. M., Mori, A., Moran, B., Fitzgerald, K. A., Tschopp, J., Petrilli, V., Andrew, P. W., Kadioglu, A. & Lavelle, E. C. 2010. Pneumolysin Activates the NLRP3 Inflammasome and Promotes Proinflammatory Cytokines Independently of TLR4. *Plos Pathogens,* 6.

Mehr, S. & Wood, N. 2012. *Streptococcus pneumoniae*--a review of carriage, infection, serotype replacement and vaccination. *Paediatr Respir Rev,* 13**,** 258-64.

Meijer, C., Wiezer, M. J., Diehl, A. M., Schouten, H. J., Meijer, S., Van Rooijen, N., Van Lambalgen, A. A., Dijkstra, C. D. & Van Leeuwen, P. A. 2000. Kupffer cell depletion by Cl2MDP-liposomes alters hepatic cytokine expression and delays liver regeneration after partial hepatectomy. *Liver,* 20**,** 66-77.

Melin, M. M., Hollingshead, S. K., Briles, D. E., Lahdenkari, M. I., Kilpi, T. M. & Kayhty, H. M. 2008. Development of antibodies to PspA families 1 and 2 in children after exposure to *Streptococcus pneumoniae*. *Clinical and vaccine immunology : CVI,* 15**,** 1529-35.

Mertens, J., Fabri, M., Zingarelli, A., Kubacki, T., Meemboor, S., Groneck, L., Seeger, J., Bessler, M., Hafke, H., Odenthal, M., Bieler, J. G., Kalka, C., Schneck, J. P., Kashkar, H. & Kalka-Moll, W. M. 2009. *Streptococcus pneumoniae* Serotype 1 Capsular Polysaccharide Induces CD8(+)CD28(-) Regulatory T Lymphocytes by TCR Crosslinking. *Plos Pathogens,* 5.

Milner, J. D., Brenchley, J. M., Laurence, A., Freeman, A. F., Hill, B. J., Elias, K. M., Kanno, Y., Spalding, C., Elloumi, H. Z., Paulson, M. L., Davis, J., Hsu, A., Asher, A. I., O'shea, J., Holland, S. M., Paul, W. E. &

Douek, D. C. 2008. Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature,* 452**,** 773-6.

Mitchell, A. M. & Mitchell, T. J. 2010. *Streptococcus pneumoniae*: virulence factors and variation. *Clinical microbiology and infection* 16**,** 411-8.

Moens, L., Wuyts, G., Boon, L., Den Hartog, M. T., Ceuppens, J. L. & Bossuyt, X. 2008. The human polysaccharide- and protein-specific immune response to *Streptococcus pneumoniae* is dependent on CD4(+) T lymphocytes, CD14(+) monocytes, and the CD40-CD40 ligand interaction. *The Journal of allergy and clinical immunology,* 122**,** 1231-3.

Moffitt, K. L., Gierahn, T. M., Lu, Y. J., Gouveia, P., Alderson, M., Flechtner, J. B., Higgins, D. E. & Malley, R. 2011. T(H)17-based vaccine design for prevention of *Streptococcus pneumoniae* colonization. *Cell Host Microbe,* 9**,** 158-65.

Moffitt, K. L., Malley, R. & Lu, Y. J. 2012. Identification of protective pneumococcal T(H)17 antigens from the soluble fraction of a killed whole cell vaccine. *PLoS One,* 7**,** e43445.

Mogensen, T. H., Paludan, S. R., Kilian, M. & Ostergaard, L. 2006. Live *Streptococcus pneumoniae*, Haemophilus influenzae, and Neisseria meningitidis activate the inflammatory response through Toll-like receptors 2, 4, and 9 in species-specific patterns. *J Leukoc Biol,* 80**,** 267-77.

Mold, C., Rodic-Polic, B. & Du Clos, T. W. 2002. Protection from *Streptococcus pneumoniae* infection by C-reactive protein and natural antibody requires complement but not Fc gamma receptors. *Journal of immunology,* 168**,** 6375-81.

Morona, J. K., Miller, D. C., Morona, R. & Paton, J. C. 2004. The effect that mutations in the conserved capsular polysaccharide biosynthesis genes cpsA, cpsB, and cpsD have on virulence of *Streptococcus pneumoniae. Journal of Infectious Diseases,* 189**,** 1905-1913.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. & Coffman, R. L. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *Journal of immunology,* 136**,** 2348-57.

Mukerji, R., Mirza, S., Roche, A. M., Widener, R. W., Croney, C. M., Rhee, D. K., Weiser, J. N., Szalai, A. J. & Briles, D. E. 2012. Pneumococcal surface protein A inhibits complement deposition on the pneumococcal surface by competing with the binding of C-reactive protein to cell-surface phosphocholine. *J Immunol,* 189**,** 5327-35.

Munoz-Almagro, C., Selva, L., Sanchez, C. J., Esteva, C., De Sevilla, M. F., Pallares, R. & Orihuela, C. J. 2010. PsrP, a protective pneumococcal antigen, is highly prevalent in children with pneumonia and is strongly associated with clonal type. *Clinical and vaccine immunology : CVI,* 17**,** 1672-8.

Mureithi, M. W., Finn, A., Ota, M. O., Zhang, Q., Davenport, V., Mitchell, T. J., Williams, N. A., Adegbola, R. A. & Heyderman, R. S. 2009. T cell memory response to pneumococcal protein antigens in an area of high pneumococcal carriage and disease. *J Infect Dis,* 200**,** 783-93.

Murray, D. & Jackson, C. 2002. A conjugate vaccine for the prevention of pediatric pneumococcal disease. *Mil Med,* 167**,** 671-7.

Musher, D. M., Groover, J. E., Rowland, J. M., Watson, D. A., Struewing, J. B., Baughn, R. E. & Mufson, M. A. 1993. Antibody to capsular polysaccharides of *Streptococcus pneumoniae*: prevalence, persistence, and response to revaccination. *Clin Infect Dis,* 17**,** 66-73.

Nabors, G. S., Braun, P. A., Herrmann, D. J., Heise, M. L., Pyle, D. J., Gravenstein, S., Schilling, M., Ferguson, L. M., Hollingshead, S. K., Briles, D. E. & Becker, R. S. 2000. Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. *Vaccine,* 18**,** 1743-54.

Nakamura, K., Doi, K., Okamoto, K., Arai, S., Ueha, S., Matsushima, K., Nakajima, S., Yahagi, N. & Noiri, E. 2013. Specific Antibody in IV Immunoglobulin for Postsplenectomy Sepsis. *Crit Care Med,* 41**,** e163-70.

Nakasone, C., Yamamoto, N., Nakamatsu, M., Kinjo, T., Miyagi, K., Uezu, K., Nakamura, K., Higa, F., Ishikawa, H., O'brien R, L., Ikuta, K., Kaku, M., Fujita, J. & Kawakami, K. 2007. Accumulation of gamma/delta T cells in the lungs and their roles in neutrophil-mediated host defense against pneumococcal infection. *Microbes Infect,* 9**,** 251-8.

Navarre, W. W. & Schneewind, O. 1999. Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiology and molecular biology reviews : MMBR,* 63**,** 174-229.

Neill, D. R., Fernandes, V. E., Wisby, L., Haynes, A. R., Ferreira, D. M., Laher, A., Strickland, N., Gordon, S. B., Denny, P., Kadioglu, A. & Andrew, P. W. 2012. T regulatory cells control susceptibility to invasive pneumococcal pneumonia in mice. *PLoS Pathog,* 8**,** e1002660.

O'brien, K. L., Wolfson, L. J., Watt, J. P., Henkle, E., Deloria-Knoll, M., Mccall, N., Lee, E., Mulholland, K., Levine, O. S., Cherian, T. & Dis, H. P. G. B. 2009. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet,* 374**,** 893-902.

Ogunniyi, A. D., Mahdi, L. K., Trappetti, C., Verhoeven, N., Mermans, D., Van Der Hoek, M. B., Plumptre, C. D. & Paton, J. C. 2012. Identification of Genes That Contribute to Pathogenesis of Invasive Pneumococcal Disease by In vivo Transcriptomic Analysis. *Infect Immun*.

Okinaga, K., Giebink, G. S., Rich, R. H., Baesl, T. J., Krishnanaik, D. & Leonard, A. S. 1981. The Effect of Partial Splenectomy on Experimental Pneumococcal Bacteremia in an Animal-Model. *Journal of Pediatric Surgery,* 16**,** 717-724.

Oksenhendler, E., Gerard, L., Fieschi, C., Malphettes, M., Mouillot, G., Jaussaud, R., Viallard, J. F., Gardembas, M., Galicier, L., Schleinitz, N., Suarez, F., Soulas-Sprauel, P., Hachulla, E., Jaccard, A., Gardeur, A., Theodorou, I., Rabian, C., Debre, P. & Grp, D. S. 2008. Infections in 252 patients with common variable immunodeficiency. *Clinical Infectious Diseases,* 46**,** 1547-1554.

Olaya-Abril, A., Gomez-Gascon, L., Jimenez-Munguia, I., Obando, I. & Rodriguez-Ortega, M. J. 2012. Another turn of the screw in shaving Gram-positive bacteria: Optimization of proteomics surface protein identification in *Streptococcus pneumoniae*. *Journal of Proteomics,* 75**,** 3733-3746.

Olliver, M., Hiew, J., Mellroth, P., Henriques-Normark, B. & Bergman, P. 2011. Human monocytes promote Th1 and Th17 responses to *Streptococcus pneumoniae*. *Infection and immunity,* 79**,** 4210-7.

Olliver, M., Spelmink, L., Hiew, J., Meyer-Hoffert, U., Henriques-Normark, B. & Bergman, P. 2013. Immunomodulatory effects of vitamin D on innate and adaptive immune responses to *Streptococcus pneumoniae*. *J Infect Dis*.

Opitz, B., Puschel, A., Schmeck, B., Hocke, A. C., Rosseau, S., Hammerschmidt, S., Schumann, R. R., Suttorp, N. & Hippenstiel, S. 2004. Nucleotide-binding oligomerization domain proteins are innate

immune receptors for internalized *Streptococcus pneumoniae*. *Journal of Biological Chemistry,* 279**,** 36426-36432.

Orange, J. S., Grossman, W. J., Navickis, R. J. & Wilkes, M. M. 2010. Impact of trough IgG on pneumonia incidence in primary immunodeficiency: A meta-analysis of clinical studies. *Clinical Immunology,* 137**,** 21-30.

Orihuela, C. J., Radin, J. N., Sublett, J. E., Gao, G., Kaushal, D. & Tuomanen, E. I. 2004. Microarray analysis of pneumococcal gene expression during invasive disease. *Infect Immun,* 72**,** 5582-96.

Paats, M. S., Bergen, I. M., Hanselaar, W. E., Van Zoelen, E. C., Verbrugh, H. A., Hoogsteden, H. C., Van Den Blink, B., Hendriks, R. W. & Van Der Eerden, M. M. 2013. T helper 17 cells are involved in the local and systemic inflammatory response in community-acquired pneumonia. *Thorax,* 68**,** 468-74.

Pabst, O. 2012. New concepts in the generation and functions of IgA. *Nat Rev Immunol,* 12**,** 821-32.

Palacios, G., Hornig, M., Cisterna, D., Savji, N., Bussetti, A. V., Kapoor, V., Hui, J., Tokarz, R., Briese, T., Baumeister, E. & Lipkin, W. I. 2009. *Streptococcus pneumoniae* Coinfection Is Correlated with the Severity of H1N1 Pandemic Influenza. *Plos One,* 4.

Pandya, G. A., Mcellistrem, M. C., Venepally, P., Holmes, M. H., Jarrahi, B., Sanka, R., Liu, J., Karamycheva, S. A., Bai, Y., Fleischmann, R. D. & Peterson, S. N. 2011. Monitoring the long-term molecular epidemiology of the pneumococcus and detection of potential 'vaccine escape' strains. *PloS one,* 6**,** e15950.

Paradiso, P. R. 2012. Pneumococcal conjugate vaccine for adults: a new paradigm. *Clin Infect Dis,* 55**,** 259-64.

Paterson, G. K. & Orihuela, C. J. 2010. Pneumococci: immunology of the innate host response. *Respirology,* 15**,** 1057-63.

Pebody, R. G., Leino, T., Nohynek, H., Hellenbrand, W., Salmaso, S. & Ruutu, P. 2005. Pneumococcal vaccination policy in Europe. *Euro surveillance : bulletin europeen sur les maladies transmissibles = European communicable disease bulletin,* 10**,** 174-8.

Pittet, L. A., Hall-Stoodley, L., Rutkowski, M. R. & Harmsen, A. G. 2010. Influenza virus infection decreases tracheal mucociliary velocity and clearance of *Streptococcus pneumoniae*. *Am J Respir Cell Mol Biol,* 42**,** 450-60.

Prevaes, S. M., Van Wamel, W. J., De Vogel, C. P., Veenhoven, R. H., Van Gils, E. J., Van Belkum, A., Sanders, E. A. & Bogaert, D. 2012. Nasopharyngeal colonization elicits antibody responses to staphylococcal and pneumococcal proteins that are not associated with a reduced risk of subsequent carriage. *Infection and immunity*.

Quinti, I., Soresina, A., Guerra, A., Rondelli, R., Spadaro, G., Agostini, C., Milito, C., Trombetta, A. C., Visentini, M., Martini, H., Plebani, A. & Fiorilli, M. 2011. Effectiveness of immunoglobulin replacement therapy on clinical outcome in patients with primary antibody deficiencies: results from a multicenter prospective cohort study. *J Clin Immunol,* 31**,** 315-22.

Reinert, R. R., Paradiso, P. & Fritzell, B. 2010. Advances in pneumococcal vaccines: the 13-valent pneumococcal conjugate vaccine received market authorization in Europe. *Expert review of vaccines,* 9**,** 229-36.

Ren, B., Szalai, A. J., Thomas, O., Hollingshead, S. K. & Briles, D. E. 2003. Both family 1 and family 2 PspA proteins can inhibit complement deposition and confer virulence to a capsular serotype 3 strain of *Streptococcus pneumoniae*. *Infection and Immunity,* 71**,** 75-85.

Renegar, K. B., Small, P. A., Jr., Boykins, L. G. & Wright, P. F. 2004. Role of IgA versus IgG in the control of influenza viral infection in the murine respiratory tract. *J Immunol,* 173**,** 1978-86.

Ricci, S., Janulczyk, R., Gerlini, A., Braione, V., Colomba, L., Iannelli, F., Chiavolini, D., Oggioni, M. R., Bjorck, L. & Pozzi, G. 2011. The factor H-binding fragment of PspC as a vaccine antigen for the induction of protective humoral immunity against experimental pneumococcal sepsis. *Vaccine*.

Richards, L., Ferreira, D. M., Miyaji, E. N., Andrew, P. W. & Kadioglu, A. 2010. The immunising effect of pneumococcal nasopharyngeal colonisation; protection against future colonisation and fatal invasive disease. *Immunobiology,* 215**,** 251-63.

Rijneveld, A. W., Lauw, F. N., Schultz, M. J., Florquin, S., Te Velde, A. A., Speelman, P., Van Deventer, S. J. & Van Der Poll, T. 2002. The role of interferon-gamma in murine pneumococcal pneumonia. *J Infect Dis,* 185**,** 91-7.

Roche, A. M., King, S. J. & Weiser, J. N. 2007. Live attenuated *Streptococcus pneumoniae* strains induce serotype-independent mucosal and systemic protection in mice. *Infect Immun,* 75**,** 2469-75.

Roche, A. M. & Weiser, J. N. 2010. Identification of the targets of cross-reactive antibodies induced by *Streptococcus pneumoniae* colonization. *Infect Immun,* 78**,** 2231-9.

Rodgers, G. L., Arguedas, A., Cohen, R. & Dagan, R. 2009. Global serotype distribution among *Streptococcus pneumoniae* isolates causing otitis media in children: potential implications for pneumococcal conjugate vaccines. *Vaccine,* 27**,** 3802-10.

Rodriguez-Ortega, M. J., Norais, N., Bensi, G., Liberatori, S., Capo, S., Mora, M., Scarselli, M., Doro, F., Ferrari, G., Garaguso, I., Maggi, T., Neumann, A., Covre, A., Telford, J. L. & Grandi, G. 2006. Characterization and identification of vaccine candidate proteins through analysis of the group A Streptococcus surface proteome. *Nat Biotechnol,* 24**,** 191-7.

Romero-Steiner, S., Pilishvili, T., Sampson, J. S., Johnson, S. E., Stinson, A., Carlone, G. M. & Ades, E. W. 2003. Inhibition of pneumococcal adherence to human nasopharyngeal epithelial cells by anti-PsaA antibodies. *Clinical and diagnostic laboratory immunology,* 10**,** 246-51.

Rose, L., Shivshankar, P., Hinojosa, E., Rodriguez, A., Sanchez, C. J. & Orihuela, C. J. 2008. Antibodies against PsrP, a novel *Streptococcus pneumoniae* adhesin, block adhesion and protect mice against pneumococcal challenge. *The Journal of infectious diseases,* 198**,** 375-83.

Rosen, F. S., Cooper, M. D. & Wedgwood, R. J. 1995. The primary immunodeficiencies. *N Engl J Med,* 333**,** 431-40.

Rosenow, C., Ryan, P., Weiser, J. N., Johnson, S., Fontan, P., Ortqvist, A. & Masure, H. R. 1997. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. *Molecular microbiology,* 25**,** 819-29.

Rudner, X. L., Happel, K. I., Young, E. A. & Shellito, J. E. 2007. Interleukin-23 (IL-23)-IL-17 cytokine axis in murine Pneumocystis carinii infection. *Infection and Immunity,* 75**,** 3055-3061.

Rukke, H. V., Hegna, I. K. & Petersen, F. C. 2012. Identification of a functional capsule locus in Streptococcus mitis. *Mol Oral Microbiol,* 27**,** 95-108.

Ruuskanen, O., Nurkka, A., Helminen, M., Viljanen, M. K., Kayhty, H. & Kainulainen, L. 2013. Specific antibody deficiency in children with recurrent respiratory infections: a controlled study with follow-up. *Clin Exp Immunol,* 172**,** 238-44.

Salha, D., Szeto, J., Myers, L., Claus, C., Sheung, A., Tang, M., Ljutic, B., Hanwell, D., Ogilvie, K., Ming, M., Messham, B., Van Den Dobbelsteen, G., Hopfer, R., Ochs, M. M. & Gallichan, S. 2012. Neutralizing antibodies elicited by a novel detoxified pneumolysin derivative, PlyD1, provide protection against both pneumococcal infection and lung injury. *Infect Immun,* 80**,** 2212-20.

Sampson, J. S., Furlow, Z., Whitney, A. M., Williams, D., Facklam, R. & Carlone, G. M. 1997. Limited diversity of *Streptococcus pneumoniae* psaA among pneumococcal vaccine serotypes. *Infection and immunity,* 65**,** 1967-71.

Sanchez, C. J., Hinojosa, C. A., Shivshankar, P., Hyams, C., Camberlein, E., Brown, J. S. & Orihuela, C. J. 2011. Changes in capsular serotype alter the surface exposure of pneumococcal adhesins and impact virulence. *PloS one,* 6**,** e26587.

Sanchez, C. J., Shivshankar, P., Stol, K., Trakhtenbroit, S., Sullam, P. M., Sauer, K., Hermans, P. W. & Orihuela, C. J. 2010. The pneumococcal serine-rich repeat protein is an intra-species bacterial adhesin that promotes bacterial aggregation in vivo and in biofilms. *PLoS pathogens,* 6**,** e1001044.

Schaller-Bals, S., Schulze, A. & Bals, R. 2002. Increased levels of antimicrobial peptides in tracheal aspirates of newborn infants during infection. *Am J Respir Crit Care Med,* 165**,** 992-5.

Schutze, G. E., Mason, E. O., Jr., Barson, W. J., Kim, K. S., Wald, E. R., Givner, L. B., Tan, T. Q., Bradley, J. S., Yogev, R. & Kaplan, S. L. 2002. Invasive pneumococcal infections in children with asplenia. *Pediatr Infect Dis J,* 21**,** 278-82.

Schwab, I. & Nimmerjahn, F. 2013. Intravenous immunoglobulin therapy: how does IgG modulate the immune system? *Nature Reviews Immunology,* 13**,** 176-189.

Segal, A. W. 2005. How neutrophils kill microbes. *Annual Review of Immunology,* 23**,** 197-223.

Seiberling, M., Bologa, M., Brookes, R., Ochs, M., Go, K., Neveu, D., Kamtchoua, T., Lashley, P., Yuan, T. & Gurunathan, S. 2012. Safety and immunogenicity of a pneumococcal histidine triad protein D vaccine candidate in adults. *Vaccine*.

Sharma, S. K., Casey, J. R. & Pichichero, M. E. 2012. Reduced Serum IgG Responses to Pneumococcal Antigens in Otitis-Prone Children May Be Due to Poor Memory B-Cell Generation. *The Journal of infectious diseases*.

Shinefield, H. R., Steinberg, C. R. & Kaye, D. 1966. Effect of splenectomy on the susceptibility of mice inoculated with Diplococcus pneumoniae. *J Exp Med,* 123**,** 777-94.

Silverpil, E., Wright, A. K., Hansson, M., Jirholt, P., Henningsson, L., Smith, M. E., Gordon, S. B., Iwakura, Y., Gjertsson, I., Glader, P. & Linden, A. 2013. Negative feedback on IL-23 exerted by IL-17A during pulmonary inflammation. *Innate Immun*.

Simell, B., Lahdenkari, M., Reunanen, A., Kayhty, H. & Vakevainen, M. 2008. Effects of ageing and gender on naturally acquired antibodies to pneumococcal capsular polysaccharides and virulence-associated proteins. *Clin Vaccine Immunol,* 15**,** 1391-7.

Simell, B., Vuorela, A., Ekstrom, N., Palmu, A., Reunanen, A., Meri, S., Kayhty, H. & Vakevainen, M. 2011. Aging reduces the functionality of anti-pneumococcal antibodies and the killing of *Streptococcus pneumoniae* by neutrophil phagocytosis. *Vaccine,* 29**,** 1929-34.

Singer, M. & Sansonetti, P. J. 2004. IL-8 is a key chemokine regulating neutrophil recruitment in a new mouse model of Shigella-induced colitis. *Journal of Immunology,* 173**,** 4197-4206.

Sjostrom, K., Spindler, C., Ortqvist, A., Kalin, M., Sandgren, A., Kuhlmann-Berenzon, S. & Henriques-Normark, B. 2006. Clonal and capsular types decide whether pneumococci will act as a primary or opportunistic pathogen. *Clin Infect Dis,* 42**,** 451-9.

Sleeman, K. L., Griffiths, D., Shackley, F., Diggle, L., Gupta, S., Maiden, M. C., Moxon, E. R., Crook, D. W. & Peto, T. E. 2006. Capsular serotype-specific attack rates and duration of carriage of *Streptococcus pneumoniae* in a population of children. *The Journal of infectious diseases,* 194**,** 682-8.

Sommerfleck, P. A., Bernaldez, P. C., Hernandez, C. M., Reijtman, V. R. & Lopardo, H. A. 2012. Acute otitis media: prevalence of ear pathogens in patients at a public hospital. *Acta Otorrinolaringol Esp*.

Song, J. Y., Moseley, M. A., Burton, R. L. & Nahm, M. H. 2013. Pneumococcal vaccine and opsonic pneumococcal antibody. *J Infect Chemother*.

Standiford, T. J., Kunkel, S. L., Basha, M. A., Chensue, S. W., Lynch, J. P., 3rd, Toews, G. B., Westwick, J. & Strieter, R. M. 1990. Interleukin-8 gene expression by a pulmonary epithelial cell line. A model for cytokine networks in the lung. *J Clin Invest,* 86**,** 1945-53.

Standish, A. J. & Weiser, J. N. 2009. Human neutrophils kill *Streptococcus pneumoniae* via serine proteases. *J Immunol,* 183**,** 2602-9.

Steinmoen, H., Knutsen, E. & Havarstein, L. S. 2002. Induction of natural competence in *Streptococcus pneumoniae* triggers lysis and DNA release from a subfraction of the cell population. *Proc Natl Acad Sci U S A,* 99**,** 7681-6.

Stillie, R., Farooq, S. M., Gordon, J. R. & Stadnyk, A. W. 2009. The functional significance behind expressing two IL-8 receptor types on PMN. *J Leukoc Biol,* 86**,** 529-43.

Sun, K. & Metzger, D. W. 2008. Inhibition of pulmonary antibacterial defense by interferon-gamma during recovery from influenza infection. *Nat Med,* 14**,** 558-64.

Sun, K., Salmon, S. L., Lotz, S. A. & Metzger, D. W. 2007. Interleukin-12 promotes gamma interferon-dependent neutrophil recruitment in the lung and improves protection against respiratory *Streptococcus pneumoniae* infection. *Infect Immun,* 75**,** 1196-202.

Sutcliffe, I. C. & Harrington, D. J. 2002. Pattern searches for the identification of putative lipoprotein genes in Gram-positive bacterial genomes. *Microbiology,* 148**,** 2065-77.

Swaidani, S., Bulek, K., Kang, Z., Liu, C., Lu, Y., Yin, W., Aronica, M. & Li, X. 2009. The critical role of epithelial-derived Act1 in IL-17- and IL-25-mediated pulmonary inflammation. *J Immunol,* 182**,** 1631-40.

Szu, S. C., Schneerson, R. & Robbins, J. B. 1986. Rabbit antibodies to the cell wall polysaccharide of *Streptococcus pneumoniae* fail to protect mice from lethal challenge with encapsulated pneumococci. *Infect Immun,* 54**,** 448-55.

Tettelin, H., Nelson, K. E., Paulsen, I. T., Eisen, J. A., Read, T. D., Peterson, S., Heidelberg, J., Deboy, R. T., Haft, D. H., Dodson, R. J., Durkin, A. S., Gwinn, M., Kolonay, J. F., Nelson, W. C., Peterson, J. D., Umayam, L. A., White, O., Salzberg, S. L., Lewis, M. R., Radune, D., Holtzapple, E., Khouri, H., Wolf, A. M., Utterback, T. R., Hansen, C. L., Mcdonald, L. A., Feldblyum, T. V., Angiuoli, S., Dickinson, T., Hickey, E. K., Holt, I. E., Loftus, B. J., Yang, F., Smith, H. O., Venter, J. C., Dougherty, B. A., Morrison, D. A., Hollingshead, S. K. & Fraser, C. M. 2001. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae. Science,* 293**,** 498-506.

Tokunaga, M., Tokunaga, H. & Wu, H. C. 1982. Post-translational modification and processing of Escherichia coli prolipoprotein in vitro. *Proceedings of the National Academy of Sciences of the United States of America,* 79**,** 2255-9.

Trzcinski, K., Thompson, C., Malley, R. & Lipsitch, M. 2005. Antibodies to conserved pneumococcal antigens correlate with, but are not required for, protection against pneumococcal colonization induced by prior exposure in a mouse model. *Infect Immun,* 73**,** 7043-6.

Trzcinski, K., Thompson, C. A. & Lipsitch, M. 2003. Construction of otherwise isogenic serotype 6B, 7F, 14, and 19F capsular variants of *Streptococcus pneumoniae* strain TIGR4. *Applied and Environmental Microbiology,* 69**,** 7364-7370.

Turner, P., Turner, C., Jankhot, A., Helen, N., Lee, S. J., Day, N. P., White, N. J., Nosten, F. & Goldblatt, D. 2012. A longitudinal study of *Streptococcus pneumoniae* carriage in a cohort of infants and their mothers on the Thailand-Myanmar border. *PLoS One,* 7**,** e38271.

Van Der Poll, T., Marchant, A., Keogh, C. V., Goldman, M. & Lowry, S. F. 1996. Interleukin-10 impairs host defense in murine pneumococcal pneumonia. *J Infect Dis,* 174**,** 994-1000.

Van Der Poll, T. & Opal, S. M. 2009. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. *Lancet,* 374**,** 1543-56.

Van Rooijen, N. & Van Nieuwmegen, R. 1984. Elimination of phagocytic cells in the spleen after intravenous injection of liposome-encapsulated dichloromethylene diphosphonate. An enzyme-histochemical study. *Cell Tissue Res,* 238**,** 355-8.

Van Rossum, A. M., Lysenko, E. S. & Weiser, J. N. 2005. Host and bacterial factors contributing to the clearance of colonization by *Streptococcus pneumoniae* in a murine model. *Infection and immunity,* 73**,** 7718-26.

Verkaik, N. J., De Vogel, C. P., Boelens, H. A., Grumann, D., Hoogenboezem, T., Vink, C., Hooijkaas, H., Foster, T. J., Verbrugh, H. A., Van Belkum, A. & Van Wamel, W. J. B. 2009. Anti-Staphylococcal Humoral Immune Response in Persistent Nasal Carriers and Noncarriers of Staphylococcus aureus. *Journal of Infectious Diseases,* 199**,** 625-632.

Vernacchio, L., Romero-Steiner, S., Martinez, J. E., Macdonald, K., Barnard, S., Pilishvili, T., Carlone, G. M., Ambrosino, D. M. & Molrine, D. C. 2000. Comparison of an opsonophagocytic assay and IgG ELISA to assess responses to pneumococcal polysaccharide and pneumococcal conjugate vaccines in children and young adults with sickle cell disease. *J Infect Dis,* 181**,** 1162-6.

Vignali, D. a. A., Collison, L. W. & Workman, C. J. 2008. How regulatory T cells work. *Nature Reviews Immunology,* 8**,** 523-532.

Vitharsson, G., Jonsdottir, I., Jonsson, S. & Valdimarsson, H. 1994. Opsonization and antibodies to capsular and cell wall polysaccharides of *Streptococcus pneumoniae*. *J Infect Dis,* 170**,** 592-9.

Waghorn, D. J. 2001. Overwhelming infection in asplenic patients: current best practice preventive measures are not being followed. *J Clin Pathol,* 54**,** 214-8.

Wang, X. Y., Kilgore, P. E., Lim, K. A., Wang, S. M., Lee, J., Deng, W., Mo, M. Q., Nyambat, B., Ma, J. C., Favorov, M. O. & Clemens, J. D. 2011. Influenza and bacterial pathogen coinfections in the 20th century. *Interdiscip Perspect Infect Dis,* 2011**,** 146376.

Wani, J. H., Gilbert, J. V., Plaut, A. G. & Weiser, J. N. 1996. Identification, cloning, and sequencing of the immunoglobulin A1 protease gene of *Streptococcus pneumoniae*. *Infection and Immunity,* 64**,** 3967-3974.

Watson, D. A. & Musher, D. M. 1990. Interruption of capsule production in Streptococcus pneumonia serotype 3 by insertion of transposon Tn916. *Infect Immun,* 58**,** 3135-8.

Watson, D. A., Musher, D. M., Jacobson, J. W. & Verhoef, J. 1993. A Brief-History of the Pneumococcus in Biomedical-Research - a Panoply of Scientific Discovery. *Clinical Infectious Diseases,* 17**,** 913-924.

Weber, S. E., Tian, H. & Pirofski, L. A. 2011. CD8+ cells enhance resistance to pulmonary serotype 3 *Streptococcus pneumoniae* infection in mice. *J Immunol,* 186**,** 432-42.

Weinberger, D. M., Dagan, R., Givon-Lavi, N., Regev-Yochay, G., Malley, R. & Lipsitch, M. 2008. Epidemiologic evidence for serotype-specific acquired immunity to pneumococcal carriage. *J Infect Dis,* 197**,** 1511-8.

Weinberger, D. M., Trzcinski, K., Lu, Y. J., Bogaert, D., Brandes, A., Galagan, J., Anderson, P. W., Malley, R. & Lipsitch, M. 2009. Pneumococcal capsular polysaccharide structure predicts serotype prevalence. *PLoS Pathog,* 5**,** e1000476.

Weiser, J. N., Austrian, R., Sreenivasan, P. K. & Masure, H. R. 1994. Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. *Infection and immunity,* 62**,** 2582-9.

Wessels, M. R., Butko, P., Ma, M. H., Warren, H. B., Lage, A. L. & Carroll, M. C. 1995. Studies of Group-B Streptococcal Infection in Mice Deficient in Complement Component C3 or C4 Demonstrate an Essential Role for Complement in Both Innate and Acquired-Immunity. *Proceedings of the National Academy of Sciences of the United States of America,* 92**,** 11490-11494.

Wiertsema, S. P., Baynam, G., Khoo, S. K., Veenhoven, R. H., Van Heerbeek, N., Zhang, G., Laing, I. A., Rijkers, G. T., Goldblatt, J., Sanders, E. A. & Le Souef, P. N. 2007. Impact of genetic variants in IL-4, IL-4 RA and IL-13 on the anti-pneumococcal antibody response. *Vaccine,* 25**,** 306-13.

Williams, J. H., Jr., Pahl, M. V., Kwong, D., Zhang, J., Hatakeyama, D., Ahmad, K., Naderi, M., Kim, M. & Vaziri, N. 2003. Modulation of neutrophil complement receptor 3 expression by pneumococci. *Clin Sci (Lond),* 104**,** 615-25.

Winter, C., Taut, K., Srivastava, M., Langer, F., Mack, M., Briles, D. E., Paton, J. C., Maus, R., Welte, T., Gunn, M. D. & Maus, U. A. 2007. Lung-specific overexpression of CC chemokine ligand (CCL) 2 enhances the host defense to *Streptococcus pneumoniae* infection in mice: role of the CCL2-CCR2 axis. *J Immunol,* 178**,** 5828-38.

Wizemann, T. M., Heinrichs, J. H., Adamou, J. E., Erwin, A. L., Kunsch, C., Choi, G. H., Barash, S. C., Rosen, C. A., Masure, H. R., Tuomanen, E., Gayle, A., Brewah, Y. A., Walsh, W., Barren, P., Lathigra, R., Hanson, M., Langermann, S., Johnson, S. & Koenig, S. 2001. Use of a whole genome approach to identify vaccine molecules affording protection against *Streptococcus pneumoniae* infection. *Infect Immun,* 69**,** 1593-8.

Wren, B. W. 1991. A family of clostridial and streptococcal ligand-binding proteins with conserved C-terminal repeat sequences. *Molecular microbiology,* 5**,** 797-803.

Wright, A. K., Bangert, M., Gritzfeld, J. F., Ferreira, D. M., Jambo, K. C., Wright, A. D., Collins, A. M. & Gordon, S. B. 2013. Experimental human pneumococcal carriage augments IL-17A-dependent T-cell defence of the lung. *PLoS Pathog,* 9**,** e1003274.

Wu, H. Y., Virolainen, A., Mathews, B., King, J., Russell, M. W. & Briles, D. E. 1997. Establishment of a *Streptococcus pneumoniae* nasopharyngeal colonization model in adult mice. *Microb Pathog,* 23**,** 127-37.

Yamada, M., Gomez, J. C., Chugh, P. E., Lowell, C. A., Dinauer, M. C., Dittmer, D. P. & Doerschuk, C. M. 2011. Interferon-gamma Production by Neutrophils during Bacterial Pneumonia in Mice. *American Journal of Respiratory and Critical Care Medicine,* 183**,** 1391-1401.

Yano, M., Gohil, S., Coleman, J. R., Manix, C. & Pirofski, L. A. 2011. Antibodies to *Streptococcus pneumoniae* capsular polysaccharide enhance pneumococcal quorum sensing. *MBio,* 2.

Ye, P., Garvey, P. B., Zhang, P., Nelson, S., Bagby, G., Summer, W. R., Schwarzenberger, P., Shellito, J. E. & Kolls, J. K. 2001. Interleukin-17 and lung host defense against Klebsiella pneumoniae infection. *American Journal of Respiratory Cell and Molecular Biology,* 25**,** 335-340.

Yoshida, T., Mei, H., Dorner, T., Hiepe, F., Radbruch, A., Fillatreau, S. & Hoyer, B. F. 2010. Memory B and memory plasma cells. *Immunological reviews,* 237**,** 117-39.

Yother, J. & Briles, D. E. 1992. Structural properties and evolutionary relationships of PspA, a surface protein of *Streptococcus pneumoniae*, as revealed by sequence analysis. *Journal of bacteriology,* 174**,** 601-9.

Young, C. C. & Huntoon, F. M. 1926. Laboratory: Antipneumococcic Serums; Purification and Concentration. *Am J Public Health (N Y),* 16**,** 1247-9.

Yuste, J., Botto, M., Bottoms, S. E. & Brown, J. S. 2007. Serum amyloid P aids complement-mediated immunity to *Streptococcus pneumoniae*. *PLoS Pathog,* 3**,** 1208-19.

Yuste, J., Botto, M., Paton, J. C., Holden, D. W. & Brown, J. S. 2005. Additive inhibition of complement deposition by pneumolysin and PspA facilitates *Streptococcus pneumoniae* septicemia. *J Immunol,* 175**,** 1813-9.

Yuste, J., Khandavilli, S., Ansari, N., Muttardi, K., Ismail, L., Hyams, C., Weiser, J., Mitchell, T. & Brown, J. S. 2010. The effects of PspC on complement-mediated immunity to *Streptococcus pneumoniae* vary with strain background and capsular serotype. *Infection and immunity,* 78**,** 283-92.

Yuste, J., Sen, A., Truedsson, L., Jonsson, G., Tay, L. S., Hyams, C., Baxendale, H. E., Goldblatt, F., Botto, M. & Brown, J. S. 2008. Impaired opsonization with C3b and phagocytosis of *Streptococcus pneumoniae* in sera from subjects with defects in the classical complement pathway. *Infect Immun,* 76**,** 3761-70.

Zandvoort, A. & Timens, W. 2002. The dual function of the splenic marginal zone: essential for initiation of anti-TI-2 responses but also vital in the general first-line defense against blood-borne antigens. *Clinical and Experimental Immunology,* 130**,** 4-11.

Zhang, Q., Bagrade, L., Bernatoniene, J., Clarke, E., Paton, J. C., Mitchell, T. J., Nunez, D. A. & Finn, A. 2007. Low CD4 T cell immunity to pneumolysin is associated with nasopharyngeal carriage of pneumococci in children. *J Infect Dis,* 195**,** 1194-202.

Zhang, Q., Bernatoniene, J., Bagrade, L., Paton, J. C., Mitchell, T. J., Hammerschmidt, S., Nunez, D. A. & Finn, A. 2006a. Regulation of production of mucosal antibody to pneumococcal protein antigens by T-cell-derived gamma interferon and interleukin-10 in children. *Infection and immunity,* 74**,** 4735-43.

Zhang, Q., Bernatoniene, J., Bagrade, L., Pollard, A. J., Mitchell, T. J., Paton, J. C. & Finn, A. 2006b. Serum and mucosal antibody responses to pneumococcal protein antigens in children: relationships with carriage status. *European journal of immunology,* 36**,** 46-57.

Zhang, W., Liu, G., Tang, F., Shao, J., Lu, Y., Bao, Y., Yao, H. & Lu, C. 2011. Pre-absorbed immunoproteomics: a novel method for the detection of Streptococcus suis surface proteins. *PloS one,* 6**,** e21234.

Zhang, Z., Clarke, T. B. & Weiser, J. N. 2009. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. *J Clin Invest,* 119**,** 1899-909.

Zhu, J., Yamane, H. & Paul, W. E. 2010. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol,* 28**,** 445-89.

Zhu, L. & Lau, G. W. 2011. Inhibition of Competence Development, Horizontal Gene Transfer and Virulence in *Streptococcus pneumoniae* by a Modified Competence Stimulating Peptide. *PLoS pathogens,* 7**,** e1002241.