Colonisation-induced protection

against *Streptococcus pneumoniae* disease

presented by

Jonathan Marc Cohen

A thesis submitted for the degree of

Doctor of Philosophy

UCL

December 2010

Infectious Diseases & Microbiology Unit

UCL Institute of Child Health

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

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisors, Dr Helen Baxendale and Dr Jerry Brown for their support and direction, and their ongoing belief in me through this project. It is always a major commitment to take on a student, especially a clinician entering the world of the laboratory. I am so grateful to Helen for her constant guidance from the planning stages of a Fellowship application right through to the present. Her mentorship has instilled in me a passion for addressing the fundamental biological question with rigor, always accompanied with a sense of fun. Jerry has taught me to how to find a path forwards, whatever the data suggests, and to think strategically about the next steps, and where current work could lead.

This thesis is the result of work undertaken both at the Institute of Child Health and the Centre for Respiratory Research, and there are so many friends, colleagues and mentors I wish to thank in both labs. It is not possible to mention all, but special thanks go to the 'pneumo group' at the CRR, Suneeta, Jose, Cat, Emilie, Shilpa, Lindsey and Tracey for being such a wonderful team to work with, and to Alejandro, Chris, Paul and Steve for all their help and advice. At the ICH, both Professors Nigel Klein and David Goldblatt have been sources of wisdom and guidance at key stages over recent years. I am so grateful to Marina, Marianne and Lindsay for taking me under their wings, and sharing time, ideas (and reagents) along the way. Also at the ICH, Sheila, Hannah, Mitch, Kiran, Halima, Hemlata, Patricia, Holly, Nazila and of course Vania, I thank for their company, thoughts and all their help.

None of this work would have been possible without the support of the Medical Research Council who funded my Clinical Research Training Fellowship. I am grateful to Dr Carmen Giefing and Dr Eszter Nagy of Intercell AG and to Prof Alex van Belkum and Dr Corné de Vogel of Erasmus Medical Centre both for reagents

supplied and for establishing fruitful collaborations. I would also like to thank Prof Claudia Mauri and Dr Natalie Carter at UCL for the kind gift of the μMT mice used in this work. I am also indebted to Mick, Barry and colleagues at the UCL Biological Services Unit for all their assistance.

Nothing I have achieved in my life would have been possible without the constant love, support and guidance of my parents. My daughters, Elia and Adina, have ensured that this work ends at the laboratory door, and that our home is a place of fun, colour and lots of noise. Finally, my deepest thanks go to Rimona who continues to amaze, inspire, and encourage me. Rimona has taught me that with passion, tenacity and a good share of ingenuity, all problems can be overcome. I am blessed and truly grateful for such a loving and inspirational family.

ABSTRACT

Streptococus pneumoniae is an important human pathogen, yet in most individuals it establishes only transient nasopharyngeal colonisation without causing disease. Using murine models, this thesis explores the hypothesis that colonisation induces acquired immune responses which protect against subsequent pneumonia.

Colonisation models with wild-type (WT) and mutant *S. pneumoniae* were established in outbred CD1 mice. Mutants lacked either capsule or lipoproteins, or were auxotrophs unable to replicate *in vivo*. WT colonisation protected against subsequent pneumonia. Mutants were cleared more rapidly than WT, were not immunogenic and did not protect. When the auxotroph was supplemented, colonisation, immunogenicity and protection were improved, suggesting duration of a colonisation event is an important factor in determining immunogenicity. This may be one factor explaining the poor immunogenicity of the other mutants.

The mechanism by which previous colonisation protected against subsequent lethal pneumonia was then defined in a series of studies in inbred CBA/Ca mice. Colonisation induced both mucosal and systemic antibody responses to bacterial surface antigens but not capsule. There was also evidence of more robust cytokine production during subsequent pneumonia, including systemic and mucosal IL-17 responses dependant on the presence of CD4-cells. Protection was primarily against systemic invasion following pneumonia. Passive transfer studies and experiments using genetically modified mice demonstrated that systemic antibody was both necessary and sufficient to protect, and in vitro and in vivo models showed this to be via opsonophagocytosis and bloodstream clearance of bacteria. Antigenic protein targets of protective serum were defined using Western blotting and multiplex bead immunoassay techniques.

Overall this thesis demonstrates that nasopharyngeal colonisation can protect against lethal pneumonia in mice via opsonophagocytic antibody against surface proteins thus preventing bacteraemia.

PUBLICATIONS, ABSTRACTS AND PRIZES

Publications

Basavanna S, Khandavilli S, Yuste J, Cohen JM, Hosie AHF, Webb A, Thomas GH and Brown JS. Screening of *Streptococcus pneumoniae* ABC transporter mutants demonstrates that LivJHMGF, a branched chain amino acid ABC transporter, is necessary for disease pathogenesis. Infect Immun, Aug 2009, 77:3412-23.

Oral Presentations

Cohen JM, Khandavilli S, Camberlein E, Catherine Hyams, Corne de Vogel, Carmen Giefing, Estzer Nagy, Alex van Belkum, Baxendale H, Brown JS. Previous colonisation protects against pneumococcal pneumonia by inducing antibodies to surface protein antigens. 7th International Symposium on Pneumococci & Pneumococcal Disease, Tel Aviv, Israel, March 2010.

Cohen JM, Khandavilli S, Camberlein E, Baxendale H, Brown JS. Previous colonisation protects against pneumococcal pneumonia: learning from natural immunity. British Thoracic Society Winter Meeting, London, December 2009.

Poster Presentations

Cohen JM, Khandavilli S, Camberlein E, Hyams C, Brown JS. Virulence and nasopharyngeal colonisation with the Δ*pabB Streptococcus pneumoniae* strain is conditional on para-amino-benzoic acid supplementation. 7th International Symposium on Pneumococci & Pneumococcal Disease, Tel Aviv, Israel, March 2010.

Cohen JM, Khandavilli S, Camberlein C, Baxendale H, Brown JS. Immunising effect of pneumococcal naso-pharyngeal colonisation in mice: previous wild-type D39 colonisation protects against death from pneumonia. 9th European Meeting on the Molecular Biology of the Pneumococcus, Bern, Switzerland, June 2009.

Khandavilli S, Cohen JM, Camberlein C, Brown JS. *Para*-aminobenzoic acid synthase and *S. pneumoniae* : a possible tool for studying pathogenesis. 9th European Meeting on the Molecular Biology of the Pneumococcus, Bern, Switzerland, June 2009.

Prizes

1st Prize, Oral Presentation & Pushpa Chopra Scholarship, AMS/MRS Clinician Scientists in Training Day, London, 2010.

1st Prize, PhD Student Poster Competition. UCL Institute of Child Health, 2009.

Young Respiratory Investigator Award, Royal Society of Medicine, 2009.

CONTENTS

FIGURE LIST

TABLE LIST

ABBREVIATION LIST

ABC , ATP-binding cassette

AM, alveolar macrophage

ANOVA, analysis of variance

APC, antigen presenting cell

BAL, bronchoalveolar lavage

BALF, bronchoalveolar lavage fluid

BSA, bovine serum albumin

CAP, community acquired pneumonia

CBP, choline binding protein

CFU, colony forming units

CPS, capsular polysaccharide

CR, complement receptor

CT, cholera toxin

CWPS, cell wall polysaccharide

DNA, deoxyribonucleic acid

ELISA, enzyme linked immunosorbent assay

FAM-SE, 5-carboxyfluorescein, succinimidyl ester

FcR, Fc-receptor

FcγR, Fcγ receptors

FITC, fluorescein isothiocyanate

GAS, group A Streptococcus

h, hour

HBSS, Hank's buffered saline solution

HIV, human immunodeficiency virus

HRP, horseradish peroxidase

i.n., intranasal

i.p., intraperitoneal

i.v., intravenous

IFN, interferon

Ig, immunoglobulin

IL, interleukin

IPD, invasive pneumococcal disease

IQR, inter-quartile range

IRAK-4, interleukin-1 receptor associated kinase 4

LBP, lipopolysaccharide binding protein

Lgt, diacylglyceryl transferase

Lsp, lipoprotein signal peptidase

LTA, lipoteichoic acid

LytA, autolysin

mAb, monoclonal antibody

MAPK, mitogen-activated protein kinase

MARCO, macrophage receptor with collagenous structure

MBL, mannose binding lectin

MFI, median fluorescence intensity

MHC, major histocompatability complex

min, minute

MIP, macrophage inflammatory protein

MNC, mononuclear cell

MOI, multiplicity of infection

MW, molecular weight

MyD88, myeloid differentiation factor 88

NALT, nasal-associated lymphoid tissue

NanA, neuraminidase A

NET, neutrophil extracellular trap

NF-κB, nuclear factor κB

NLR, Nucleotide-binding domain, Leucine-Rich repeat containing (also known as Nod-like receptors)

NOD, nucleotide oligomerisation domain

PAF, platelet activating factor

PAFr, platelet activating factor receptor

PAMP, pathogen associated molecular pattern

PavA, pneumococcal adherence and virulence factor A

PBMC, peripheral blood mononuclear cells

PBS, phosphate buffered saline

PC, phosphorylcholine

PCR, polymerase chain reaction

PCV, pneumococcal conjugate vaccine

PE, phycoerythrin

PFA, paraformaldehyde

pIgR, polymeric immunoglobulin receptor

Ply, pneumolysin

PMN, polymorphonuclear cells

PPS, plain pneumococcal polysaccharide

PPS2, type 2 pneumoocccal polysaccharide

PRR, pathogen recognition receptor

PsaA, pneumococcal surface adhesion A

PspA, pneumococcal surface protein A

RBC, red blood cell

Rpm, revolutions per minute

RT, room temperature

SD, standard deviation

SEM, standard error of the mean

SNP, single nucleotide polymorphism

TA, teichoic acid

TBST, tris-buffered saline with Tween-20

TCR, T-cell receptor

TGFβ, transforming growth factor β

TLR, Toll-like receptor

TMB, tetramethylbenzadine

TNF, tumour necrosis factor

Tw, Tween-20

WT, wild type

1 GENERAL INTRODUCTION

1.1 *STREPTOCOCCUS PNEUMONIAE*

1.1.1 Historical overview

Streptococcus pneumoniae is a major bacterial pathogen responsible for significant mortality and morbidity globally. It was discovered in 1881 by Pasteur and Sternberg, and has been renamed several times reflecting advances in microbiological understanding. Such names include *Microbe septicèmique du salive* (Pasteur), reflecting the nature of this organism which is recoverable from the oro/nasopharynx and has the capacity on occasion to invade and kill. The young, old and immunocompromised are most vulnerable. Most morbidity is attributable to pneumococcal pneumonia. Its recognition as the major cause of lobar pneumonia earned it the label 'pneumococcus', whilst its mortality was ironically reflected in the moniker 'Old Man's Friend' attributed to Canadian physician William Osler. Its morphology was reflected in the subsequent appellation *Diplococcus,* until 1974, when it was renamed *Streptococcus pneumoniae* (Watson et al., 1993) reflecting its pattern of growth in liquid media.

Studies of this prototypic extracellular bacterial pathogen led to many important biological discoveries especially in the field of humoral immunity. In the last century, immunisation of African mine workers with killed *S. pneumoniae* led to large reductions in the mortality caused by epidemic lobar pneumonia (Austrian et al., 1976). Further studies identified the polysaccharide capsule as an important antigen against which serotype specific protection could be induced (Smillie et al., 1938). Perhaps the most remarkable discovery to arise from work on *S. pneumoniae* was when Avery demonstrated that deoxyribonucleic acid (DNA) is the molecule that

conveys heritable phenotypic characteristics (Avery et al., 1944), explaining the transformation of bacterial strains previously described by Griffith (Griffith, 1928).

1.1.2 Microbiology

S. pneumoniae is a gram-positive chain-forming coccus. It produces large amounts of hydrogen peroxide and growth is optimal in the presence of a source of catalase. Hence, in the laboratory it is often grown on blood agar, where it forms grey-white colonies. Release of the toxin pneumolysin (Ply) leads to degradation of haemoglobin, resulting in a green coloration referred to as α-haemolysis. *S. pneumoniae* is differentiated from other gram-positive chain-forming α-haemolytic organisms by its sensitivity to optochin and its solubility in bile salts. Recent identification of optochin-resistant *S. pneumoniae* has increased the need for genetic identification methods where precision is required. Such approaches include testing by polymerase chain reaction (PCR) for the presence of the *pia* locus (Brown et al., 2001a).

1.1.3 Anatomy and physiology

The components of the *S. pneumoniae* cell surface are illustrated in Figure 1.1 (Jedrzejas, 2004). Every invasive isolate of *S. pneumoniae* and nearly every isolate associated with mucosal infection has a polysaccharide capsule. The only exceptions have been occasional cases of pneumococcal conjunctivitis (Ertugrul et al., 1997). Capsules consist of repeating oligosaccharide units which are polymerized, and transported to the cell surface. Capsular polysaccharide (CPS) is covalently bound to cell wall peptidoglycan and cell wall polysaccharide. These processes are controlled by a genetic cassette consisting of up to 15 genes (Morona et al., 1997). The precise monosaccharides within the oligosaccharide units and the bonds between them

provide the epitopes recognised by specific antisera used to distinguish different capsular serotypes (Park et al., 2007). On this basis, at least 91 serotypes can be identified, falling into a number of groups within which there are varying degrees of cross-reactivity. The Quellung reaction detects increased refractility of bacterial cells to light following incubation with specific anti-capsular antisera (Neufeld, 1902). This technique is used to serotype isolates of *S. pneumoniae*, and initially came to the fore when anti-sera where used therapeutically. It is again of great importance in the application of capsular polysaccharide-based vaccines to prevent *S. pneumoniae* disease. *S. pneumoniae* have the ability to internalise and incorporate DNA from other *S. pneumoniae* strains or even other species, a process known as competence (Tomasz, 1966). This process itself is under genetic control and responds to environmental signals, particularly quorum sensing (Lee and Morrison, 1999). By these means, *S. pneumoniae* can switch capsular type in a natural setting (Nesin et al., 1998).

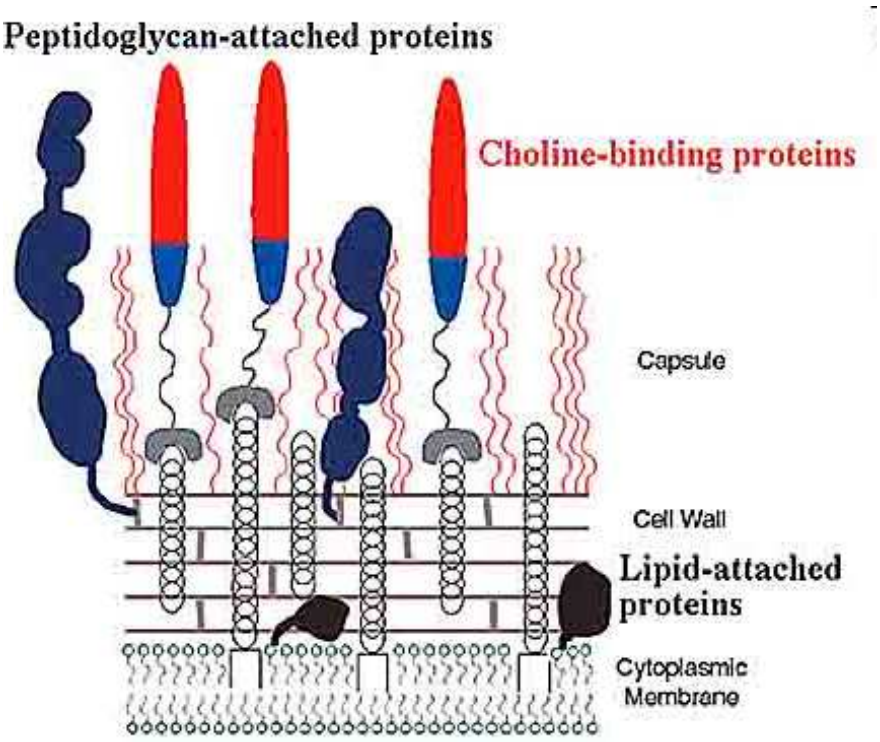

Figure 1.1. *S. pneumoniae* cell surface components

Proteins are attached to the surface of *S. pneumoniae* by at least three mechanisms. (1) proteins with choline binding domains attach to phosphocholine residues in cell wall teichoic and lipoteichoic acids; (2) proteins with appropriate motifs including LPxTG are covalently attached to cell wall peptidoglycan by sortases; (3) lipoproteins are attached to the cell membrane by acylation of apolipoprotein (also see Figure 1.2). (Jedrzejas, 2004).

In common with other streptococci, the bacterial cell wall of *S. pneumoniae* consists of peptidoglycan and teichoic acids (TA) (Tomasz et al., 1975). Peptidoglycan is formed by the cross-linking of long chains of the saccharides N-acetyl-D-glucosamine and N-acetylmuramic acid. Stem peptides, four to six amino acids in length, serve as cross-linkers, and are themselves cross-linked by pentaglycine bridges, providing substantial strength to the overall structure. Teichoic acids are phosphorylcholine (PC) containing polysaccharides which are covalently linked to peptidogylcan.

Lipoteichoic acids (LTA) have a lipid moiety and are attached to the cell membrane in addition. The PC residues in TA and LTA are major antigenic determinants of C-polysaccharide, or cell wall polysaccharide (CWPS), common to all *S. pneumoniae* strains (Jennings et al., 1980). Proteins are attached to the cell wall by a variety of mechanisms (as illustrated in Figure 1.1). Certain proteins are capable of binding the choline moieties of teichoic acids (Bergmann and Hammerschmidt, 2006). There are 10-15 such choline binding proteins (CBPs) including pneumococcal surface protein A (PspA), PspC, also known as CbpA, LytA (autolysin) and LytC (lysozyme). Other proteins are covalently anchored to peptidoglycan after cleavage of a LPxTG sequence by a transpeptidase known as a sortase (Kharat and Tomasz, 2003). There are approximately 10-20 such proteins, including neuraminidase A (NanA) and β-galactosidase. For many of these proteins, important functions have been identified in establishing colonisation or in obfuscating host defense mechanisms, as described further below. Other surface-located proteins act enzymatically on host structures to enhance binding (e.g. the action of neuraminidase A (NanA) on host sialic acid structures) or to facilitate tissue invasion (e.g. hyaluronidase).

Lipoproteins are important components of the ATP-binding cassette (ABC) transporters responsible for influx and efflux of molecules important for nutrition, signalling and stress response (Garmory and Titball, 2004). They are attached to the cell membrane by a mechanism that is conserved amongst gram-positive organisms (Sutcliffe and Harrington, 2002). Prolipoproteins containing an N-terminal signal peptide are secreted out of the cell by the general secretory pathway. The enzyme diacylglyceryl transferase (Lgt) catalyses the attachment of a universally conserved cysteine residue within a 'lipobox' domain to the membrane phospholipid diacylglycerol (Sutcliffe and Harrington, 2002). Finally, a type II lipoprotein signal peptidase (Lsp) cleaves the N-terminal signal peptide to form the mature membrane-

attached lipoprotein (Khandavilli et al., 2008). Pneumococcal surface adhesion A (PsaA) is a typical surface lipoprotein that plays an essential role in manganese and zinc transport (Lawrence et al., 1998). Further lipoproteins important to *S. pneumoniae* physiology include iron transporters PiaA and PiuA (Brown et al., 2001a), and PpmA (Cron et al., 2009) and SlrA (Hermans et al., 2006) which have roles in adherence to nasopharyngeal epithelium and evasion of phagocytosis. There are predicted to be approximately 40-50 *S. pneumoniae* lipoproteins. Lipoproteins are important ligands of Toll-like receptor 2 (TLR2) (Travassos et al., 2004). Many proteins have been predicted to be surface-attached as their genes encode lipobox domains, LPxTG motifs or choline-binding domains. Other proteins have shown to be surface-located by more empiric methods.

S. pneumoniae produces a single exotoxin, pneumolysin (Ply). It has homology to other streptococcal cytolysins (Jefferies et al., 2007). It plays important an role in evading host defences at mucosal surfaces. Through polymerisation it may form pores in eukaryotic host cell membranes leading to cytolysis (Tilley et al., 2005), and may trigger apoptosis of a range of host cell types (Littmann et al., 2009). However, it also triggers host defence mechanisms as it is a TLR4 ligand (Malley et al., 2003).

1.1.4 Colonisation and diseases

S. pneumoniae can transmit between individuals via aerosol and droplet spread (Bogaert et al., 2004a). Following inhalation into the upper respiratory tract, if the conditions are appropriate, bacteria may establish viable colonies on the nasopharyngeal surface. Multiple factors dictate the competition between the various organisms that vie for occupancy of this limited niche (Bogaert et al., 2004a). The state of colonisation, or carriage, will continue until the bacteria are actively cleared

by various host response mechanisms, as described below. In this thesis, the terms colonisation and carriage will be used interchangeably to describe the period during which viable bacteria can be cultured from samples collected from the nasopharynx. Following acquisition and colonisation of the nasopharynx, *S. pneumoniae* can spread to various sites and lead to disease, as illustrated in Figure 1.2 (Bogaert et al., 2004a). Bacteria ascending the eustachian tube can establish infection of the middle ear causing otitis media (Rodgers et al., 2009). Whilst this has minimal associated mortality, it probably represents the largest number of disease cases caused by *S. pneumoniae* (Cripps et al., 2005). Most cases are not microbiologically diagnosed, and resolve either through spontaneous resolution or with the assistance of a course of antibiotics. Individuals with structurally abnormal airways can develop *S. pneumoniae* colonisation of the bronchial tree, as part of a chronic bronchitis. This is most commonly recognised as part of chronic obstructive pulmonary disease (Patel et al., 2002), but probably occurs in other airway disorders as well.

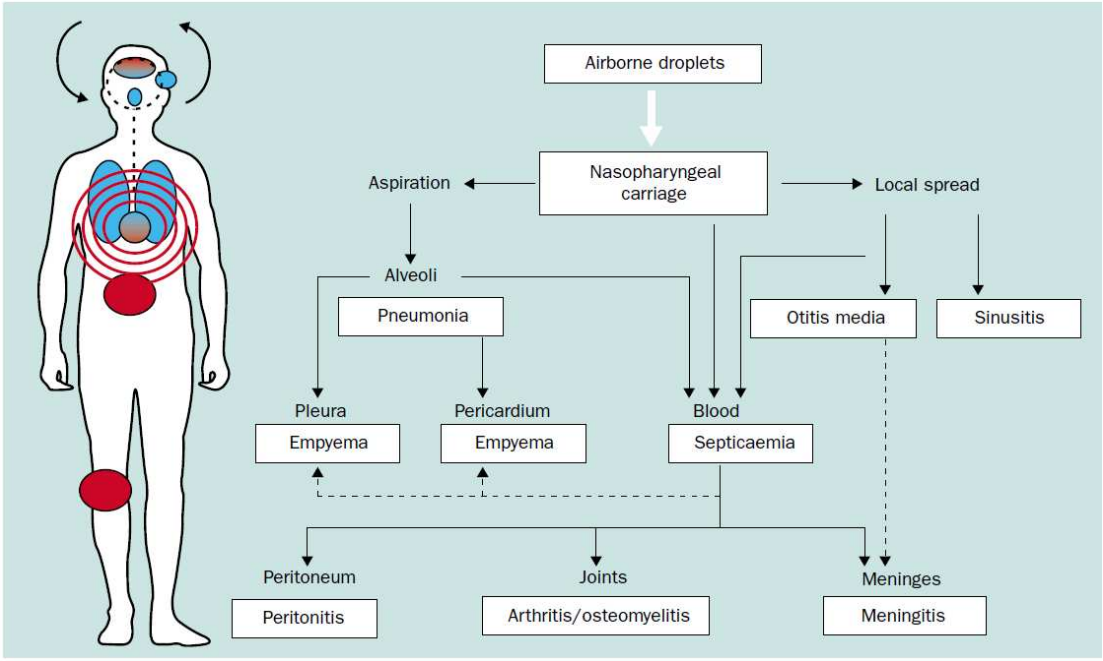

Figure 1.2. Routes for the spread of *S. pneumoniae* leading to disease

Nasopharyngeal acquisition from airborne droplets may be asymptomatic. Mucosal spread can lead to diseases such as otitis media or sinusitis. Aspiration into the alveoli can lead to pneumonia which may be complicated by pleural empyema or rarely pericardial effusion (empyema). Pneumonia can lead to septicaemia but this can also arise through direct invasion following nasopharyngeal colonisation. Haematogenous spread can seed distant sites leading to peritonitis, bone and joint infection or meningitis. Pneumonia can also arise from haematogenous spread. (Bogaert et al., 2004a)

Aspiration of small numbers of bacteria probably occurs constantly during colonisation (van der and Opal, 2009). However, there are abundant physical, chemical and immunological mechanisms which limit the capacity of inhaled bacteria to establish pulmonary infection. Studies in mice would suggest that a threshold inoculum is required before these mechanism are overwhelmed (Gingles et al., 2001b, Chiavolini et al., 2008), but that the size of this inoculum varies between individuals and serotypes. The innate and adaptive immune defences which protect the host against pneumonia are discussed in more detail below. Epidemiological data would suggest that prior exposure to *S. pneumoniae* may enhance the response to subsequent exposure (Lipsitch et al., 2005). Thus, those components of the immune system that can adapt may become more important following previous exposure. Aspiration of bacteria may lead to either a more focal lobar pneumonia or to more diffuse bronchopneumonia. Only rarely is pneumonia believed to develop directly from haematogenous spread. Hallmark clinical features include fever, cough, production of purulent sputum and difficulty in breathing (van der and Opal, 2009). Untreated, this can lead to hypoxia, respiratory failure, or septicaemia. Specific complications include the development of parapneumonic pleural effusions, empyema, and rarely lung abscess or necrosis. However, clinically complete resolution is possible, especially with prompt antibiotic therapy.

Histopathological studies show similarity between animal models and human disease (Bergeron et al., 1998). Specific interactions between bacterial pathogen-associated molecular patterns (PAMPs) and host soluble and cellular pathogen recognition receptors (PRRs) facilitate the recognition of infection and initiate the inflammatory response (Calbo and Garau, 2010). A range of inflammatory mediators are released, driving cellular recruitment and activation (Dallaire et al., 2001). Airspace oedema

occurs early and is followed by recruitment of leukocytes to the alveoli. These are predominantly neutrophils, especially in the earlier stages of infection, but by 72-96 h monocytes and some lymphocytes dominate. Interstitial oedema and haemorrhage follows from 24-28 h, with proliferation of type II pneumocytes. As infection progresses, tissue architecture is either progressively destroyed, or moves to a resolution phase with phagocytosis and apoptosis of inflammatory cells, and gradual restoration of a quiescent environment. The role of these inflammatory processes in limiting infection is discussed in detail below.

Many studies have identified *S. pneumoniae* as the leading pathogen causing community-acquired pneumonia (CAP) (Brown and Lerner, 1998). Diagnosis can be made from culture of sputum, which is specific but lacks sensitivity. Positive urinary antigen test for *S. pneumoniae* CWPS is a sensitive test, and specific for determining the causal pathogen of CAP when used in populations with low carriage rates such as adults in developed countries, but remains of low specificity in infants and children where it may simply reflect carriage (Charkaluk et al., 2006). In the majority of cases of *S. pneumoniae* pneumonia, blood cultures remain sterile (Werno and Murdoch, 2008). *S. pneumoniae* can invade the bloodstream either directly across the nasopharyngeal epithelium (Bogaert et al., 2004a), or secondary to the inflammation associated with pneumonia. The former is more common in young children, and in the absence of a clinical focus for the fevers it triggers, is termed occult bacteraemia (Joffe and Alpern, 2010). When bacteraemia does occur it is most commonly secondary to pneumonia and has a high mortality (Trotter et al., 2010). Once viable bacteria have entered the bloodstream they can seed distant sites giving rise to focal disease such as meningitis, or more rarely, septic arthritis, osteomyelitis or peritonitis.

1.2 EPIDEMIOLOGY

1.2.1 Colonisation

The majority of studies of the epidemiology of nasopharyngeal carriage are cross-sectional prevalence studies based on defined populations (Bogaert et al., 2004a). These show that carriage rates vary with age, geographical location, socio-economic status, immune status, and environmental features. Initial colonisation occurs at the latest within the first few months of life (Gray and Dillon, Jr., 1988, Loda et al., 1975, Vives et al., 1997). In areas of high prevalence this can be within weeks of birth (Hill et al., 2008) (Aniansson et al., 1992, Granat et al., 2007, Faden et al., 1997), and is nearly ubiquitous by six months of age. In resource-rich countries, 50% of infants have been colonised by one year of age (Syrjänen et al., 2001, Lee et al., 1995), whereas nearly 95% of infants were colonised by 6 months of age in Bangladesh (Granat et al., 2007). Repeat colonisation events take place, such that peak carriage rates occur at approximately 3-5 years of age and then wane to a stable rate of approximately 10% in adult life (Bogaert et al., 2004a, Abdullahi et al., 2008). It is not known whether carriage rates change with increasing age amongst adults. Human immunodeficiency virus (HIV) infection can increase the rate of *S. pneumoniae* colonisation in both children (Madhi et al., 2007) and adults (Gill et al., 2008). Patterns of social interaction play a large role in dictating spread between children. Thus higher carriage has been observed amongst children attending daycare (Bogaert et al., 2001) and in those living in institutions (Raymond et al., 2000). Carriage rates are also enhanced during periods of viral upper respiratory illness (Brimblecombe et al., 1958) and during the rainy season in tropical environments (Abdullahi et al., 2008). Whilst initial colonisation events may persist for up to 4 months (Gray and

Dillon, Jr., 1988), the duration appears to shorten with age. In adult populations, carriage may last 2 to 4 weeks (Ekdahl et al., 1997). The methodology of such studies limits their ability to identify very short carriage events. If carriage duration is shorter with increasing age, prevalence studies will therefore underestimate the total number of carriage events as age increases. As disease is thought to arise shortly after acquisition, this may also underestimate the protective effect of prior exposure on the development of disease following re-exposure.

Serotype specific carriage prevalence varies between different populations, with limited numbers of serotypes dominating in different communities. Prior to the introduction of the pneumococcal conjugate vaccine (PCV), serotypes 19F, 6B, 6A, 9V, and 23F were most frequently found among children under 3 years of age in several European countries (Bogaert et al., 2001, Bogaert et al., 2006) and in the United States US (Yeh et al., 2003). However, in Kenya, serotypes 13, 15, 14, 6B, and 19F are most prevalent (Mbelle et al., 1999). The serotypes carried also appear to change with increasing age (Bogaert et al., 2006, Kaltoft et al., 2000). Even when the effect of age is controlled for, differences in carriage duration for different serotypes persist (Hogberg et al., 2007). Serotype prevalence has been affected by introduction of the pneumococcal conjugate vaccine as discussed below. It is becoming apparent that methodologies based on culture and Quellung reaction alone can only identify a small number (perhaps 1-2, at most probably 3) colonizing serotypes, and that an individual may be colonised with further strains at lower density. Genetic approaches such as multiplex PCR to detect capsule synthesis genes offer the prospect of identifying larger numbers of distinct isolates and permitting more sophisticated tracking of colonisation (Rivera-Olivero et al., 2009).

1.2.2 Pneumonia

For many years, pneumonia has remained the commonest cause of death from *S. pneumoniae* disease worldwide (WHO, 1999), yet in most cases precise diagnosis is elusive. Culture of sputum or blood is often negative. It is estimated that 30-50% of adult CAP is attributable to *S. pneumoniae* (Fedson et al., 1998). Thus, epidemiological estimates of the burden of *S. pneumoniae* pneumonia are largely extrapolated from other data. In this regard, vaccine probe studies have proven helpful. The reduction in total pneumonia following the introduction of a pneumococcal conjugate vaccine which specifically protects against seven *S. pneumoniae* serotypes identifies the burden of pneumonia attributable to these or related serotypes. It is then possible to extrapolate to estimate total *S. pneumoniae* pneumonia incidence based on serotype prevalence data. Using such techniques, over 800,000 deaths in children under 5 years of age are thought to die each year from *S. pneumoniae* pneumonia (O'Brien et al., 2009). Such approaches support the concept that *S. pneumoniae* pneumonia is more common in the very young, the old and the immunocompromised (Fedson and Scott, 1999, O'Brien et al., 2009). However, epidemiological data are more robust for invasive pneumococcal disease (IPD), reflecting the ability to isolate the causative organism. Nonetheless, non-microbiologically differentiated pneumonia rates are at their highest in infants and the elderly.

1.2.3 Invasive pneumococcal disease

Studies from many countries indicate that rates of IPD are highest in children under 3 years of age, fall during childhood, and rise again in later adult life (Trotter et al., 2010, Lipsitch et al., 2005, Burman et al., 1985) to rates of approximately 50 cases per 100,000 persons. This fall during the first few years of life is shared between all

types of IPD (i.e. meningitis, invasive pneumonia, septicaemia) (Lipsitch et al., 2005). Furthermore, similar rates of decline are observed for all serotypes, irrespective of how commonly they are carried. Certain populations have higher rates of IPD, including Alaskans (Davidson et al., 1994) and Aboriginal Australians (Torzillo et al., 1995). This is associated with higher colonisation rates but the underlying reasons are not clear. Strains of *S. pneumoniae* vary in their ability to cause invasive disease following colonisation. Traditionally this has been measured as the 'invasiveness odds ratio', relating IPD data to cross-sectional carriage prevalence (Brueggemann et al., 2003, Brueggemann et al., 2004). To overcome possible bias of carriage duration, the 'attack rate' for a strain can also be calculated, using data on acquisition events as the denominator (Sleeman et al., 2006). Using this technique it appears that capsular serotype is an important determinant of both carriage duration and attack rate. However, there is still considerable variation in invasiveness between different genetic strains of a given serotype, which correlates with the strains ability to limit complement deposition (Sjostrom et al., 2006).

There is also a seasonal distribution of IPD incidence with higher rates in the winter months (Bogaert et al., 2004a), following the pattern seen for respiratory viral infection. Those individuals with viral infection such as influenza have increased risk of developing IPD (O'Brien et al., 2000). This may reflect both upper respiratory viral infection predisposing to bacterial colonisation, and to lower respiratory viral illness affecting lung defences, as discussed below.

1.2.4 Risk factors for S. pneumoniae disease

Many risk factors have been identified which predispose to *S. pneumoniae* pneumonia or IPD. These are listed in Table 1.1 (adapted from (van der and Opal, 2009)). Impaired pulmonary defence mechanisms predispose to pneumonia specifically,

whereas factors affecting immunity more generally also predispose to non-pneumonic bacteraemia and meningitis. Globally, malnutrition and HIV infection are the most significant contributors to the increased burden of *S. pneumoniae* disease in developing countries (Janoff et al., 1992). As well as increasing the risk of progression to disease once colonised, HIV positivity increases the risk of colonisation itself (Gill et al., 2008). HIV may predispose to increased levels of *S. pneumoniae* disease in many ways. These include direct effects on CD4 T-cells impacting on responses to colonisation (Malley, 2010), direct (Swingler et al., 2008) and indirect effects on antibody production, and effects of HIV infection on macrophage function (Gordon et al., 2005). Specific mechanisms of defence against *S. pneumoniae* infection are discussed in more detail below.

Table 1.1. Risk factors for *S. pneumoniae* pneumonia and invasive disease

Epidemiological
Younger than 2 years
Older than 65 years
Isolated populations
Poverty, crowding
Childhood day care
Pulmonary
Cigarette smoking
Chronic lung disease
Poor mucociliary function
Diminished cough reflex, aspiration pneumonitis
Proton-pump inhibitors and other gastric-acid inhibitors
Immunological
Asplenia or hyposplenia
Defects in humoral immunity (complement or immunoglobulin)
HIV infection
Genetic polymorphisms (e.g., complement, MBL)
Single gene defects in innate immunity (IRAK-4, MyD88)
Defects in cellular immunity and neutrophil defects
Miscellaneous
Alcoholism
Diabetes mellitus
Antecedent influenza and other viral infections
Recent acquisition of a new virulent strain
Severe liver disease
Recent exposure to antibiotics

1.3 NASOPHARYNGEAL COLONISATION

Although not a natural commensal of mice, Wu (Wu et al., 1997b) demonstrated that inoculation of the nasopharynx of mice with *S. pneumoniae* can establish stable colonisation, and this approach has provided the basis for much research into both the factors influencing colonisation and its subsequent effects on the host (Bogaert et al., 2004a).

1.3.1 Airway anatomy and the mucosal immune system

The upper respiratory tract is outside the thorax and consists of the oro- and naso-pharynx and larynx. The lower respiratory tract is intra-thoracic and consists of the trachea, bronchi, bronchioles and most distally the alveolar spaces. Specific mucosal immune processes operate complimentary to systemic immunity. There are specialized organs within the respiratory tract which act as induction and effector sites for localized adaptive immune responses. During colonisation, bacterial antigens within the lumen are in close relation to upper airway mucosa-associated lymphoid tissue (MALT) (Matthias et al., 2008). In humans, this tissue is organized into adenoidal and tonsillar structures together forming Waldeyer's ring (Figure 1.3a). In mice, discrete aggregates of nasal-associated lymphoid tissue (NALT) are found on the hard palate (Figure 1.3b) (Heritage et al., 1997). In addition, bacterial material can be transported to draining lymph nodes in the cervical region. Induction of immune responses can occur both in NALT and in the draining cervical lymph nodes (Yamada et al., 2005). Very small numbers of bacteria that enter the lungs during colonisation are rapidly cleared and may contribute to some degree to induction of responses within bronchial-associated lymphoid tissue (the lower respiratory MALT)

and draining mediastinal lymph nodes. Immune responses may also induced in the spleen following systemic trafficking.

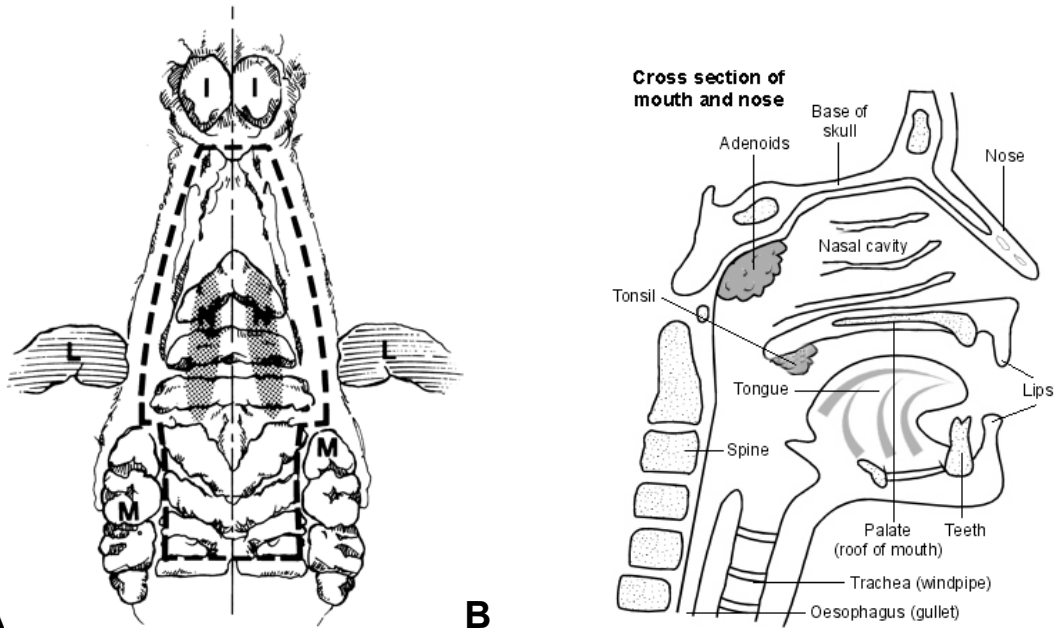

Figure 1.3. Anatomic location of upper airway mucosa-associated lymphoid tissue.

A, Mouse. Illustrated view of ventral surface of upper palate. I, incisor tooth; L, ligament; M, molar tooth; N, NALT located on the dorsal surface of upper palate within the stippled area. (Image from Heritage, 1997). B, Adenoid and tonsil location in human (Image adapted from Website of Patient UK).

During subsequent antigen re-exposure, there are possible effector sites for the mucosal immune system, depending on the location of the infectious challenge. If organisms are inhaled but not significantly aspirated into the lower respiratory tract, the nasopharynx itself is the effector site. If effective, adaptive immune responses may prevent re-colonisation. On the other hand, if organisms are aspirated into the lower respiratory tract, the lung itself is the effector site of mucosal immunity. Adaptive immune responses will operate in addition to innate reponses in limiting

infection, and may prevent the development of pneumoniae. If bacteria invade the lungs, the systemic circulation and reticulo-endothelial system become the effector sites that may prevent systemic infection.

1.3.2 Establishing colonisation

The first defence mechanism that *S. pneumoniae* need to overcome on entering the nasopharynx is entrapment in lumenal mucus. Negatively charged sialic acid residues on mucus polysaccharides are repelled by the majority of negatively-charged *S. pneumoniae* polysaccharide capsules and strains lacking capsule have greater binding to mucus, and can therefore be expelled with mucus from the nasopharynx (Nelson et al., 2007). Thus the capsule is required to establish colonisation (Magee and Yother, 2001), and it has been suggested that bacteria which colonise the nasopharynx evolved capsules specifically to avoid rapid clearance in mucus with the secondary effect of avoiding phagocytosis (Nelson et al., 2007). Adherence to nasopharyngeal epithelium requires specific molecular interactions between the bacterium and the host cell (Bogaert et al., 2004a). *S. pneumoniae* undergoes phase variation characterised by variation in the levels of capsule and surface protein expression (Weiser et al., 1994), which influences invasive potential. Bacterial cells with lower levels of capsule expression (transparent phenotype) are enhanced in their capacity to adhere to respiratory epithelium compared with cells with greater amounts of capsule (opaque phenotype). Whilst transparent *S. pneumoniae* can readily be washed from the nasopharyngeal lumen, further *S. pneumoniae* identified only through homogenisation of tissues are of the opaque phenotype (Briles et al., 2005). The selective presence of opaque bacteria within the epithelium layer and deeper tissue suggests that they might be selected for by their resistance to phagocytosis.

Once mucus clearance is avoided, several specific molecular interactions enhance the capacity of bacterial cells to adhere to epithelial cells. These operate over and above non-specific physico-chemical interactions to facilitate establishment of colonisation. The bacterial neuraminidase enzyme NanA cleaves sialic acid residues from polysaccharides on the surface of host cells. This exposes N-acetyl-glucosamine residues to which PsaA can bind (Tong et al., 2002). Attachment is aided further by the CBP PspC binding sugars such as sialic acid and lacto-N-neotrehalose as well as to the polymeric immunoglobulin receptor (pIgR) on host cells (Rosenow et al., 1997). As well as facilitating attachment, these interactions lead to activation of signalling pathways within epithelial cells, resulting in production of cytokines and type I interferons (Joyce et al., 2009). Further signalling occurs through PRRs including TLR2, which recognise pathogen-associated molecular patterns such as bacterial lipoproteins (Aliprantis et al., 1999). Epithelial cell activation leads to enhancement of bacterial binding through upregulation of surface expression of pIgR and platelet activating factor (PAF) receptor (PAFr), to which PC residues in *S. pneumoniae* cell wall can bind. Pre-existing viral infection enhances the expression of PAFr and increases levels of cytokines including interleukin (IL)-1 and tumour necrosis factor (TNF)-α at the respiratory mucosa, which predisposes to bacterial transcytosis and invasive disease (Tuomanen, 1997). Immunoglobulin (Ig) A1 and IgA2 are actively secreted across epithelial cells into the lumen, and can bind specifically to *S. pneumoniae* antigens. However, the ability of IgA1 to enhance opsonophagocytosis is impaired as *S. pneumoniae* encodes an IgA protease, which cleaves the functional domain of IgA1. This results in enhanced bacterial binding to epithelium but impaired opsonic clearance (Weiser et al., 2003). Thus, through a range of specific and dynamic interactions, *S. pneumoniae* attach and can enter into nasopharyngeal epithelial cells.

Colonisation appears to involve a degree of superficial tissue invasion in addition to surface attachment (Briles et al., 2005). This is aided by surface located serine proteases (Mitchell, 2003) in addition to recruitment of host plasminogen to the bacterial surface by *S. pneumoniae* enolase and glyceraldehyde-3-phosphate dehydrogenase (Hammerschmidt, 2006). *S. pneumoniae* killing by mucosal apolactoferrin is prevented by its binding to PspA (Shaper et al., 2004). Various other molecules directly affect the ability of *S. pneumoniae* to establish colonisation, including pneumococcal adherence and virulence factor A (PavA) which binds fibronectin (Pracht et al., 2005), an important host extracellular matrix protein.

The nasopharynx is a competitive niche in terms of limited resources to bacteria, and the presence of longer term commensal species may inhibit the establishment of colonisation. In both humans and mice (Borthen et al., 1987, Bernstein, 1992), viridans streptococci, lactobacilli and in some cases *Staphylococcus aureus* form important parts of the resident flora. Viridans streptococci compete with *S. pneumoniae*, *Haemophilus influenzae* and *S. aureus* for occupancy of this niche as revealed in studies of the effects of antibiotics on nasopharyngeal flora (Faden et al., 1990, GHAFFAR et al., 1999, Ghaffar et al., 2002). Furthermore, direct competition takes place in the nasopharynx where hydrogen peroxide produced by *S. pneumoniae* kills lysogenic *S. aureus* (Selva et al., 2009). This may explain why *S. aureus* colonisation rates increase as *S. pneumoniae* carriage falls (Bogaert et al., 2004a). Direct competition between individual strains of *S. pneumoniae* has also been shown in mice (Lipsitch et al., 2000). Intra-species competition may involve pneumococcal bacteriocins whose expression is regulated by quorum sensing mechanisms (Dawid et al., 2007, Dawid et al., 2009).

1.3.3 Host immune response to colonisation

Following colonisation of the nasopharynx, there is a brisk influx of neutrophils to the paranasal spaces (van Rossum et al., 2005, Nelson et al., 2007). Whilst this is not sufficient to clear colonisation, this neutrophil influx is important in degradation of whole bacteria and release of antigenic material (Matthias et al., 2008). This is enhanced through lysis of neutrophils by Ply. M-cells sample luminal contents and traffic it to underlying nasal associated lymphoid tissue (NALT) for induction of immune responses. Bacterial antigens can be taken up from the lumen by this mechanism. Although direct invasion of M-cells by *S. pneumoniae* has not been described, this has been shown to occur with *Streptococcus pyogenes* (Park et al., 2003). Both antigen-specific B-cell and T-cell responses can be elicited from murine NALT (Matthias et al., 2008, Richards et al., 2010), and equivalent responses have been shown in the human equivalent, adenoidal mononuclear cells (MNCs) (Zhang et al., 2002, Zhang et al., 2007, Ivarsson et al., 2004).

1.3.3.1 Antibody responses to colonisation

The early success of vaccines inducing anti-CPS antibody responses established the belief that CPS was the only protective *S. pneumoniae* antigen against *S. pneumoniae* disease. The presence of anti-CPS antibody became equated with immunity to *S. pneumoniae*, and its absence was taken to indicate lack of natural immunity following colonisation (Musher et al., 1986). However, studies in both humans and animal models have revealed that multiple surface and intracellular antigens induce responses following natural exposure, many of which can be protective even in the absence of an anti-CPS response (Bogaert et al., 2004a). Studies looking at anti-CPS IgG levels have been performed in several countries prior to the introduction of vaccines (Balmer et al., 2007, Kayhty et al., 2005, Shinefield et al., 1999). Using highly-specific

enzyme-linked immunosorbant assay (ELISA) techniques with pre-absorption of non-specific antibodies, pre-existing antibody responses have been demonstrated which are likely to reflect natural exposure. For most capsular serotypes, levels remain low in the first year of life, rise in early childhood, and then rise further in the 20-30 age group. This may reflect boosting following further exposure during parenthood. Observation of outbreaks in military camps has shown that natural exposure can induce anti-capsular antibodies to serotypes 7F and 8 (Musher et al., 1997). Other studies have demonstrated that colonisation with only certain serotypes induces anti-CPS serum IgG. Carriage of serotypes 9V, 14, 18C, 19F and 23F by an individual or family member was associated with a rise in serotype-specific serum IgG, but this did not occur with serotype 6B (Goldblatt et al., 2005). Children exposed to serotypes 11A and 14 developed serum IgG responses, but those exposed to serotypes 6B, 19F and 23F did not (Soininen et al., 2001). There is very little data on anti-CPS levels in the elderly, but one study suggests that serum IgG to only 2 of 6 serotypes studied falls with increasing age, whereas IgM to all serotypes falls (Simell et al., 2008). Salivary IgA specific to several serotypes has also been shown to rise following natural exposures or mucosal disease (Simell et al., 2002). Thus, natural exposure to *S. pneumoniae* can induce both systemic and mucosal anti-CPS antibodies, but this appears to vary with serotype, and levels only rise significantly after the first year of life for most serotypes.

There is now abundant data demonstrating anti-protein antibody responses develop following natural exposure. Unlike anti-capsular responses, serum anti-protein IgG is rapidly detectable following *S. pneumoniae* exposure in the first year of life. Serum IgG to PspA, PsaA and Ply is detectable from 6 months of age, and correlates with exposure or previous mucosal disease (Rapola et al., 2000). Indeed, in Kenya, 100% of infants under one year of age had serum IgG to these protein antigens (Laine et al.,

2004). Antibodies to pneumococcal proteins in early infancy may be due to passive placental transfer. Levels of antibodies to PhtD, PspC and LytC were found to fall after birth, but then rise again following natural exposure from just 4 to 5 months of age (Holmlund et al., 2009). In addition to induction in infancy, there is also evidence that levels may be boosted following exposure in adult life. In a longitudinal household study, serum anti- PsaA and anti-Ply rose amongst adults following carriage, although there was no change in anti-PspA (Goldblatt et al., 2005). In the only published report of anti-*S. pneumoniae* protein IgG levels in the elderly, levels of serum IgG against all protein antigens investigated fell with increasing age (Simell et al., 2008), correlating with increasing risk of *S. pneumoniae* pneumonia and IPD. Allelic differences between *S. pneumoniae* strains such as for PspA can limit the cross-reactivity of antibodies, restricting the interpretation of some studies that link colonisation with immunogenicity. Recent data suggests that adults have broader anti-PspA responses probably reflecting multiple exposures to different protein variants (Melin et al., 2008). Salivary IgA to PspA, PsaA and Ply is also detectable from 6 months of age, and again correlates with exposure or previous mucosal disease (Simell et al., 2001). Mononuclear cells isolated from human adenoidectomy samples have been shown to secrete antibodies against PspA, Ply, PsaA and CbpA (Zhang et al., 2002) suggesting that mucosal lymphoid tissue is an important site of antibody production. These approaches to identify serum correlates of protective immunity have selected antigens based on evidence of protective efficacy in animal models or on the basis that they are surface expressed and thus antibody targeting them may be opsonophagocytic. An alternative unbiased approach at the genomic scale has identified further antigens that are immunogenic in humans following natural exposure (Giefing et al., 2008), some of which are protective in animal models and are being developed as novel vaccine candidates. Thus, natural exposure to *S.*

pneumoniae induces serum and mucosal anti-protein antibodies in infancy, with levels boosted during adult life, but probably waning in the elderly.

From such human observational studies, it is not possible to disaggregate the immunising contribution of colonisation per se from that of milder disease such as otitis media or undiagnosed pneumonias. Furthermore, due to the near universal prevalence of *S. pneumoniae,* it is not possible to identify never-colonised controls. Given the cross-reactivity between certain *S. pneumoniae* antigens and those of related species (Jefferies et al., 2007), such naturally acquired responses in some cases may also reflect exposure to other species. In the only published human colonisation experiment to date, adult volunteers were colonised with serotype 23F or 6B strains of *S. pneumoniae* (McCool et al., 2002, McCool et al., 2003) and the antibody response to a range of putative antigens was assessed. Colonisation induced serum IgG and salivary IgA against PspA and serum IgG against PspC, but not against several other protein antigens or the homologous CPS. Pre-existing serum IgG against PspA correlated with protection against experimental colonisation in this model. Colonisation with the 23F strain lasted for 42 days, similar to mouse models using this strain.

There is limited data on murine antibody responses to experimental nasopharyngeal colonisation as a model of first exposure in humans. Experimental colonisation of BALB/c mice with a serotype 23 strain induced serum IgG to unidentified bacterial antigens, but none specific to the type 23 CPS. Colonisation with a serotype 19 strain (Palaniappan et al., 2005) also induced non-capsular responses, with both serum IgG and mucosal IgA against PspA, PsaA, PspC and PdB (a Ply toxoid derivative (Paton, 1996). Colonisation of MF1 mice with D39, a serotype 2 strain, induced serum IgG against PspA, a weak serum IgM response to the type 2 capsule but again no serum anti-CPS IgG. Colonisation of BALB/c mice with a serotype 14 strain did not induce

significant serum IgG against type 14 CPS, but did prime B-cells for more robust responses to pneumococcal conjugate vaccine (PCV) (Rabquer et al., 2007), perhaps reflecting a need for multiple B-cell antigen encounters prior to induction of anti-CPS responses. Most recently, using unbiased analysis of antibody binding, the dominant responses to colonisation of C57/BL6 mice appeared to vary depending on colonizing bacterial strain (Roche and Weiser, 2010). An unencapsulated serotype 4 strain (TIGR4) induced serum IgG to PspA and PpmA, unencapsulated 6A induced IgG to PsaA, and a wild-type 23F strain induced IgG only to PpmA. IgG to other antigens was also induced but in lesser amounts. Thus, colonisation may induce both systemic and mucosal antibodies specific to capsule or protein antigens, but there appears to be much variation both between various animal models and between differing human studies. Although studies are limited, it appears that a single colonisation event in mice is sufficient to induce specific antibody responses but not to CPS antigens.

1.3.3.2 T-cell responses to colonisation

In addition to inducing antibody responses to bacterial antigens, colonisation with *S. pneumoniae* can induce the generation of antigen-specific memory T-cells (Zhang et al., 2009). The polarisation of the T-cell response is influenced by the PRR signals received by the antigen presenting cell (APC) and the cytokines it produces in response (Gerosa et al., 2008). Thus, IL-12 induces Th1 responses leading to interferon (IFN)-γ on recall; IL-1β, IL-6 and transforming growth factor (TGF)-β prime for Th17 cells responses leading to production of the signature cytokines IL-17A, IL-17F and IL-22; IL-4 primes for Th2 responses leading to production of IL-4, IL-5 and IL-13. In other circumstances, TGF-β can induce regulatory T-cells which produce IL-10 (Zhu and Paul).

There appears to be a propensity towards the development of Th17 responses at mucosal surfaces in response to antigen exposure, perhaps reflecting the relative abundance of TGF-β at such sites (Zygmunt et al., 2009, Pepper et al., 2009). APC signals can also influence the generation of Th17 cells. TLR2 (which recognises lipoproteins) (Reynolds et al., 2010) and nucleotide oligomerisation domain (NOD)-2 (which recognises peptidoglycan) (van Beelen et al., 2007) signalling in the APC can polarise naïve T cells towards the Th17 lineage. Cells of the Th17 lineage can rapidly expand in response to IL-23 production by various cells at the time of challenge (Khader et al., 2007). The effects of IL-17A, IL-17F and IL-22 are discussed in the context of lung defence mechanisms below. Nasopharyngeal colonisation of mice with live wild-type *S. pneumoniae* can induce a Th17-cell response both mucosally and systemically (Zhang et al., 2009). This response was dependant on TLR2, perhaps necessary for induction of IL-6 in APCs. Such responses can also be elicited using killed bacteria (Lu et al., 2008) or bacterial antigens alone (Basset et al., 2007) with appropriate adjuvants as described below. Such Th17-cell responses are critical for both primary clearance of colonizing bacteria and in limiting subsequent re-colonisation in mice.

Evidence supporting such processes in humans is currently more limited. Adenoidal tissue from healthy children undergoing adenoidectomy contains T-cells which proliferate in response to *S. pneumoniae* antigens such as Ply, and this appears to negatively correlate with carriage (Zhang et al., 2007). Similarly, human peripheral blood mononuclear cells (PBMCs) from Gambian adults proliferate in response to as range of *S. pneumoniae* protein antigens, but such responses did not correlate with carriage (Mureithi et al., 2009). IL-17 can be elicited in antigen-specific manner from culture of human PBMC's with *S. pneumoniae* antigens but not from cells from newborn babies who are antigen naïve (Lu et al., 2008). To date there is no reported

experimental evidence of an *in vivo* Th17 response following a colonisation event in humans.

1.3.4 Clearance of colonisation

1.3.4.1 Mice

The mechanisms responsible for primary clearance of *S. pneumoniae* colonisation in mice have recently been elucidated. It was noted several years ago that the time taken for three immunocompetent naïve mouse strains (BALB/c, CBA/J and C57Bl/6) to clear carriage of a serotype 23F *S. pneumoniae* strain was similar (McCool and Weiser, 2004). Furthermore, this time was similar to that taken to clear the same strain in human colonisation experiments (McCool et al., 2002). This time period (approximately 6 weeks) was suggestive of the need to induce a primary adaptive response. Antibody-deficient mice cleared carriage at a similar rate to WT mice, but this was markedly delayed in mice lacking CD4 T-cells, suggesting a T-helper cell mediated mechanism (McCool and Weiser, 2004). This clearance was subsequently shown to be mediated by mucosal Th17 cells (Zhang et al., 2009). IL-17 produced by antigen-specific Th17 cells induces chemokine release from cells including nasopharyngeal epithelium (Roussel et al., 2010). In turn this leads to both neutrophil and monocyte/macrophage recruitment to paranasal spaces and thus enhances the phagocytic clearance of colonizing bacteria (Zhang et al., 2009). This clearance of primary colonisation is mediated primarily by monocyte/macrophages rather than neutrophils. However, on re-challenge, a robust Th17 recall response leads to rapid recruitment of neutrophils to the nasopharynx leading to clearance of bacteria within two days.

Such Th17-cell responses can also be primed using heat-killed whole *S. pneumoniae* cells if the adjuvant cholera toxin is included (Malley et al., 2005). This protects

against subsequent wild-type *S. pneumoniae* colonisation in a non-serotype specific manner, dependant on a Th17-cell response (Trzcinski et al., 2008, Lu et al., 2008). Protective responses can be induced using single antigens such as CWPS (Malley et al., 2006) or combinations of purified *S. pneumoniae* proteins (Basset et al., 2007). CWPS is a zwitterionic PS, and such PS are processed and presented by the major histocompatibility complex (MHC) and recognised by the T-cell receptor (Duan et al., 2008). In this regard it behaves similar to a protein antigen. Induction and recall of Th17-cell responses in this system are antigen-specific**.** Protection against colonisation correlates with but was not mediated by serum antibody responses to PsaA, PspA and CWPS (Trzcinski et al., 2005).

A murine model of Group A *Streptococcus* (GAS) nasopharyngeal infection has also been established (Park et al., 2003). In this model, rapid clearance of re-colonisation is also dependent on an antigen-specific Th17-cell response (Wang et al., 2010a). This raises the possibility that such mechanisms may be broadly important in control of colonisation at this site. No data have been reported to date for other organisms which occupy this niche. Features of rheumatic fever and post-streptococcal glomerulonephritis, complications of GAS infection, bear similarities with auto-immune disorders in which Th17 cells play a critical pathogenic role (Paust et al., 2009, Lubberts, 2010). Whether anti-GAS Th17 cells are important in the pathology of these complications remains to be discovered.

1.3.4.2 Humans

The serotype-specific success of PCV in reducing carriage of vaccine-type strains indicates that anti-CPS antibody is capable of limiting colonisation. The precise serological correlate of PCV-induced protection against carriage is not known, but serum concentrations of specific IgG of 5µg/ml serum have been suggested (Goldblatt

et al., 2005). However, there are limited data to suggest that such protection underlies natural immunity induced through exposure. In a study of Gambian infants, previous carriage was associated with reduced risk of re-acquisition or shorter subsequent recolonisation only for serotype 14 (Hill et al., 2008). A study of children attending day-care in Israel indicated that only for serotypes 6A, 14 and 23F was previous colonisation associated with reduced likelihood of homologous recolonisation. This correlated with increased levels of CPS-specific antibody (Weinberger et al., 2008). Whilst for certain serotypes, there is evidence that naturally induced anti-CPS antibody is associated with protection against colonisation, a contrasting study from Bangladesh has shown immunity to colonisation to be serotype-independent (Granat et al., 2009). Thus, naturally acquired anti-CPS antibodies may only make a limited contribution to the adaptive immunity to colonisation that emerges with age.

Non-CPS antibody responses or T-cell mediated responses similar to the Th17 responses described in mice may be more important. In a UK study, *S. pneumoniae* colonised children were found to have lower serum and salivary IgG levels to proteins CbpA and Ply than uncolonised children (Zhang et al., 2006). In Gambian children, higher serum anti-PsaA antibody levels were found in non-carriers. However, such associations do not prove causality. In a neonatal study in Papua New Guinea, where infants are colonised at a median of 17 days of life, higher umbilical cord blood anti-Ply levels were associated with delayed acquisition, but anti-PspA was associated with earlier acquisition (Francis et al., 2009). As for the murine studies, antibody responses may only be indicators of exposure history, and it is possible that Th17 cells are the actual mediators of protection.

1.4 NATURAL IMMUNITY AGAINST *S. PNEUMONIAE* PNEUMONIA

Many mechanisms converge to prevent or limit the development of pneumonia once *S. pneumoniae* enter the lungs. Breakdown in one or more of these mechanisms renders an individual at increased risk of developing pneumococcal disease. Established risk factors are listed in Table 1.1 (van der and Opal, 2009), and reflect either abnormalities of respiratory tract structure and physiology, or of the supporting inflammatory and immunological systems. Many of the environmental risk factors, including alcoholism, malnutrition, renal insufficiency, diabetes mellitus, smoking and pre-existing lung disease affect multiple protective mechanisms. The ways in which prior or current viral infection predispose to *S. pneumoniae* infection are becoming increasingly unravelled at a molecular level (Sun and Metzger, 2008, Didierlaurent et al., 2008). There appears to be redundancy between some of these immune mechanisms, especially where multiple mediators exist with similar functional properties. However, the identification of individuals with genetic predisposition to pneumococcal pneumonia has identified where components of the immune system are essential for protection against *S. pneumoniae* and other pathogens (Picard et al., 2003).

1.4.1 Innate mechanisms of protective immunity

The bacteria entering the lung are swept along by the lining fluid which is constantly moved towards the pharynx by the ciliary escalator. This is subsequently swallowed and bacteria destroyed by the digestive system. This process of ciliary clearance can be neutralised by *S. pneumoniae* products such as hydrogen peroxide and the toxin

Ply (Feldman et al., 2002, Feldman et al., 2007). The mucus itself contains abundant substances which assist in maintaining sterility of the lower airway. These soluble factors have either intrinsic anti-microbial properties, or facilitate cellular anti-microbial responses by binding to the bacterial surface and enhancing interaction with cell surface receptors of phagocytes.

1.4.1.1 Respiratory tract soluble factors

The respiratory tract lumen contains a broad range of soluble factors which aid in defence against infection. Collectins such as surfactant proteins are found in alveolar lining fluid and mucous and can bind bacterial carbohydrate structures to assist both phagocytic clearance and triggering of innate immune responses to pathogens. Surfactant protein A facilitates *S. pneumoniae* phagocytosis mediated by alveolar macrophage (AM) scavenger receptors (Kuronuma et al., 2004), and deficiency of surfactant protein D leads to increased disease in mice (Jounblat et al., 2005). Collectins can also modulate signalling via TLRs (Shimizu et al., 2009). Other antimicrobial molecules present in alveolar lining fluid include cationic antimicrobial peptides and proteins such as lysozyme, defensins and cathelicidins which can bind and kill bacteria directly (Kolls et al., 2008). Defensins and cathelicidins operate synergistically and kill microbes through disruption of the bacterial cell membrane (Schneider et al., 2005, Zanetti, 2005). α-defensins can also block the ability of Ply to lyse host cells (Lehrer et al., 2009). Other cell types may also be important for production of antimicrobial molecules. Sub-lytic concentrations of Ply trigger human lung mast cell killing of *S. pneumoniae in vitro* via cathelicidin LL-37 (Cruse et al., 2010), although the role of mast cells in host defence against infection *in vivo* is not known. In addition to their direct antimicrobial role, certain antimicrobial proteins such as S100A8 and S100A9 play a role in facilitating phagocyte migration to alveoli

(Raquil et al., 2008). Whilst some of these molecules are constitutively present, expression of many is induced in a cytokine dependent fashion following recognition of the presence of *S. pneumoniae*. Their production by epithelial cells is closely regulated in parallel with that of chemokines in response to cytokine triggers (Kolls et al., 2008). Although this role is traditionally ascribed to cytokines belonging to the IL-1 family (Cowland et al., 2003), other cytokines are also involved. IL-17A acts upon the bronchial epithelial IL-17RA to induce production of human β-defensin-2 and the chemokine CCL20 (Kao et al., 2004). In mouse tracheal epithelium, IL-17 and IL-22 synergise to induce lipocalin-2, critical for defence against *Klebsiella pneumoniae* (Chan YR FAU - Liu et al., 2009).

S. pneumoniae has evolved mechanisms to overcome some of these defences. The lytic action of lysozyme on peptidoglycan (PG) is evaded via enzymatic modification of PG by PgdA, an N-acetylglucosamine deacetylase, and Adr, an O-acetyl transferase (Davis et al., 2008). Cationic antimicrobial peptides become entrapped within anionic CPS further limiting their ability to reach the bacterial cell membrane (Llobet et al., 2008). A further mechanism of host defence is to limit the availability of iron at mucosal surfaces. This is achieved through high-affinity sequestering molecules such as lactoferrin. *S. pneumoniae* overcomes this limitation through the ability of pneumococcal PspA to bind lactoferrin (Hammerschmidt et al., 1999). Whilst the relative ability of many of these molecules to limit *S. pneumoniae* growth *in vitro* has been described (Lee et al., 2004), their contributions *in vivo* remain unclear and there may be extensive redundancy between them. Overall, however, they contribute to a relatively hostile environment such that the majority of small aspirated inocula do not lead to established infection.

Over 30 proteins constitute the complement system, which, when activated, contribute to orchestrated defence against invading microorganisms (Walport, 2001a, Walport,

2001b). Binding and activation of complement components on the surface of *S. pneumoniae* leads to opsonophagocytosis and the induction of an inflammatory response. The complement system can be activated through three distinct pathways, which appear to play different roles in defence against *S. pneumoniae*. The classical pathway is activated by antibody–antigen complexes (Walport, 2001a) but also through microbial binding to soluble mediators such as serum amyloid protein (Yuste et al., 2007), SIGN-R1 (Kang et al., 2006), or C-reactive protein which binds PC (Mold et al., 2002). Secondly, the lectin pathway is triggered by mannose binding lectin (MBL) or ficollin recognition of surface carbohydrate residues (Garred et al., 2009). Finally, the alternative pathway is continuously activated but controlled at low levels of activation unless amplified through interaction with foreign surfaces. Using panels of knockout mice, it has been shown that the classical pathway appears most important in initial recognition of *S. pneumoniae* (Brown et al., 2002). In particular, the role of natural cross-reactive IgM antibodies in triggering classical pathway activation was demonstrated. These antibodies are known to bind PC residues in teichoic and lipoteichoic acids (Kolberg et al., 1997). Once complement deposition is triggered, secondary amplification occurs via the alternative pathway (Brown et al., 2002). These finding are supported by the observation that humans with genetic deficiency in early complement pathway proteins such as C2 (Jonsson et al., 2005) are markedly predisposed to recurrent *S. pneumoniae* infection, due to inability to enhance phagocytosis through complement deposition (Yuste et al., 2008). In contrast, the risk of infection is smaller in those with MBL deficiency (Roy et al., 2002, Brouwer et al., 2009).

S. pneumoniae has evolved several strategies to evade the deposition of complement. Most important of these is probably the polysaccharide capsule, which significantly limits the deposition of C3 on the bacterial surface (Hyams et al., 2010a). In addition,

the initial activation of the complement pathway through C1q deposition can be inhibited by the abundant *S. pneumoniae* surface protein PspA (Tu et al., 1999). The released pneumococcal exotoxin Ply also reduces the level of C3 deposition on the bacterial surface (Yuste et al., 2005). Ply activation of the complement pathway is independent of specific antibody (Mitchell et al., 1991). It has been suggested that Ply may be acting as a decoy for complement activation away from bacterial surface (Paterson and Mitchell, 2006). Where C3 is deposited, it can be degraded by the surface protein PhpA (Hamel et al., 2004). The ability of the alternative pathway to amplify complement deposition is also impeded by *S. pneumoniae* protein PspC which binds the complement regulatory protein factor H (Dave et al., 2004, Dave et al., 2001). These mechanisms act together to limit the amount of complement deposition on *S. pneumoniae*, and attenuate its clearance from the airways.

1.4.1.2 Cellular recognition of *S. pneumoniae*

There are several cell types which express surface PRRs designed to respond to the presence of pathogens in the lower airway. These include AM resident quiescently in the uninfected lung (Marriott and Dockrell, 2007) and structural cells including epithelial cells (Armstrong et al., 2004). In addition, dendritic cells have processes interdigitating into the alveolar spaces, allowing contact with aspirated bacteria. Specialised subsets of lymphocytes operating as part of a non-adaptive immune response are also involved in recognition of *S. pneumoniae*. These include both γδ-T-cells (Nakasone et al., 2007) and NKT-cells (Kawakami et al., 2003) as discussed below. The PRRs which recognise *S. pneumoniae* have recently been reviewed (van der and Opal, 2009). They include TLRs, mannose receptors and scavenger receptors such as macrophage receptor with collagenous structure (MARCO). Other soluble factors such as complement components and immunoglobulins which have bound

bacterial structures can trigger responses via complement receptors (CR) and Fc-receptors (FcR). Once internalised, further intracellular PRRs may trigger responses to the presence of bacterial products, including further TLRs, NODs receptors and other NOD-like receptors (NLRs). Expression of PRRs can be affected by recent viral infection. For example, IFN-γ produced by CD4+ and CD8+ T-cells in influenza infection leads to downregulation of MARCO expression on AMs, inhibiting clearance of *S. pneumoniae* and increasing the incidence of secondary bacterial infection (Sun and Metzger, 2008).

Several of these mechanisms have been specifically implicated in host defence against *S. pneumoniae*. TLR2 recognises lipidated bacterial motifs including lipoproteins and lipopeptides in conjunction with TLR1 (for triacylated lipoproteins) or TLR6 (for diacylated lipoproteins as found in *S. pneumoniae*) (Kang et al., 2009). TLR4 was originally identified as the recognition molecule for lipopolysaccharide (LPS), the Gram-negative endotoxin (Poltorak et al., 1998). However, it also mediates recognition of the *S. pneumoniae* toxin Ply (Malley et al., 2003). Endosomal TLR9 can recognise bacterial unmethylated CpG from *S. pneumoniae* (Albiger et al., 2007). The relative contribution of these TLRs towards in the host response to *S. pneumoniae* infection is still unclear, partly as a result of the use of differing models (Mogensen et al., 2006). TLR2 deficiency leads to more severe disease in murine *S. pneumoniae* meningitis (Echchannaoui et al., 2002, Koedel et al., 2003). Whilst *in vitro*, AM production of the important inflammatory mediator TNF-α is entirely dependent on TLR2 signalling, TLR2 signalling appears to make only a modest contribution towards host defence in murine models of *S. pneumoniae* pneumonia (Knapp et al., 2004). TLR2 signalling is modulated via *S. pneumoniae* peptidoglycan binding to lipopolysaccharide binding protein (LBP) (Weber et al., 2003). Whilst increased quantities of LBP are found in alveolar fluid during *S. pneumoniae* pneumonia, its

deficiency does not lead to more severe disease (Branger et al., 2004a). In this context, TLR2 redundancy may be due to the ability of Ply to directly activate TLR4 on the AM surface (Malley et al., 2003). Similar to TLR2, the contribution of TLR4 towards protection against *S. pneumoniae* pneumonia appears modest (Branger et al., 2004b). Other studies have shown TLR9 to be the most critical TLR for *S. pneumoniae* recognition in the lung (Albiger et al., 2005). These differences may be due to the differing strains of both mouse and bacteria used. However, the TLR adapter molecule myeloid differentiation factor 88 (MyD88) which is common to the function of all these TLRs is critical for induction of host responses irrespective of its upstream receptor (Albiger et al., 2005).

Further PRRs, in addition to TLRs, may be important for *S. pneumoniae* defence. Intracellular NOD-2 recognises bacterial peptidoglycan (Girardin et al., 2003). *S. pneumoniae* induces responses via NOD-2 *in vivo*, but its specific contribution is unclear (Opitz et al., 2004). MARCO has been implicated in the recognition of *S. pneumoniae* (Arredouani et al., 2006), although its bacterial ligand remains unidentified. *S. pneumoniae* CPS is recognised by the macrophage C-type lectin SIGN-R1 (Kang et al., 2004). This appears important for defence against invasive infection and perhaps meningitis as it mediates *S. pneumoniae* CPS uptake by microglial cells (Park et al., 2009). SIGN-R1 does not appear to be expressed on alveolar macrophages (Koppel et al., 2005). However, splenic marginal zone macrophage uptake of *S. pneumoniae* and it products by SIGN-R1 facilitates production of natural IgM which is of crucial importance in defence against subsequent pulmonary *S. pneumoniae* infection. Thus, SIGN-R1 is indirectly important in *S. pneumoniae* pulmonary defence.

PRR activation leads to signalling via several intracellular pathways which are important in orchestrating an appropriate cellular response to *S. pneumoniae*. These

include the canonical NF-κB pathway (Albiger et al., 2005) and various mitogen-activated protein kinase (MAPK) pathways, all of which respond to the presence *S. pneumoniae* (Xu et al., 2008). In theory, there appears to be a degree of redundancy between the multiple mechanisms of innate immune recognition of *S. pneumoniae*. However, clues to important pathways can be gleaned from experiments of nature where single gene mutations lead to increased susceptibility to disease. Individuals deficient in either functional interleukin-1 receptor associated kinase (IRAK)-4 or NF-κB essential modulator (NEMO) are at increased risk of bacterial infections including *S. pneumoniae* (Picard et al., 2003). IRAK-4 is part of the downstream signalling between TLRs and NF-κB (Suzuki et al., 2002). NEMO, formerly known as IKK-γ is subsequently required for NF-κB activation (Rothwarf et al., 1998). A recent meta-analysis of case-control studies investigating the contribution of single nucleotide polymorphisms (SNPs) in the genes encoding TLR2 and TLR4 did not show increased risk of *S. pneumoniae* infection (Brouwer et al., 2009). This may be limited by sample size and the authors advocate a pooled biobank to overcome such challenges. Thus, of the many PRRs capable of responding to the presence of *S. pneumoniae* in the lungs, at the least TLR signalling plays a non-redundant role in activating mechanisms of host defence.

1.4.1.3 Gamma-delta T-cells and NKT cells

Up to 10% of T-lymphocytes present in the lung under resting conditions express the γδ-T-cell receptor (TCR) (Wands et al., 2005). Unlike classical αβ-TCR bearing T-lymphocytes, TCR gene expression is oligoclonal (Takagaki et al., 1989). Specific genotypes are enriched at different mucosal sites, with a preponderance of $V_\gamma1^+$, $V_\gamma4^+$ and $V_\gamma6^+$ cells within the lung (Wands et al., 2005). They make intimate contact with macrophages and dendritic cells. The number of γδ-T-cells generally and $V_\gamma4^+$

specifically increases by 3 h following *S. pneumoniae* pulmonary infection in mice (Nakasone et al., 2007). Survival and lung bacterial clearance of mice deficient in TCR-$V_\gamma 4$ is reduced, and associated with impaired neutrophil recruitment and reduced levels of pulmonary TNF-α and the cytokine MIP-2 (Nakasone et al., 2007). TCR-$\gamma^{-/-}$ mice have lower levels of IL-17 present in lung tissue and higher numbers of *S. pneumoniae* 4 h following infection (Ma et al., 2009).

The *S. pneumoniae* ligands and their respective receptors triggering IL-17 production by γδ-T-cells are not known. However, a subset of γδ-T-cells expressing TLR1 and TLR2 have been shown to produce the cytokines IL-17 and IL-22 in response to direct pathogenic stimulation with *C. albicans* or *E. coli* (Martin et al., 2009). IL-17 production in response to *S. pneumoniae* is further enhanced by the presence of IL-23, which may be released by APCs upon appropriate stimulation (see below). Thus, γδ-T-cells they form a potentially rich source of IL-17 prior to the recruitment of adaptive Th17 cells. γδ-T-cells have also been reported to professionally phagocytose, process and present opsonised antigen on MHC class II (Wu et al., 2009). Whether this is the case for *S. pneumoniae* is unknown.

Invariant NKT-cells expressing TCR-Vα14 have also been implicated in mediating pulmonary neutrophil recruitment following *S. pneumoniae* infection (Kawakami et al., 2003). Their genetic deficiency leads to reduced levels of MIP-2 and TNFα in response to infectious challenge. This defect can be overcome with exogenous administration of IFNγ (Nakamatsu et al., 2007). The interpretation of such data is limited by the broader effects of specific gene knockouts on the intertwined elements of the developing immune system. Thus, although defective in a single gene, other cell types may be affected in underappreciated ways, and the effects of deficiency in certain cell types on pathogenesis of *S. pneumoniae* pneumonia may not be as direct as suggested.

1.4.1.4 Inflammatory mediators

Activation of AMs in early *S. pneumoniae* pneumonia leads to abundant production of TNF-α (Bergeron et al., 1998, Kirby et al., 2005). Reciprocal cytokine signalling between AMs and epithelial cells leads to regulated production and release of cytokines. Whilst both TNF-α and IL-1 lead to NF-κB activation, which is necessary and sufficient for neutrophil recruitment in pneumococcal pneumonia, there appears to be some redundancy between them (Jones et al., 2005, Quinton et al., 2007). Whilst there are extensive reports of increased risk of *Mycobacterium tuberculosis* infection in individuals receiving anti-TNF therapy for auto-immune disease, reports of *S. pneumoniae* pneumonia are fewer, despite greater exposure in populations receiving such therapy (Colombel et al., 2004). Nonetheless, in animal models, administration of anti-TNF-α antibody worsened the course of pneumococcal pneumonia (Takashima et al., 1997), and regulation of such inflammatory factors may be crucial to outcome in human infection. Other cytokines play important roles in defence against *S. pneumoniae* pneumonia. IL-6 is an important inducer of acute phase protein production by the liver (Deban et al., 2009), include CRP and SAP which are important in opsonic enhancement of *S. pneumoniae* phagocytosis (Mold et al., 1982, Yuste et al., 2007). IL-6 also delays neutrophil apoptosis and enhances neutrophil cytotoxic function (Biffl et al., 1996). An IL-6 promoter polymorphism which enhances IL-6 production protects against bacterial dissemination during *S. pneumoniae* infection (Schaaf et al., 2005). The counterbalancing role of IL-10, a regulatory anti-inflammatory cytokine is crucial to avoid excessive inflammation including macrophage deactivation (Bogdan et al., 1991). However, the highest IL-10 levels are associated with worst outcome in CAP (Gallagher et al., 2003), suggesting that a fine dynamic balance exists in the regulation of these processes to achieve bacterial clearance and inflammatory resolution with the least lasting tissue damage.

The cytokines IL-17 (IL-17A), IL-17F and IL-22 are produced by inducible memory Th17 cells on antigen recall. IL-17 is also produced by a range of innate cells in an otherwise naïve host, including γδ-T-cells and NKT-cells as discussed above (Cua and Tato, 2010). In addition to this inducible lineage, a non-inducible naturally occurring CD4+ Th17 producing cell type known as nTh17 (as opposed to the now canonical iTh17 cell) has recently been implicated in airway responses to antigens (Tanaka et al., 2009). Its contribution to host defence remains poorly understood. The effects of IL-17 and its lesser characterised homologue IL-17F (Tesmer et al., 2008) are pleiotropic and have been extensively investigated in recent years for their pathogenic role in a range of autoimmune diseases (Ouyang et al., 2008) and their protective role against infection at the mucosa (Dubin and Kolls, 2008). IL-17 induces TNF-α, IL-1 and MMP-9 production by macrophages (Jovanovic et al., 1998). Neutrophils are activated and produce more myeloperoxidase and MMP-9 (Zelante et al., 2007). Both epithelial cells and fibroblasts (Aujla et al., 2007) produce a broad range of anti-microbial peptides, chemokines and other inflammatory mediators (reviewed in (Iwakura et al., 2008)). Through induction of granulocyte colony stimulating factor (G-CSF), neutrophils are released from the bone marrow (Schwarzenberger et al., 2000). These traffic to the lung under the influence of chemokines which ligate CXCR1 and CXCR2 as described in further detail below (Laan et al., 1999). IL-17 induces expression of the polymeric Ig receptor enhancing transportation of IgA and IgM into the airway (Jaffar et al., 2009). IL-22 has been specifically implicated in airway mucosal protection. It increases lung epithelial cell proliferation and enhances transepithelial resistance to injury during *Klebsiella pneumoniae* infection (Aujla et al., 2008). Innate and adaptive sources of IL-17 in the context of lung defence are illustrated in Figure 1.4 (Image from (Matsuzaki and Umemura, 2007)).

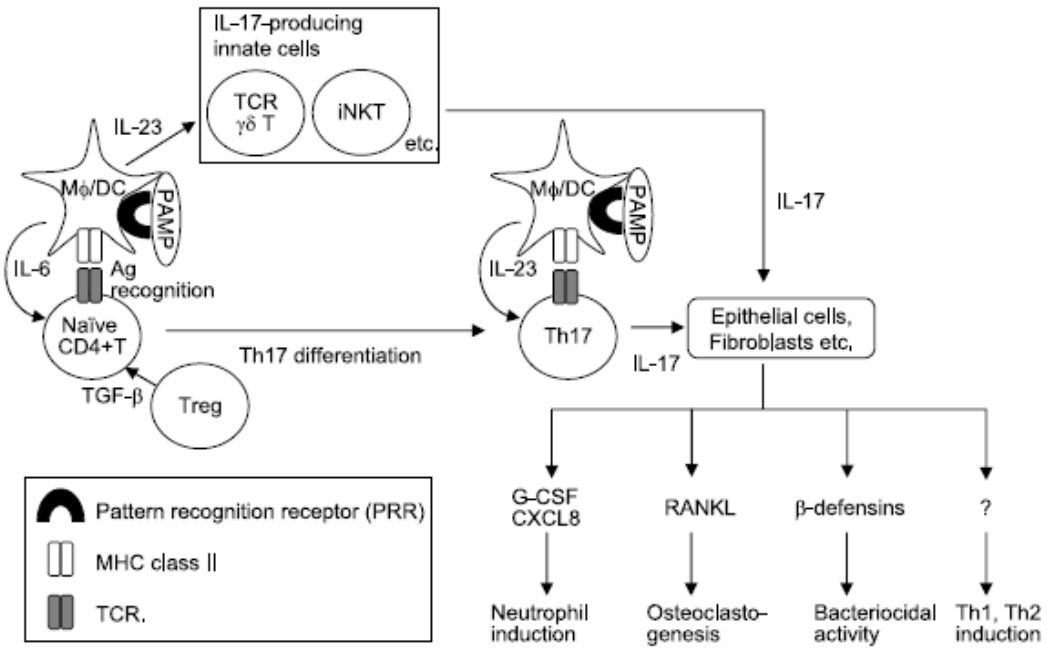

Figure 1.4. Innate and adaptive mechanisms of IL-17 production

IL-23 production by antigen presenting cells (APCs) including macrophages and dendritic cells stimulates IL-17 production from innate cells including γδ-T-cells and NKT cells. Certain innate cells may also produce IL-17 in direct response to bacterial pathogen-associated molecular patterns (PAMPs). Memory CD4+ Th17 cells are induced through presentation of antigen by APCs under the influence of cytokines including TGF-β and IL-6. Re-presentation of the cognate antigens at the time of infection in the context of IL-23 production by APCs leads to further IL-17 production. Amongst its diverse roles, IL-17 can enhance the response to infection by enhancing neutrophil recruitment through stimulation of chemokine (e.g. CXCL8) and G-CSF production and by inducing the production of bacteriocidal molecules such as β-defensins. (Matsuzaki and Umemura, 2007).

Chemokines which assist in the recruitment of leukocytes to sites of inflammation are critical for the early infiltration of neutrophils into alveolar spaces (Matsuzaki and Umemura, 2007). Neutrophils expressing CXCR1 and CXCR2 receptors are attracted to chemokines including CXCL1 (also known as Gro-α in humans and KC in mice) and CXCL8 (also known as IL-8 in humans with murine functional homologue MIP-

2) (Holmes et al., 1991). They are expressed by epithelial cells in response to TNF-α, IL-1 and IL-17 as a result of NF-κB activation. Further mediators including G-CSF and GM-CSF enhance bone marrow production of granulocytes and monocytes, leading to increased numbers in peripheral blood (Fossiez et al., 1996). These are discussed under cellular recruitment below.

1.4.1.5 Cellular recruitment

In the absence of infection, alveolar fluid contains resident AMs maintaining a quiescent state through crosstalk with pulmonary epithelium (Hussell and Goulding, 2010). Resident AMs are of critical importance both for early recognition of *S. pneumoniae*, activation of further host defence mechanisms, and initial clearance of bacteria through phagocytosis (Xu et al., 2008). By 4 h following infection there is a massive influx of neutrophils into the alveolar spaces (Dallaire et al., 2001, Bergeron et al., 1998), and these rapidly become the dominant phagocyte clearing bacteria (Knapp et al., 2003). They continue to increase in number for 24 to 48 h (Dallaire et al., 2001). Both host and pathogen-derived factors promote the massive neutrophil recruitment into the alveolar spaces. Host factors produced in response to *S. pneumoniae* infection include CXC-chemokines (including IL-8 and GRO-α in humans or their counterparts macrophage inflammatory protein 2 [MIP-2] and KC in mice), in addition to complement component C5a, leukotriene B_4 and PAF (Ali et al., 1999). These CXC-chemokines bind to the CXC receptors CXCR1 and CXCR2 on neutrophils. CXCR2 is also expressed on monocytes, and appears necessary in preventing lethal pneumonia (Herbold et al., 2010). Whether this requirement for CXCR2 reflects the specific importance of neutrophil recruitment or monocyte recruitment is not clear. Bacterial factors promoting neutrophil recruitment and activation include N-formyl peptides such as N-formyl-methionyl-leucyl-

phenylalanine (fMLP) which binds to formyl peptide receptors on neutrophils (Selvatici et al., 2006). There appears to be redundancy between the various factors which recruit neutrophils, although this may depend on the relative number of bacteria. When low bacterial inocula are present, the host-derived CXC chemokines are critical to neutrophil recruitment. However, when the inoculum is large, these become redundant as bacterial N-formyl peptides are sufficient to achieve neutrophil chemotaxis (Gauthier et al., 2007). Thus, enhanced chemokine responses would facilitate more rapid responses early in infection. The precise mechanism of neutrophil recruitment from the vasculature into the alveolar spaces in *S. pneumoniae* infection is not fully understood. It requires the soluble lectin galectin-3, but is independent of classical β_2-integrin mediated adhesion (Nieminen et al., 2008).

Peripheral neutropenia is a specific risk factor for enteric gram-negative sepsis, but not *S. pneumoniae* disease (Morrison, 2005). However, neutropenic patients have an increased risk of all cause pneumonia (Rolston, 2001), and neutropenia worsens the prognosis in established IPD (Kumashi et al., 2005). Studies using cyclophosphamide to render mice neutropenic prior to infection have suggested that neutrophils are essential in defence against *S. pneumoniae* pneumonia. However, cyclophosphamide has much broader immunosuppressive actions. Where neutrophils were selectively depleted by monoclonal antibody, there was little effect on lung bacterial clearance or cytokine responses, but enhanced inflammation and higher levels of bacteraemia and associated mortality. Thus, unlike for *Pseudomonas aeruginosa* (Koh et al., 2009), neutrophils may not be entirely essential in protecting against in *S. pneumoniae* pneumonia. Furthermore, excessive or uncontrolled neutrophil influx may lead to adverse effects. Production of reactive oxygen species and release of matrix metalloproteinases from neutrophils (Cockeran et al., 2009) leads to host tissue damage as a bystander effect of the attempt to control infection.

Neutrophil recruitment is followed by monocyte recruitment as part of the orchestrated inflammatory response. As neutrophils adhere to the blood vessel wall, azurocidin is released from granules, leading to endothelial cell activation and capillary leak, contributing to the development of pulmonary oedema (Linder et al., 2010). Released cathelicidins, defensins, cathepsin G and azuricidin promote the recruitment of monocytes which follow the neutrophil influx into lung tissue and alveolar spaces (Soehnlein and Lindbom, 2010). The neutrophil granular protein proteinase-3 further induces chemokine release from neighbouring cells. These chemokines then undergo N-terminal modification by released neutrophil serine proteases greatly enhancing their potency adding to the drive to monocyte recruitment (Soehnlein and Lindbom, 2010). Once inflammation is established and in the face of a large bacterial burden, monocyte apoptosis leads to reduced production of inflammatory cytokines and limits neutrophil recruitment (Marriott et al., 2006), important for the eventual resolution. Several factors contribute to actively switch off neutrophil recruitment to sites of inflammation. A switch in the lipid mediators produced by platelets and epithelial cells with prostaglandin E2 and D2 (Levy et al., 2001) promoting production of lipoxins and resolvins. These can limit neutrophil recruitment (Chiang et al., 2006) and inhibit TNF signalling (Arita et al., 2007). Annexin 1 released by apoptotic neutrophils enhances their phagocytosis by monocytes and macrophages (Scannell et al., 2007). This is supported by a change in the inflammatory milieu towards higher levels of anti-inflammatory IL-10, TGF-β and PGE2. Neutrophil phagocytosis by macrophages limits macrophage production of IL-23 (Stark et al., 2005), which drives neutrophil production and recruitment through IL-17 released by CD4+ T-cells, γδ-T-cells and NKT cells. Thus, once neutrophils are phagocytosed, mechanisms driving further recruitment to sites of inflammatory are terminated.

Recruitment of lymphocytes also occurs during *S. pneumoniae* pneumonia. Numbers of $V_\gamma1^+$, $V_\gamma4^+$ and $V_\gamma6^+$ γδ-T-cells quickly rise following *S. pneumoniae* infection, and continue to do so for up to 10 days, after which time they slowly reduce (Kirby et al., 2007). This is in contrast to CD4+ and CD8+ T-lymphocytes for which changes are more modest (Kadioglu et al., 2000). Numbers of AM and pulmonary dendritic cells also increase over this time period (Kirby et al., 2006). Both pulmonary dendritic cells and AM appear capable of transferring antigenic material from the lungs to the draining lymph nodes facilitating the adaptive immune responses which occur following non-lethal pneumonia (Kirby et al., 2009).

1.4.1.6 Neutrophil killing

Neutrophils may kill bacteria in their extracellular environment by first trapping them in DNA and histone protein containing neutrophil extracellular traps (NETs) (Brinkmann et al., 2004). However, *S. pneumoniae* CPS limits trapping by NETs, and subsequent killing by antimicrobial components is impeded by bacterial D-alanylated LTA (Wartha et al., 2007). Bacterial phagocytosis by neutrophils is enhanced by recognition of complement or immunoglobulin G or M on the bacterial surface by neutrophil CRs or Fcγ receptors (FcγR) respectively (Berger et al., 1994). The presence of the capsule inhibits both of these interactions (Hyams et al., 2010a). Once internalised, the phagosome merges with intracellular granules which contain a range of antimicrobial substances. Unlike several other bacterial species, *S. pneumoniae* are not killed by reactive oxygen species or mechanisms dependant upon their generation (Kaplan et al., 1968). Killing appears to be mediated by a range of antimicrobial proteins and peptides. The presence of negatively charged surface CPS sensitises the bacterial cells to lysis by the α-defensins human neutrophil proteins 1 to 3 (Beiter et al., 2008). In addition, serine proteases contained in neutrophil

azurophilic granules including neutrophil elastase, cathepsin G and proteinase-3 can kill *S. pneumoniae* (Standish and Weiser, 2009). Thus, neutrophils possess several mechanisms by which internalised bacteria can be killed. Similar processes may be involved in macrophage killing of internalised *S. pneumoniae*, since they can kill *S. aureus* by means of proteinases such as elastase (Houghton et al., 2009). There are probably degrees of synergy and redundancy between these various mechanisms.

1.4.2 Adaptive mechanisms of protective immunity

The relative contributions of specific antibody and cell mediated immunity to *S. pneumoniae* have recently been reviewed (Malley, 2010). These responses may be induced naturally by *S. pneumoniae* carriage, mucosal disease, survival following infection, cross-protection from other organisms, or artificially induced through vaccination. Vaccine-mediated protection is specifically discussed later. Adaptive immunity against extracellular bacteria has traditionally been attributed to opsonic antibodies enhancing neutrophil phagocytosis. This paradigm has recently been challenged by data demonstrating the ability of non-CPS antibodies to effect protection. The place of Th17 cells in protection against *S. pneumoniae* nasopharyngeal colonisation (Zhang et al., 2009, Lu et al., 2008) has already been discussed. It is not currently known whether adaptive Th17 cells have a role in augmenting protection against *S. pneumoniae* pneumonia. It is of note, however, that Th17 cells can mediate protection against lung disease caused by *Bordatella pertussis* (Higgins et al., 2006) and *M. tuberculosis* (Khader et al., 2007).

1.4.2.1 Antibody-mediated adaptive immunity

Pre-existing IgA and IgG within alveolar fluid may enhance protection by opsonising bacteria, thereby enhancing *S. pneumoniae* phagocytosis by AMs and recruited

neutrophils. Although traditionally ascribed only to antibodies against CPS, it is now clear that antibody to surface proteins (Jomaa et al., 2005) can enhance phagocytosis of bacteria, at least *in vitro*. Antibody may further limit disease progression by neutralising virulence factors that aid invasion e.g. Ply (Garcia-Suarez et al., 2004), or those which subvert host defences such as PspA (Shaper et al., 2004, Tu et al., 1999). Finally, opsonic serum IgG can facilitate clearance of bacteria from the blood via macrophages of the reticulo-endothelial system (Holdsworth et al., 1989). All of these mechanisms depend on pre-existing levels of mucosal or systemic antibody as secondary recall responses from memory B-cells re-exposed to *S. pneumoniae* antigens during disease are too slow to assist in protection against rapidly progressive *S. pneumoniae* pneumonia. The importance of antibody-mediated protection against *S. pneumoniae* disease is reflected in the strong tendency to develop sino-pulmonary *S. pneumoniae* infection in patients with antibody deficiency syndromes. These include X-linked agammaglobulinaemia and common variable immune deficiency (Picard et al., 2003). The incomplete protection against recurrent pulmonary infection in common variable immune deficiency patients receiving prophylactic intravenous immune globulin has been attributed to the need for natural IgM to protect against such infection, which is lacking in commercial intravenous immunoglobulin preparations (Carsetti et al., 2005). IgA deficiency is common affecting 1/400 individuals, but not consistently associated with predisposition to *S. pneumoniae* infection. Since *S. pneumoniae* produces an IgA1ase enzyme (Wani et al., 1996), IgA1 may be redundant in defence against *S. pneumoniae* at the mucosal surface where it is the dominant IgA subclass (Kett et al., 1986). The specific contribution of IgA2 (the dominant subclass in the serum) is therefore not clear. Variability in tendency toward *S. pneumoniae* infection in these susceptible IgA-deficient patients may reflect associated deficiency of IgG subclasses (Picard et al., 2003). Finally,

polymorphisms of FcγR may affect the ability of phagocytes to recognise and respond to antibody-opsonised *S. pneumoniae* (Endeman et al., 2009), although the proven association between such polymorphisms and predisposition to *S. pneumoniae* infection remains weak to date.

The success of capsule type-specific serum therapy to treat *S. pneumoniae* pneumonia historically demonstrates efficacy against disease, but may be operating to limit septicaemia and give time for other innate mechanisms to resolve the pulmonary infection (Austrian, 1984). Immunisation with CPS antigens offers strong protection against serotype-specific IPD, but weaker protection against non-invasive pneumonia. In mouse models IgM against PC residues, either natural IgM or induced through exposure to other bacterial species such as *H. influenzae* may also offer protection against *S. pneumoniae* IPD (Goldenberg et al., 2004, Brown et al., 2002, Briles et al., 1981, Baxendale et al., 2008, Carsetti et al., 2005), although its role in protecting humans is not known. There is now abundant evidence from murine models that antibodies against *S. pneumoniae* proteins can protect against both pneumonia and invasive disease. IgG binding PspA (Briles et al., 2003), PhtE (Hamel et al., 2004), Ply-derivatives (Alexander et al., 1994), the combination of SlrA and IgA1 protease (Audouy et al., 2007) or the combination or PiaA and PiuA (Jomaa et al., 2006) are protective against pneumonia. These and additional antigens will be discussed further in the context of protein vaccination below.

1.4.2.2 T-cell-mediated adaptive immunity

S. pneumoniae antigen-specific memory T-cell responses may also offer protection against pneumonia. Such T-cells may be located within the lung tissue or in draining lymph nodes. The role of IFN-γ producing Th1 cells in acquired protection against *S. pneumoniae* is unclear, and individuals with defects of the IFN-γ / IL-12 / Th1 axis

are not predisposed to *S. pneumoniae* infections (Picard and Casanova, 2004). Th2 cell production of cytokines such as IL-4, IL-5 and IL-13 is of great importance in directing an appropriate B-cell response following initial antigen encounter (Mosmann et al., 1986), but it is not clear how recall production of these cytokines during subsequent disease would assist in protection. Th17 cells could assist in protection by enhancing levels of mucosal and systemic IL-17 and IL-22 during early infection. The roles of these cytokines have been discussed above in their innate context, in terms of phagocyte recruitment and activation, epithelial cell activation, chemokine and antimicrobial peptide production. Adaptive responses leading to their further production would augment levels of these cytokines already produced in the innate response to infection by other cell types.

The role of Th17 cytokines in vaccine-mediated protection against infection has been recently reviewed (Lin et al., 2010). Pulmonary Th17-cell responses induced by prior systemic immunisation with *Bordetella pertussis* antigens are sufficient to protect against pulmonary *B. pertussis* disease (Higgins et al., 2006). Th17 cell responses to *M. tuberculosis* antigens can protect against pulmonary *M. tuberculosis* infection by enhancing recruitment of protective Th1 cells into lung (Khader et al., 2007). Induction of a pulmonary Th17-cell response by nasal immunisation with live attenuated *Pseudomonas aeruginosa* was recently shown to protect against *P. aeruginosa* pneumonia (Priebe et al., 2008). In contrast, an over-exuberant adaptive Th17 response can also be detrimental in response to a micro-organism, leading to excessive inflammation, as recently shown for *Helicobacter pylori* (DeLyria et al., 2009).

The importance of T cells for adaptive immunity against *S. pneumoniae* is supported by the association of several T-cell immunodeficiency syndromes with increased risk of *S. pneumoniae* disease in humans. Severe combined immune deficiency and other

T-cell immunodeficiencies affecting CD4+ T-cells predispose at least through effects on antibody production (Picard et al., 2003). MHC class I deficiency which leads to a lack of CD8+ T-cells has also been associated with *S. pneumoniae* disease but the mechanism is not clear. Deficiency of the signalling molecule STAT3, a cause of hyper-IgE syndrome (also known as Job syndrome) (Minegishi et al., 2007), is associated with mucosal infections (primarily caused by *Staphylococcus aureus* and fungal including *Candida*) and pulmonary infections (primarily caused by *S. aureus*) (Milner et al., 2008). As a result of signal transduction and activator of transcription 3 (STAT3) deficiency, there is failure of APCs to produce IL-6, essential for developing Th17 cell responses to these specific pathogens. There are weaker associations between hyperIgE syndrome and *S. pneumoniae* infection (Buckley and Becker, 1978, Geha and Leung, 1989, Grimbacher et al., 2008). The effect of lack of specific Th17 cells on nasopharyngeal carriage of bacteria has not been reported. The induction of Th17-cell responses following nasal colonisation with *S. pneumoniae* has been discussed previously. Whilst this response is sufficient and necessary to clear recolonisation of a previously colonised mouse, its role in protection against invasive disease is still not clear.

1.4.2.3 Protective responses following colonisation with live attenuated *S. pneumoniae*

There are no published reports to date on the ability of nasopharyngeal colonisation with potentially virulent wild-type *S. pneumoniae* to induce protection against subsequent pneumonia. There are two reports utilising attenuated mutant strains of *S. pneumoniae* to induce protection (Roche et al., 2007, Richards et al., 2010). In these models, carriage with either Ply-deficient or capsule-deficient strains is attenuated in terms of colonisation density and duration. Although rapidly cleared from the

nasopharynx, unencapsulated strains of *S. pneumoniae* are immunogenic in C57Bl/6 mice, inducing both serum IgG and mucosal IgA to several *S. pneumoniae* proteins (Roche et al., 2007). Such vaccination is protective against subsequent lethal pneumonia, including in IgA deficient mice (Roche and Weiser, 2010), but not in μMT mice (which lack all classes of antibody), suggesting that antibody is required for this protection. In this study, it was observed that nasal vaccination of $MHCII^{-/-}$ mice (that congenitally lack CD4 cells) with an unencapsulated strain was also not protective (Roche et al., 2007). It is incorrect to conclude from this, however, that memory CD4 T-cells (including Th17-cells) necessarily play a role in colonisation-induced protection against lethal pneumonia, since the lack of CD4 cells at the time of colonisation will impact on the nature of the antibody responses that develop. In this model, vaccination with a PspA/Ply-deficient double mutant was also immunogenic and protective.

Nasal vaccination with a single mutant Ply-deficient strain of D39 can also protect against subsequent lethal pneumonia challenge (Richards et al., 2010). This strain was carried for one week, whereas its parent WT strain was carried for over four weeks. Previous data has suggested Ply-deficient strains are carried for longer than their parent WT (van Rossum et al., 2005), attributed to reduced lysis of neutrophils following bacterial phagocytosis leading to a weaker Th17-cell response (Matthias et al., 2008). Mouse and bacterial strain differences may explain the more rapid clearance observed by Richards (Richards et al., 2010). Weak serum anti-capsule IgM and anti-PspA IgG responses were also reported to be induced by the Ply deficient mutant. In both the work of Roche and Richards, cross-protection against a heterologous serotype was demonstrated. Can this be extrapolated to imply that natural exposure to a WT strain of *S. pneumoniae* induces adaptive protective immunity against pneumonia? Lack of either capsule or Ply affects the interaction of

S. pneumoniae with the host immune system. This includes interactions with phagocytic cells (Hyams et al., 2010a), complement (Tu et al., 1999) and T-cell responses (Kadioglu et al., 2004). Since direct comparison with WT strains was not made in these studies, it is not clear whether similar protective responses would occur in a more natural WT setting. Furthermore, a direct comparison of the effects of colonisation with mutant versus WT strains may elucidate important factors required in naturally acquired immunity.

1.5 VACCINE-MEDIATED IMMUNITY AGAINST *S. PNEUMONIAE* PNEUMONIA

1.5.1 Vaccination aims and approaches

Systemic immunisation with CPS-based vaccines originally demonstrated efficacy in preventing IPD (Smillie et al., 1938). In this context, opsonic IgG to CPS is an established serotype-specific correlate to protection against IPD (Jokinen et al., 2004). Conjugation of CPS to carrier proteins led to improved immunogenicity and protection in infants (Black et al., 2000). This included evidence of protection against pneumonia and changes in colonisation dynamics leading to herd immunity (Lipsitch, 2001). However, the serum correlates of anti-CPS antigen-induced protection against pneumonia and colonisation are not clear. Other vaccine approaches under intense investigation include *S. pneumoniae* protein-based vaccines, heat-killed whole bacterial cells and live attenuated organisms (both *S. pneumoniae* and other organisms) carrying *S. pneumoniae* antigens. It is important to recognise that responses to vaccination may be influenced by pre-existing natural immunity to *S. pneumoniae*, due to either passively transferred maternal antibody in infancy (Holmlund et al., 2009), or following natural exposure (Baxendale et al., 2000).

Whilst current vaccines are only licensed for systemic administration by intramuscular injection, protection against mucosal disease and colonisation may be greater following mucosal administration. These approaches along with their successes and limitations are discussed further below.

The ultimate aim of vaccination against *S. pneumoniae* is to prevent disease. However, certain vaccination approaches may lead to protection against subsequent colonisation in addition to protection against disease (O'Brien et al., 2007).

Elimination of colonisation by common *S. pneumoniae* strains could have major effects on the bacterial ecology of the nasopharynx, allowing increased colonisation with other potentially more virulent *S. pneumoniae* strains or even other bacterial pathogens. Alternatively, this ecological niche could be filled by weakly invasive *S. pneumoniae* strains hence resulting in a new stable but lower incidence of IPD. The dynamics of colonisation are complex and it is difficult to predict the outcome of such a strategy.

1.5.2 Pneumococcal plain polysaccharide vaccines

Immunisation with purified *S. pneumoniae* CPS antigens, known as plain pneumococcal polysaccharide (PPS) has been known to protect against lethal disease for nearly one century (Smillie et al., 1938). A 23-valent PPS vaccine (Pneumovax™) and its derivatives have been in use for over 20 years. It contains CPS from serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F (Summary of Product Characteristics, Sanofi Pasteur MSD Ltd, Berkshire, England). Its efficacy is greatest (approximately 50-80%) in protecting immunocompetent adults against IPD (Fedson and Scott, 1999) (Mangtani et al., 2003, Fine et al., 1994). However, it does not appear to protect against non-bacteraemic pneumonia (Jackson et al., 2003) or other mucosal disease such as chronic bronchitis or otitis media. As a T-independent vaccine, PPS is not immunogenic or protective in infants (Fedson and Scott, 1999). This has been attributed to several factors relating to immunological immaturity, including function of B-cells, the splenic marginal zone, germinal centre immaturity and limited bone marrow niches to establish plasma cells (Pollard et al., 2009, Siegrist and Aspinall, 2009). A combination of intrinsic B-cell factors and those relating to supporting microenvironments also lead to poor immunogenicity in the later adult life (Siegrist

and Aspinall, 2009). When PPS is immunogenic, it does not prime for memory, requiring repeat doses every five years (Butler et al., 1993). It is recommended for use in developed countries in high-risk adults, including those over 65 years of age, with predisposing cardiopulmonary disease or who are immunocompromised including HIV infection and sickle-cell disease. However, evidence of efficacy in these specific populations is weak, particularly in resource-poor countries. Thus, PPS vaccines are not a priority recognised by the World Health Organisation (WHO, 2008), which instead supports more widespread use of pneumococcal conjugate vaccines (PCV) in infancy to reduce the burden of colonisation in high risk communities. PCV were developed to overcome many of these obstacles and are discussed below.

1.5.3 Pneumococcal conjugate vaccines

The ability to conjugating a T-cell independent PS antigen to a carrier protein to improve immunogenicity in infants was first demonstrated for the pathogen *Haemophilus influenzae type b* (Anderson et al., 1985). Using the same strategy and indeed same carrier protein (the diphtheria toxoid CRM_{197}), 7-valent PCV were developed and found to be immunogenic (O'Brien et al., 1996, Ahman et al., 1998). In a landmark study involving 38,000 children in California, *S. pneumoniae* vaccine serotype bacteraemia and meningitis were nearly eliminated (Black et al., 2000). This has been replicated in further studies (Pavia et al., 2009). More modest reductions have also been seen for mucosal diseases including non-bacteraemic pneumonia and otitis media (Shinefield and Black, 2000), including a decline in radiologically confirmed pneumonia of up to 35%. PCV has also been shown to be immunogenic in other high risk groups such as following bone marrow transplantation (Molrine et al., 2003) (Meisel et al., 2007). Unlike PPS, PCV is also effective in preventing IPD in

HIV-infected adults in areas of high *S. pneumoniae* burden (French et al., 2010). The WHO Strategic Advisory Group of Experts has recently recommended the introduction of PCV into developing countries and significant funds are being made available to support this effort (O'Brien et al., 2009). In a recent immunogenicity and safety study, PCV was more immunogenic in terms of antibody levels and opsonophagocytic killing than PPS in the elderly (de Roux et al., 2008). Responses to PCV are different in the elderly compared to younger adults, particularly in terms of recruitment of memory B cells (Baxendale et al., 2010b, Baxendale et al., 2010a). The efficacy of PCV in the elderly remains to be tested. In addition to reduction in disease, PCV use has led to significant falls in carriage rates in the immunised population (Dagan et al., 2002). As strains are often transferred between family members, this has led to changes in carriage amongst non-immunised persons, with consequential falls in disease rates due to herd immunity (Whitney et al., 2003, Givon-Lavi et al., 2003). An unanticipated further benefit has been reduction in carriage of antibiotic-resistant strains in vaccines recipients, since the most commonly carried strains are most exposed to antibiotics (Kyaw et al., 2009).

The cost and physical bulk of these glycoconjugates limits the number of serotypes that can be included in a vaccine formulation. However, work to improve the synthesis of PS for use in PCV is ongoing and may facilitate the inclusion of larger numbers of serotypes in the future. The 7 serotypes included in the most widely used vaccine Prevenar™ (4, 6B, 9V, 14, 18C, 19F, 23F) were chosen as they represent over 80% of IPD causing strains in many developed countries (Butler et al., 1995). However, they represent only 50% IPD isolates at most in many developing countries (Hausdorff et al., 2000b, Hausdorff et al., 2000a), which inherently limits their impact. To partly overcome this, newer PCV containing either 10 or 13 PCV have

recently been licenced, offering protection against serotypes 1, 3, 5, 6A, 7F, 19A in addition (Bryant et al., 2010).

Loss of vaccine strains from their nasopharyngeal ecological niche has been associated with an increase in carriage and invasive disease by non-vaccine serotypes (Hanage, 2008, Melegaro et al., 2010). This may be due to a combination of direct replacement, genetic capsule switching between strains and pre-existing trends. The impact of this has been reported to differ in the USA and the UK. In the USA, there appears to be a sustained reduction in IPD over an 8-year period since PCV introduction (Pilishvili et al., 2010). In the UK, replacement disease caused by non-vaccine serotypes is believed to be the cause of little overall change in IPD rates compared to before PCV introduction (Kaye et al., 2010). These differences may reflect operational factors underlying data collection, but concern exists as to the longer term utility of this approach and the feasibility of reformulating such vaccines on an ongoing basis and for different geographical locations. Furthermore, prior natural exposure to CPS antigens affects the nature of the immune response to subsequent immunisation (Baxendale et al., 2000) There is emerging evidence natural exposure to CPS as a T-independent antigen can lead to hyporesponsiveness to subsequent immunisation with PCV, reducing its efficacy (Dagan et al., 2010). This may limit the efficacy of PCV in population with high neonatal exposure rates where need is greatest. To enhance mucosal protection in addition to systemic, the immunogenicity of mucosally administered PCV has been investigated. Whilst PCV is immunogenic via this route, protection against invasive disease may be improved by the inclusion of various adjuvants such as the cytokine IL-12 (Lynch et al., 2003). Thus, PCV have been and continue to be highly effective vaccines. Shortcomings remain and there is therefore much interest in alternative approaches to preventing *S. pneumoniae* disease.

1.5.4 Peptide mimotopes

In an attempt to overcome some of the limitations of CPS-based vaccines described above, peptide mimotopes of CPS antigens have been investigated (Valadon et al., 1996). Using either rational design of antigens (Westerink et al., 1995) or phage display technology (Oldenburg et al., 1992), peptides are identified which immunologically mimic the parent CPS. Immunisation leads to antibodies which not only bind the peptide antigen but also the CPS structure that it mimics (Westerink et al., 1995). Thus, the mechanism of inducing protection differs to that of the parent CPS, but the method of effecting protection is the same, namely, opsonophagocytic anti-CPS antibody. The advantage over using CPS antigens themselves is that the peptide mimotopes will be T-dependent antigens and thereby immunogenic in infancy. In addition, they would be cheaper and simpler to manufacture than CPS vaccines (Valadon et al., 1996). Furthermore, inclusion of multiple peptides representing broad capsular serotypes would be more feasible than for CPS antigens whether as PPS or PCV. To date, only a limited number of *S. pneumoniae* CPS mimotopes have been identified (Buchwald et al., 2005, Smith et al., 2009), and there are no current human trials. They may have a role in inducing antibodies against carbohydrate antigens that are otherwise tolerated by the immune system, such as *Neisseria meningitides* type B capsular antigen (Park et al., 2004) or tumour antigens (Kieber et al., 1997).

1.5.5 Protein vaccines

To overcome serotype-specificity of vaccine antigens, many *S. pneumoniae* species-wide proteins have been investigated. In addition to their ability to overcome serotype restriction, protein vaccines are easier and cheaper to manufacture that CPS based vaccines (Bernatoniene and Finn, 2005). Protein vaccines are immunogenic in

infants and responses to them are boosted upon repeat immunisation. For certain abundant surface proteins such as PspA, genetic variation leads to families and clades of proteins, with some limitations to cross-protection (Hollingshead et al., 2000). Other protein antigens, in particular lipoproteins such as PsaA, PiuA and PiaA, are heavily conserved between *S. pneumoniae* strains (Brown et al., 2001b)and could potentially provide full cross-protection. Proteins may need modification to render them non-toxic, as is the case with PdB, a derivative of Ply (Briles et al., 2003). Whilst concerns have been expressed regarding the accessibility of surface proteins to antibody *in vivo* due to CPS (Gor et al., 2005), there are now abundant reports of protective efficacy of this approach in murine models of *S. pneumoniae* disease, in particular when proteins are used in combination (Briles et al., 2003).

Antibodies binding *S. pneumoniae* proteins may protect in several ways. They may opsonise the bacteria for enhanced phagocytosis (e.g. PiaA and PiuA (Jomaa et al., 2005)). Disease may be prevented by antibodies blocking *S. pneumoniae* surface proteins required for either colonisation (e.g. PsaA (Pimenta et al., 2006)) or in establishing disease (e.g. PspA(Briles et al., 2003), PsrP (Rose et al., 2008). Antibodies can neutralise the toxin Ply (Alexander et al., 1994). In addition to this, neutralisation of mechanisms by which *S. pneumoniae* evades the host immune response to infection may shift the balance in the host's favour, e.g. by preventing PspA interfering with complement activation (Tu et al., 1999). It appears that immunisation with combinations of proteins leads to stronger protection. The combination of PspA and PsaA protects better against colonisation than either antigen alone whereas the combination of PspA and Ply protects better against invasive disease than either antigen alone (Briles et al., 2000a). This may be due to both synergy in opsonophagocytosis and between other mechanisms of antibody-mediated protection. Other *S. pneumoniae* proteins protective in animal models include the

choline binding protein PspC (Ogunniyi et al., 2001), lipoproteins SlrA (Audouy et al., 2007) and other cell wall anchored proteins including NanA (Long et al., 2004) and IgA1 protease (Audouy et al., 2007). Antibodies to the surface located protein PhtD (Adamou et al., 2001) are also protective and this may involve blockade of complement evasion (Melin et al., 2010). Immunisation with the more recently characterised *S. pneumoniae* pilus proteins RrgA and RrgB is also protective against disease (Gianfaldoni et al., 2007).

S. pneumoniae proteins have also been administered mucosally either as purified proteins with accompanying adjuvant (Wu et al., 1997a) or expressed on other avirulent organisms including *Lactobacillus casei* (Ferreira et al., 2009) and genetically engineered live attenuated *Salmonella enterica* serovar typhimuirum (Xin et al., 2009, Wang et al., 2010b). The mucosal route induces both mucosal IgA and systemic IgG(Wu et al., 1997a). An alternative expression system is to display proteins on the surface of a non-recombinant, killed *Lactococcus lactis*-derived delivery system called Gram-positive Enhancer Matrix (GEM) (Audouy et al., 2007). The GEM particles induce the production of macrophage TNF-α and enhance the maturation of dendritic cells. Other strategies under investigation include immunisation with both PspA DNA and protein (Moore et al., 2006). *S. pneumoniae* proteins have also been used as the carrier proteins for PCV, and induce anti-protein antibody-based cross-protection in mice (Lee et al., 2001). To date, such proteins have not been used for carriers in human vaccine studies.

Based on the concept of that antibody neutralisation of virulence factors would aid protection, many of these proteins were initially screened on the basis that genetic mutant *S. pneumoniae* strains which lacked them had reduced virulence. An alternative direction of investigation has been to identify surface-located proteins by more empiric means (Hamel et al., 2004). Genomic data has been scrutinised to

identify proteins likely to be surface expressed based on the presence of secretion motifs including cell wall anchors, signal peptidase signals, choline binding domains and integrin binding domains (Wizemann et al., 2001). Whilst not exhaustive, this has led to the identification of several immunogenic and protective proteins, including the Pht family of surface expressed proteins (Adamou et al., 2001). Of these, PhtA, PhtB and PhtD protected against sepsis (Adamou et al., 2001). PhtE, formerly known as BvH-3, protected against both sepsis and pneumonia (Hamel et al., 2004). Other have adopted an entirely unbiased approach and used pooled serum from humans exposed to or surviving *S. pneumoniae* infection to screen an antigen display library expressed in *E. coli* (Giefing et al., 2008). This has led to the discovery of several further *S. pneumoniae* proteins which are surface expressed, immunogenic following natural exposure, lead to opsonophagocytic antibodies, and which protect against disease in animal models. These include the proteins SktP and PcsB (Giefing et al., 2008). Whilst immunisation with many *S. pneumoniae* proteins induces protection in models of sepsis, only a limited number of proteins have been shown to protect specifically against pneumonia. These include systemic administration of either PspA (Briles et al., 2003), PhtE (Hamel et al., 2004), PhtD (PATH, 2010) or Ply-derivatives (Alexander et al., 1994), or nasal administration of the combination of SlrA and IgA1 protease in the GEM system (Audouy et al., 2007) or the combination or PiaA and PiuA (Jomaa et al., 2006).

Human studies of the immunogenicity of *S. pneumoniae* proteins are limited to date. Immunisation of adult human volunteers with PspA led to serum antibody against this protein which when passively transferred into naïve mice was able to protect against lethal disease (Briles et al., 2000b). Other protein vaccines currently in phase I/II clinical trials include the combination of StkP, PcsB and PspA (PATH, 2010) and PhtD (PATH, 2010). In addition to inducing antibodies, protein immunisation could

potentially elicit protective T-cell responses to *S. pneumoniae*. Whilst this can prevent colonisation, the ability of anti-protein T-cell responses to protect against disease independently of antibody has not been reported to date. Based on murine data demonstrating Th17-cell mediated protection against colonisation, others are using T-cell antigen discovery approaches to identify the *S. pneumoniae* protein antigens which lead to the strongest Th17 cell responses for use in a protein vaccine engineered to induce both protective antibody and T-cell responses (PATH, 2010).

1.5.6 Nasopharyngeal immunisation with whole bacteria

As an alternative to generating subunit vaccines, investigators have introduced either killed or live attenuated whole bacterial cells into the nasopharynx of mice as a means of vaccinating against disease. Inspired by the observations that natural mucosal exposure can induce antibodies and Th-17 cells responses to a range of bacterial antigens, this approach also exposes nasopharynx to the bacteria, and allows the host-bacteria interaction to dictate the antigens which dominate the immune response. Such approaches have identified protective immunisation regimes. Furthermore, they offer insights into the mechanisms by which the naturally-induced immune response may offer protection against subsequent disease. However, genetic modification of *S. pneumoniae* or use of adjuvants may affect the immune response to the bacteria. Thus, the immunity they induce may differ to that generated by nasopharyngeal exposure to non-adjuvanted live wild-type bacteria.

1.5.6.1 Killed whole cell *S. pneumoniae*

Nasal administration of killed whole *S. pneumoniae* is protective only when given with cholera toxin (CT) (Malley et al., 2001). The bacterial strain used was acapsular, lacked autolysin and was ethanol killed prior to administration. Th17 cell responses

induced with this vaccine were both necessary and sufficient to protect against subsequent colonisation (Malley et al., 2005, Lu et al., 2008). Passive transfer of serum from immunised mice protected naïve recipients against lethal pneumonia in an infant rat model (Malley et al., 2001). Furthermore, when administered systemically, this vaccine was protective in murine models of colonisation, pneumonia and sepsis (PATH, 2010). Clinical trials of systemic administration of this vaccine are planned to commence in 2011 (PATH, 2010).

1.5.6.2 Live attenuated *S. pneumoniae*

Nasal immunisation with live genetically attenuated strains of *S. pneumoniae* lacking either capsule, PspA or both (Roche et al., 2007) or lacking Ply (Richards et al., 2010) have also been investigated for their ability to induce protection against colonisation and disease. Using live bacteria offers the prospect of onward transmission of the vaccine enhancing possible protection. This approach has the further advantage of presenting a large number of B-cell antigens to the immune system, and overcoming the potential limitation of MCH restriction of T-cell epitopes. As discussed in detail in the context of adaptive immunity against pneumonia, mice were protected against both colonisation and lethal disease. However, no comparison has been made with the parent WT strains, and many questions remains as to the precise mechanisms of protection induced through colonisation with these strains.

1.5.6.3 Importance of capsule in colonisation-induced protection

Despite more rapid clearance from the nasopharynx, colonisation with unencapsulated strains of *S. pneumoniae* may still protect against subsequent pneumonia (Roche et al., 2007), via non-capsular immunity. It is possible that the presence of capsule would impede the presentation of sub-capsular antigens to the adaptive immune system and thus an encapsulated strain may be less immunogenic and less protective

than its unencapsulated mutant. Furthermore, lack of capsule can lead to enhanced pro-inflammatory responses (Hyams et al., 2010a), affecting the immune response further. Direct comparison of isogenic encapsulated and unencapsulated strains could explore how this balance influences immunogenicity and protection. D39-DΔ (Morona et al., 2004) and TIGR4Δ*cps* (Trzcinski et al., 2003) are unencapsulated mutants of the virulent type 2 strain D39 and the virulent type 4 strain TIGR4, respectively. They are used in this thesis to address this question. Absence of capsule has been previously confirmed by electron microscopy and the Stains-all techniques (Hyams et al., 2010b). D39-DΔ was generated by deletion of just the type 2 *cpsD* gene (Morona et al., 2004) and so differs from AM1000, an unencapsulated D39 strain previously reported to have attenuated colonisation capacity in which the entire type 2 capsule locus *cpsABCDETFGHI* has been deleted (Magee and Yother, 2001). TIGR4Δ*cps* was generated by deletion of the entire capsule locus(Trzcinski et al., 2003). Both D39-DΔ and TIGR4Δ*cps* are avirulent in mouse models of pneumonia, attributable to rapid opsonophagocytosis compared to their WT parent strains (Hyams et al., 2010a).

1.5.6.4 Importance of lipoproteins in colonisation-induced protection

The contribution of surface lipoproteins to colonisation-induced protection is not known. Lack of surface lipoproteins may affect responses to colonisation in several ways. Their roles in nutrient transport and cellular adhesion may be important in establishing and maintaining colonisation in the nasopharynx. Thus, the presence of surface lipoproteins may be essential to induce colonisation-induced immunity. In addition, lipoproteins are important TLR2 agonists. Whilst TLR2 signalling is known to be essential for colonisation-induced Th17 cell responses, its role in antibody responses to colonisation is not known. Comparison of responses to colonisation with

wild-type and lipoprotein-deficient mutant strains of *S. pneumoniae* would enable these questions to be addressed. To facilitate this, a lipoprotein-deficient D39Δ*lgt* mutant has been used in this thesis. This mutant was generated and characterised by Dr Suneeta Khandavilli in the laboratory of Dr Jeremy Brown (Centre for Respiratory Research, Rayne Building, 5 University Street, London, WC1E 6JF) by in frame mutagenesis and replacement of the SP1412 gene with a chloramphenicol resistance cassette, as shown in Figure 1.5 (unpublished data). Absence of the *lgt* gene and presence of the chloramphenicol resistance cassette were confirmed by PCR (data not shown). Coomassie staining of a Triton-X extract of lysed Δ*lgt* mutants confirmed absence of lipoproteins from the lipid phase (Figure 1.6a), and immunoblotting bacterial lysates demonstrated retention of the prolipoproteins within the aqueous phase (Figure 1.6b). Absence of the lipoprotein PpmA from the bacterial surface was also demonstrated by immuno-electron microscopy (data not shown). The Δ*lgt* mutant was attenuated in growth *in vitro* (Figure 1.7a) and there was complete loss of virulence *in vivo* (Figure 1.7b).

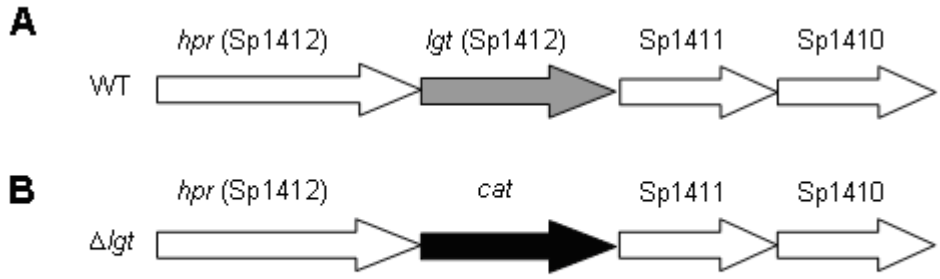

Figure 1.5. Generation of *S. pneumoniae* Δ*lgt* deletional mutants

(A-B) The *lgt* locus Sp1412 present in WT (A) was replaced with a chloramphenicol resistance cassette to create a Δ*lgt* mutant (B). Experiments performed and figures kindly supplied by Dr S Khandavilli.

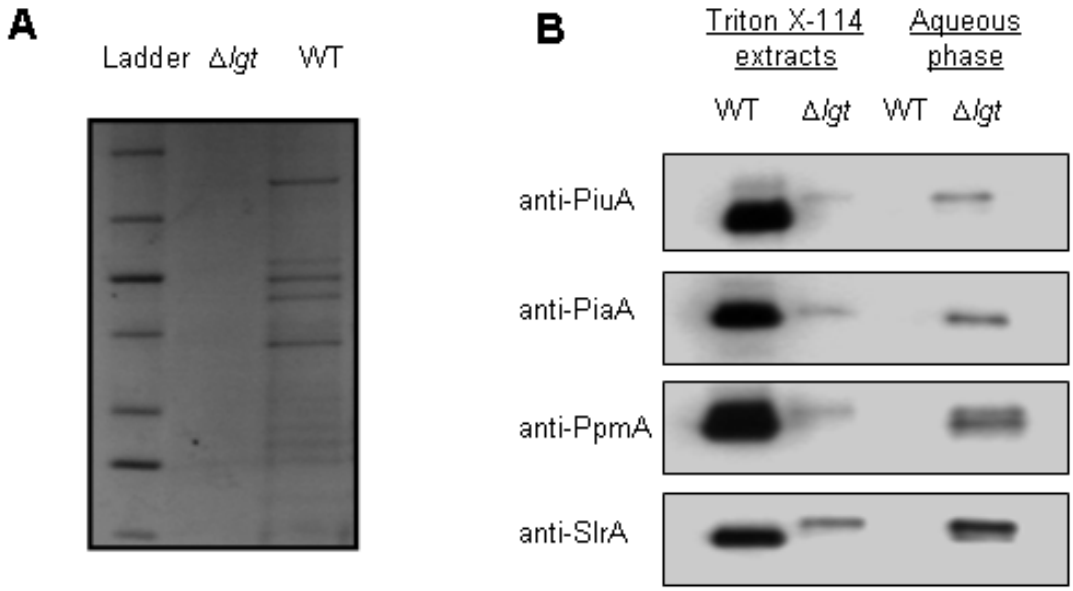

Figure 1.6. Absence of lipoproteins in Δ*lgt* mutant.

Coomassie blue stained SDS-PAGE separated proteins present in Triton-X extract of WT and Δ*lgt* mutant *S. pneumoniae*. (B) Immunoblots of lipoproteins PiuA, PiaA, PpmA and SlrA in Triton-X and aqueous extracts of WT and Δ*lgt* mutant *S. pneumoniae*. Experiments performed and figures kindly supplied by Dr S Khandavilli.

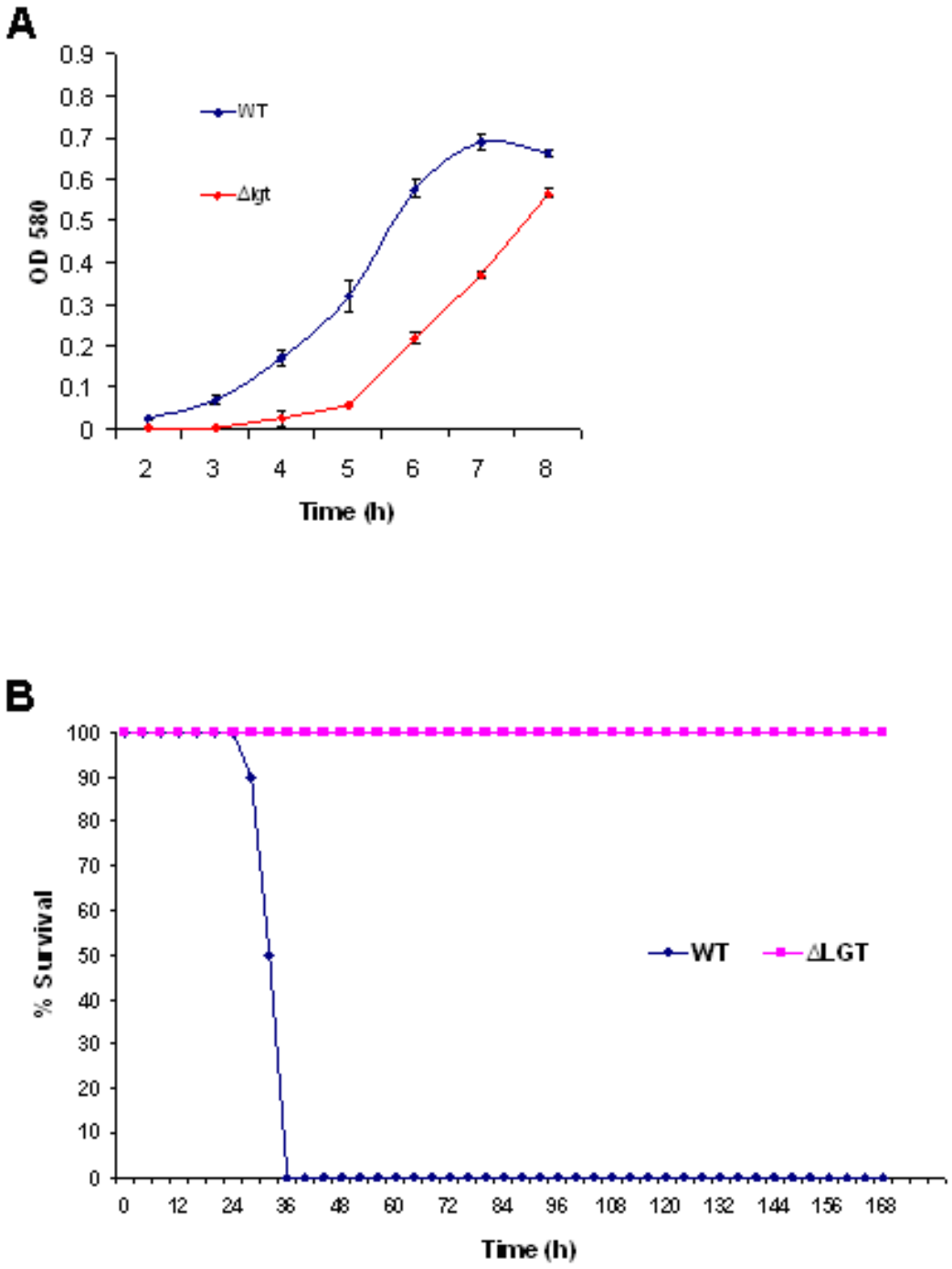

Figure 1.7. Growth and virulence of of Δ*lgt* mutant.

(A) Growth curves of WT and Δ*lgt S. pneumoniae* in THY medium. (B) Survival curves of CD1 mice challenged i.p. with either WT of Δ*lgt S. pneumoniae*. Experiments performed and figures kindly supplied by Dr S Khandavilli.

1.5.6.5 Importance of colonisation duration in colonisation-induced protection

Lack of capsule or surface lipoprotein may affect the ability of a *S. pneumoniae* strain to colonise the nasopharynx, with resulting effects on colonisation-induced immunity. However, this will also be affected by the inflammatory and immunological effects of capsule or lipoprotein deficiency. To address the question of the impact of colonisation duration per se on immunogenicity and colonisation-induced protection, it is necessary to control colonisation duration without impacting on these other factors. The growth of auxotrophic strains of bacteria is dependent upon an exogenous nutrient. Providing and withholding this nutrient may be a tool to control colonisation duration of an auxotrophic mutant strain. Comparison with the parent wild-type strain would allow examination of the impact of colonisation duration on immune responses and protection. This would allow for further interpretation of the data generated with the other mutants. The *S. pneumoniae pabB* gene (SP0665) encodes para-amino-benzoic acid (PABA) synthetatse, an enzyme required by *S. pneumoniae* for folate synthesis (Figure 1.8). Deletion mutants on the D39 and TIGR4 backgrounds were generated by Dr Suneeta Khandavilli in the laboratory of Dr Jeremy Brown by in frame mutagenesis and replacement of the Sp0665 gene with a kanamycin resistance cassette, as shown in Figure 1.9 (unpublished data). Absence of the *pabB* gene and presence of the kanamycin resistance cassette were confirmed by PCR (data not shown). The strains were characterised by Dr Khandavilli. The Δ*pab* strain grew well in complete medium (data not shown) but not in serum unless supplemented with PABA (Figure 1.10a). Virulence in a mouse sepsis model was dependant on supplementation of PABA at 1 mg/ml to mouse drinking water.

These mutant strains are used in this thesis to address underlying questions about how colonisation with WT *S. pneumoniae* strains may induce protection against

pneumonia. In this sense they are live attenuated vaccines. However, there are strong safety considerations before introducing such vaccines into a human population, and any such vaccine would require multiple attenuations to guarantee lack of virulence whilst maintaining immunogenicity.

1.5.7 Summary

Whilst PCV and PPS have led to significant reductions in IPD and contributions towards reduced pneumonia, newer approaches to protection are under intense investigation. Protein vaccines may offer species-wide protection, overcoming the limitations of CPS-based vaccines. Both rational and empiric approaches to selection of optimal proteins for inclusion have been pursued. Aside from their potential as vaccination strategies, studies using heat-killed and live attenuated *S. pneumoniae* have led to the identification of novel mechanisms of protection against colonisation. However, the mechanisms by which they protect against pneumonia are still not clear.

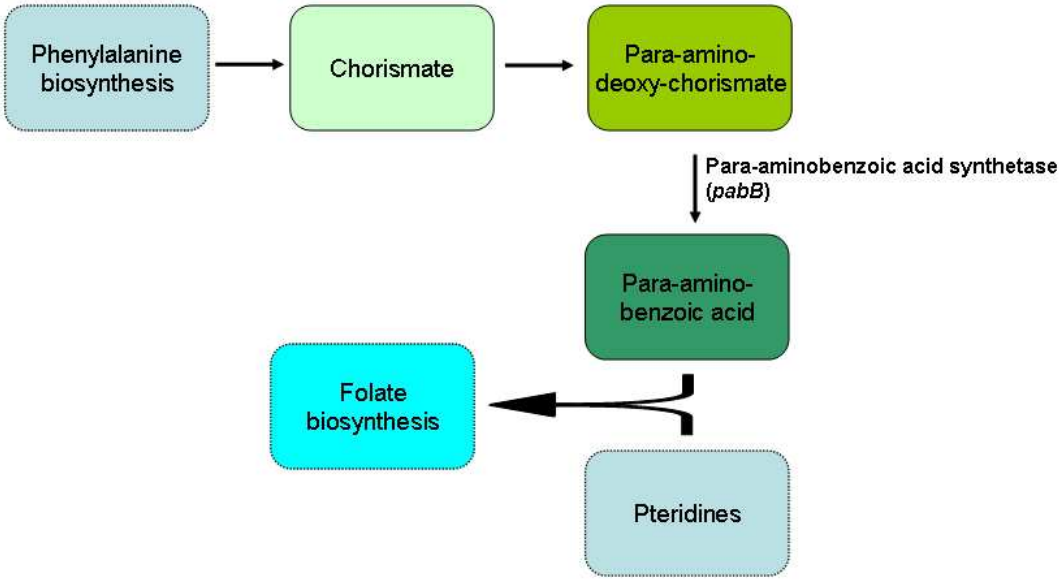

Figure 1.8. *S. pneumoniae* folate biosynthesis pathway

Chorismate is produced during phenylalanine biosynthesis. It is converted via para-amino-deoxychorismate to para-aminobenzoic acid (PABA) by the enzyme PABA synthetase, which is encoded by *pabB*. Reaction with pteridines feeds into folate biosynthesis. In the absence of PABA synthetase, an exogenous source of PABA would be required to support folate biosynthesis.

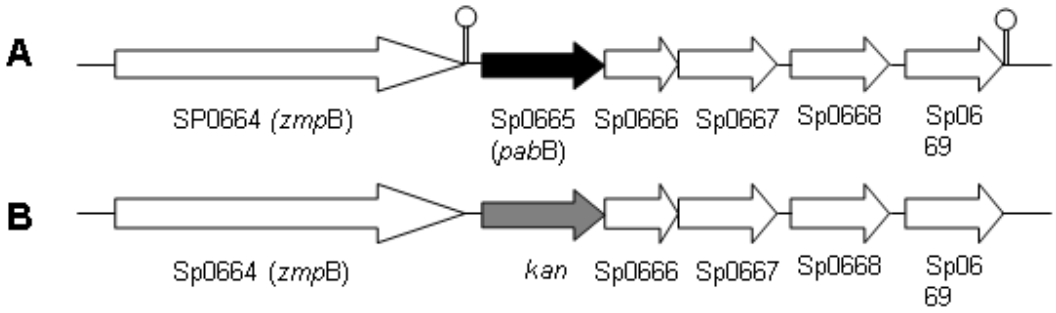

Figure 1.9. Generation of *S. pneumoniae* Δ*pab* deletional mutants

The *pabB* locus Sp0665 present in WT (A) was replaced with a kanamycin resistance cassette creating a Δ*pab* mutant (B). Experiments performed and figures kindly supplied by Dr S Khandavilli.

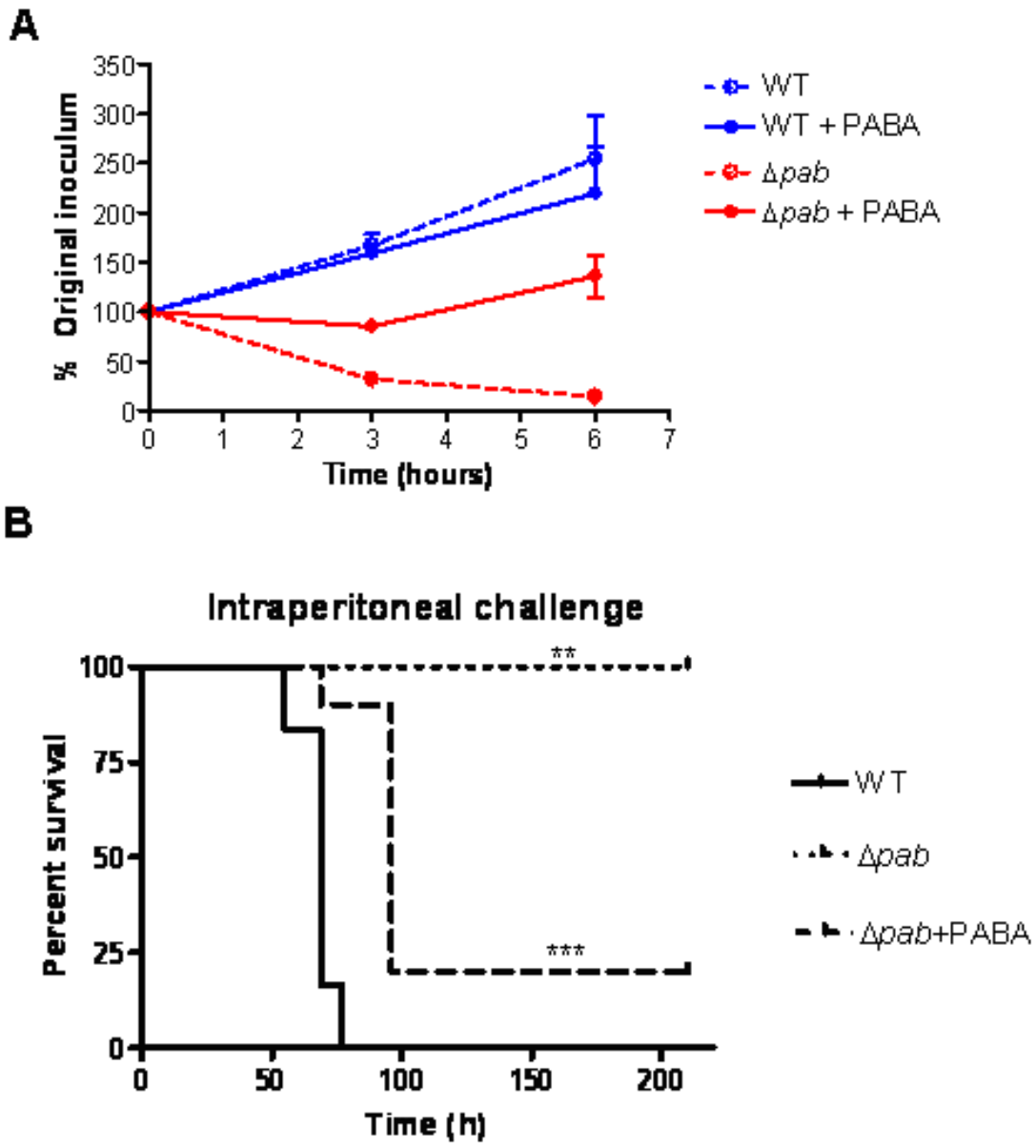

Figure 1.10. Growth and virulence of Δ*pab* mutant

(A) Growth curves of WT and Δ*pab* *S. pneumoniae* in serum with or without supplementation with PABA. (B) Survival of CD1 mice challenged by i.p. route with either WT or Δ*pab* *S. pneumoniae*, with or without PABA supplementation. Experiments performed by Dr S Khandavilli.

1.6 SUMMARY

S. pneumoniae is a major pathogen and the second commonest cause of fatal bacterial infection worldwide. Following acquisition through aerosol and droplet spread, bacteria can establish nasopharyngeal colonisation through a series of molecular interactions with host epithelial cells. This requires avoidance of mucosal clearance which is assisted by the presence of a polysaccharide capsule. This capsule also serves to reduce opsonophagocytic clearance. Colonisation can occur from early infancy, and occurs earlier in areas of high prevalence. Most infants have recurrent episodes of *S. pneumoniae* carriage. Colonisation rates fall to 10% by late childhood. Most *S. pneumoniae* deaths are due to pneumonia which results from aspiration of nasopharyngeal bacteria into the lungs. Pneumonia can subsequently progress to septicaemia. Colonisation can also lead to direct haematogenous spread and seeding of distal sites. Whilst nasopharyngeal colonisation is a pre-requisite for infection, it may also induce adaptive immune responses that could be protective against disease following subsequent nasopharyngeal re-exposure. Despite the ubiquity of *S. pneumoniae* exposure, the majority of individuals do not develop disease as a combined result of innate and adaptive immune response.

Serological studies in human populations suggest that natural nasopharyngeal exposure induces mucosal and systemic anti-protein antibody responses already detectable in infancy. Such exposure also may also induce antibody responses to CPS antigens, but this appears to vary with capsular serotype and is often not detectable until the second year of life, by which age disease rates are already significantly lower than in infancy. Although limited, data from murine studies suggests that multiple antigen encounters may be required for colonisation to induce a systemic anti-CPS IgG response. In addition to antibody responses, murine models have demonstrated

that colonisation induces a TLR2-dependant Th17-cell response to *S. pneumoniae* antigens, and that this response is required for both clearance of primary nasopharyngeal colonisation and protection against subsequent recolonisation. However, there is no evidence to date that Th17 cells play a role in directly preventing *S. pneumoniae* disease. Peripheral blood and mucosal Th17-cell responses to *S. pneumoniae* antigens are detectable in humans. They are likely to be induced through nasopharyngeal exposure but their contributions to the prevention of colonisation or disease are not known.

S. pneumoniae pneumonia results from aspiration of colonising bacteria from the nasopharynx into the lungs. The bacteria then overwhelm the combined efforts of both innate and adaptive immune defence mechanisms. Pathogen recognition through PRRs such as TLRs is critical to activation of resident AMs which can then phagocytose bacteria. Cytokine production by AMs, DCs and γδ-T-cells in response to the presence of bacteria leads to epithelial cell activation, and the production of chemokines and anti-microbial molecules. Such cytokines include TNF-α, IL-1, IL-6 and IL-17. Further anti-bacterial soluble factors including complement proteins aid augment bacterial killing and bacterial uptake by phagocytes. Chemokines such as IL-8 (MIP-2) or GRO-α (KC) are important in neutrophil recruitment to alveolar spaces to assist in bacterial clearance. If these responses are not adequate, disease progresses and can lead to lethal bacteraemia.

Adaptive immune responses have the potential to augment these innate protective mechanisms. Both anti-CPS and anti-protein antibodies can protect against lethal pneumonia in animal vaccination models using CPS and protein antigens. Vaccination-induced Th17-cells can protect against pneumonia caused by several other pathogens by enhancing IL-17 and IL-22 production over and above the innate IL-17 response to infection. The contribution of adaptive Th17-cells in protection

against *S. pneumoniae* pneumonia is not known. Infants and the elderly are particularly susceptible to *S. pneumoniae* pneumonia. Although the reasons for the changing susceptibility with age remain poorly understood, it may reflect the development and subsequent waning of adaptive immune responses. Of note, anti-protein antibody responses appear to wane with age more than those against CPS antigens. Little is known of Th17-cell responses in the elderly. Previous natural exposure may protect against subsequent disease by different immunological mechanisms to those generated through vaccination. Systemic immunisation with PPS vaccines protects adults primarily against IPD by inducing opsonic anti-CPS antibodies. Infants respond best to PCV and this reduces both disease and carriage in a serotype-specific fashion. This has positive effects such as herd immunity, but has also led to replacement serotypes occupying a vacated nasopharyngeal niche and causing replacement disease. PCVs are expensive and difficult to manufacture. There is an urgent need to develop novel *S. pneumoniae* vaccines to overcome such limitations. Species-wide protein antigens have been investigated extensively in murine models and can protect against both pneumonia and IPD. Unbiased serological studies have identified several novel immunogenic *S. pneumoniae* antigens, several of which are now in early phase clinical trials.

Studies with heat-killed and live attenuated whole *S. pneumoniae* demonstrate that nasopharyngeal exposure is sufficient to induce adaptive responses protective against pneumonia. Heat-killed bacteria are only protective if introduced with a mucosal adjuvant. This suggests that brief exposure to non-replicating bacterial antigens alone is insufficient to induce protective responses, and that in the absence of an artificial adjuvant, persistence of antigens in the nasopharynx may be important. The only studies of the protective efficacy of prior colonisation with live *S. pneumoniae* strains have utilised genetic mutants with attenuated virulence, such as lack of capsule, lack

of the surface protein PspA or lack of the toxin Ply. Such mutations will affect host-bacterial interaction, and may have specific effects on the immune response in the nasopharynx. Since no comparisons have been made between mutant and WT strains, extrapolation of these mechanisms of protection to those following WT exposure are not appropriate. Thus, it is not known whether prior exposure to a live WT strain is protective against pneumonia. Although colonisation with an acapsular strain is sufficient to protect, the specific role of capsule in colonisation-induced protection by encapsulated strains remains unclear. Bacterial lipoproteins are a further group of abundant surface molecules. They play crucial roles in nutrient uptake, and may be important in colonisation. In addition, they are bacterial antigens and TLR2 agonists. Whilst colonisation-induced Th17-cell responses are TLR2 dependant, the role of TLR2 and lipoproteins in antibody responses to colonisation are not known. Studies comparing the immunogenicity and protective efficacy of WT *S. pneumoniae* strains with acapsular mutants and Δ*lgt* mutants lacking lipoproteins could address these questions. If colonisation duration could be controlled using auxotrophic strains such as Δ*pab*, this may identify the minimal requirements of a colonisation event to induce protection. Where prior colonisation does protect against subsequent pneumonia, the specific contributions of antibody versus cell-mediated immunity remain unclear. Furthermore, whether colonisation protects through mucosal or systemic immunity is not known, and the antigenic targets of such protective immunity remain to be defined.

1.7 AIMS OF THIS THESIS

1.7.1 General aim and scope

This thesis aims to define the mechanisms by which prior colonisation can protect against subsequent lethal *S. pneumoniae* pneumonia in mice.

1.7.2 Specific aims

- To establish whether prior colonisation with potentially virulent wild-type strains of *S. pneumonia* protect against subsequent lethal pneumonia
- To determine how the inability to replicate and the absence of capsule and surface lipoproteins affects nasopharyngeal colonisation with *S. pneumoniae*
- To explore whether these factors affect the ability of previous colonisation to protect against subsequent wild-type lethal pneumonia
- To characterise how previous colonisation affects the progression of pathophysiology of lethal pneumonia
- To identify the relative contributions of antibody and T-cell responses to colonisation on the progress of subsequent pneumonia
- To determine the precise function of the protective elements of the immune responses to colonisation
- To identify antigenic targets of protective immune responses

2 METHODS

2.1 BACTERIAL METHODS

2.1.1 Bacterial strains

The following wild-type (WT) strains were used in experiments:

D39, a mouse-virulent invasive serotype 2 strain that has been genome sequenced (Lanie et al., 2007) and was a kind gift from Prof James Paton (University of Adelaide, Level 4, Molecular Life Sciences, SA5005, Adelaide, Australia). This strain was first described in experiments determining the role of DNA in transferring heritable characteristics (Avery et al., 1944).

TIGR4, a highly virulent capsular serotype 4 strain clinical isolate that has been genome sequenced (Tettelin et al., 2001) and was given as a kind gift by Prof Jeffery Weiser (Department of Micriobiology, University of Pennsylvania, 402A Johnson Pavilion, Philadelphia, PA 19104-3511, USA).

Strain 0100993, a serotype 3 human pneumonia isolate, originally maintained by SmithKlineBeecham (Lau et al., 2001). This strain was a kind gift from Prof David Holden (Centre for Molecular Microbiology and Infection, Imperial College London, Flowers Building, Exhibition Road, London, SW7 2AZ, UK). This WT strain will be referred to as ST3 within this thesis.

The following mutant strains derived from the above WT strains were used in experiments:

D39-DΔ, an unencapsulated avirulent mutant created from the D39 WT strain by in-frame deletion of capsule biosynthesis gene *cps2D* by overlap-extension PCR (Morona et al., 2004). This also confers erythromycin resistance.

D39Δ*lgt*, a mutant of WT strain D39 that lacks surface lipoproteins was generated by Dr Suneeta Khandavilli in the laboratory of Dr Jeremy Brown. The SP1412 gene (*lgt*)

encoding prolipoprotein diacylglyceryl transferase was replaced with a chloramphenicol resistance cassette.

D39Δ*pab*, an auxotrophic mutant of WT strain D39 was also generated by Dr Suneeta Khandavilli in the laboratory of Dr Jeremy Brown. The gene SP0665 (*pabB*) was replaced with a kanamycin resistance cassette.

TIGR4*cps*, an unencapsulated avirulent mutant created from the TIGR4 WT strain. It contains a Janus cassette in place of the capsule gene locus, conferring resistance to kanamycin (Trzcinski et al., 2003). This strain was a kind gift from Prof J Weiser.

TIGR4Δ*pab*, an auxotrophic mutant of WT strain TIGR4 was also generated by Dr Suneeta Khandavilli in the laboratory of Dr Jeremy Brown. As for D39 Δ*pab*, the gene SP0665 (*pabB*) was replaced with a kanamycin resistance cassette.

Characteristics of these mutant strains are summarised in Table 2.1.

Table 2.1. Mutant strains of *S. pneumoniae* used in this thesis.

Mutant strain	Wild-type background	Mutation	Phenotype	Antibiotic resistance
D39-DΔ[1]	D39	Deletion of *cpsD*	Unencapsulated	Erythromycin
D39Δ*lgt*	D39	Deletion of *lgt*	Lipoprotein deficient	Chloramphenicol
D39Δ*pab*	D39	Deletion of *pabB*	PABA auxotroph	Kanamycin
TIGR4Δ*cps*[2]	TIGR4	Deletion of whole *cps* locus	Unencapsulated	Kanamycin
TIGR4Δ*pab*	TIGR4	Deletion of *pabB*	PABA auxotroph	Kanamycin

[1] (Morona et al., 2004)
[2] (Trzcinski et al., 2003)

2.1.2 Bacterial culture

Bacteria were cultured at 37 °C in 5% CO_2 on Columbia agar (Oxoid) containing 5% defibrinated horse blood (TCS Biosciences). Working stocks were made by transferring one colony of *S. pneumoniae* to Todd-Hewitt broth (Oxoid) supplemented with 0.5% yeast extract (Oxoid) (THY) and grown to an optical density (OD_{580}) between 0.3 and 0.4. This corresponded to approximately 10^8 colony forming units (CFU) per ml. Single use aliquots of bacteria containing 10% glycerol were stored until required at -80 °C. The precise number of CFU was determined by culturing 100 µl volumes of a thawed aliquot in 10-fold serial dilution on blood agar in duplicate, and counting the number of colonies 16 h later. The mean of duplicate platings was used to derive the number of CFU per ml of the original frozen stock. To confirm the identity of mutant *S. pneumoniae* strains prior to use in experiments and whilst maintaining stocks, bacteria were also cultured on blood agar supplemented with antibiotics at the following concentrations: erythromycin, 0.2 µg/ml; kanamycin, 500 µg/ml; chloramphenicol, 4 µg/ml. Antibiotics were selected according to the sensitivity of the mutant strain according as shown in Table 2.1.

For culture of samples recovered from mice, serial 10-fold dilutions of blood, lung homogenate or broncheo-alveolar lavage (BAL) fluid (BALF) were prepared using sterile phosphate buffered saline (PBS [Sigma], containing 8 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, and 0.2 g KCl). Sufficient dilutions were plated to both maximise sensitivity and ensure numbers of CFU could be robustly counted (<500 CFU/plate). Samples recovered from respiratory tract were cultured on blood agar supplemented with gentamicin 5 µg/ml to limit overgrowth of contaminants. Samples recovered from blood were cultured on blood agar without antibiotics. Parallel plating with and

without gentamicin confirmed that at this concentration, gentamicin did not affect numbers of CFU of *S. pneumoniae*.

2.1.3 5-carboxyfluorescein, succinimidyl ester labelling

S. pneumoniae were labeled with the intra-cellular dye 6-carboxyfluorescein succinimidyl ester (FAM-SE) for use in infection experiments and phagocytosis assays (Hyams et al., 2010a, Yuste et al., 2008). Bacteria were cultured overnight on 5% blood agar at 37 °C in 5% CO_2. A single 10 µl loop (Greiner) of colonies was inoculated into 15ml THY medium and grown to late log phase (OD_{580} 0.7-0.8). Bacterial cells were harvested by centrifugation and washed once with 5ml 0.1M sterile $NaHCO_3$ buffer. Following resuspension in 1ml $NaHCO_3$ buffer with 50µl FAM-SE solution (Cambridge Bioscience, prepared at 10mg/ml in DMSO), bacteria were incubated for 1 h at 37 °C in 5% CO_2. Cells were washed six times in Hank's balanced salt solution (HBSS, containing 0.137M NaCl, 5.4mM KCl, 0.25mM Na_2HPO_4, 0.44mM KH_2PO_4, 1.3mM $CaCl_2$, 1.0mM $MgSO_4$, 4.2mM $NaHCO_3$) (GIBCO) supplemented with 0.2% bovine serum albumin (BSA) (Merck), until no further free dye appeared in the supernatant. Single use aliquots of labeled cells were frozen with 10% glycerol, protected from light at -80 °C until required.

2.1.4 Bacterial lysates

S. pneumoniae lysates were prepared for use in immunoblotting experiments. Bacteria were cultured overnight on 5% blood agar at 37 °C in 5% CO_2. A 10 µl loop of colonies was inoculated into 4ml THY medium and grown to late log phase (OD_{580} 0.7-0.8). Bacterial cells were harvested by centrifugation, washed twice with PBS, and resuspended to 200 µl with PBS. Lysates were sonicated for two min using a Sonifier 250 instrument (Branson). Lysates were frozen at -20 °C until use.

2.2 *IN VIVO* METHODS

2.2.1 Mouse strains

CD1 outbred mice and CBA/Ca inbred mice were obtained from Charles River UK Ltd. B6.129-S2-Igh-6tm1Cgn/J (μMT) mice containing a targeted mutation in the heavy chain locus of immunoglobulin M (IgM) and which do not produce mature B cells or antibody (Kitamura et al., 1991) were a kind gift of Dr Claudia Mauri, (UCL Division of Medicine, Windeyer Building, 46 Cleveland Street, London, W1T 4JF, UK). They had been bred and maintained at UCL according to institutional guidelines. All mice were female and aged 6-8 weeks at the commencement of experiments unless otherwise stated. They were housed in individually ventilated cages with no more than five mice per cage. Separate cages were used to house mice colonised with different bacterial strains. Control mice were also housed in separate cages to colonised mice. Mice were permitted food and water *ad libitum* in a temperature, humidity and light controlled environment. Experiments were performed according to the institutional guidelines for animal use and care, and all experiments conducted under Home Office (UK) license.

2.2.2 Mouse models

2.2.2.1 Inoculation for colonisation and disease models

Mice were colonised by atraumatic instillation of 10^7 CFU *S. pneumoniae* suspended in 10μl PBS into the nares under light halothane anaesthesia. This inoculum was determined as optimal to ensure consistent colonisation as described in Chapter 3 and reported by others (Wu et al., 1997b). Control mice received 10μl PBS alone. For pneumonia challenge, 10^7 CFU *S. pneumoniae* suspended in 50 μl PBS was instilled

intranasally (i.n.) under deep halothane anaesthesia (Brown et al., 2001a). Mice were held in the upright position for one min following inoculation to ensure aspiration of the inoculum. For intravenous (i.v.) challenge, mice received 5 x 10^6 CFU *S. pneumoniae* suspended in 100µl PBS via tail vein injection (procedure performed by Mr Mick Keegan (UCL Biological Services Unit)). All inocula were cultured in serial dilution on blood agar plates at the time of inoculation to determine the actual number of CFU present. Where mutant strains of *S. pneumoniae* were used *in vivo*, bacteria were also cultured on blood agar containing the appropriate antibiotic to confirm the identity of the mutant.

2.2.2.2 Survival experiments

In certain experiments, mice were monitored at regular intervals following pneumonia challenge to identify signs of terminal illness (Brown et al., 2001a, Brown et al., 2002). Mice were observed every 12 h for the first 24 h, then every six h until day seven and again 12-hourly thereafter until the end of the experiment. Mice displaying well-described signs of terminal illness (reduced mobility, haunched posture, starry coat) were culled and survival time recorded as the next observation timepoint.

2.2.2.3 Passive serum transfer

For passive serum transfer experiments, serum collected from donor mice was pooled and frozen immediately after separation. Serum was thawed immediately prior to use and brought to room temperature (RT) prior administration by intraperitoneal (i.p.) injection to recipient mice.

2.2.2.4 CD4-cell depletion

To deplete CD4+ cells, mice received doses of the monoclonal antibody (mAb) GK1.5 (eBiosciences) by i.p. injection as described in Chapter 5 (Zhang et al., 2009).

GK1.5 was stored at +4 °C until use. All vials used were from the same lot and were pooled prior to use *in vivo*.

2.2.3 Sample Processing

2.2.3.1 Whole blood and serum

For experiments requiring sampling of lower respiratory samples in addition to blood, mice were anaesthetised with 2 mg pentobarbital i.p. (Euthatal, Rhone-Merieux) followed by exanguination via severing the femoral artery. This prevented the contamination of respiratory samples with blood whilst maximising the quantity of blood obtained. When lower respiratory samples were required without blood samples, a more rapidly lethal dose of 7 mg pentobarbital was administered. When only nasopharyngeal wash was to be collected, mice were culled by asphyxiation with CO_2. When only blood samples were required, but not respiratory samples, cardiac puncture and terminal exsanguination under isofluorane anaesthesia was performed to maximise the yield. For non-terminal sampling, blood was collected via tail vein bleeding. Whole blood was collected for bacterial culture into heparinised collection tubes. 50 µl heparin (Sigma) was used per 0.5ml tube at 1000 U/ml. Subsequent dilution calculations took account of the volume of heparin. Blood samples intended for serum were collected into plain tubes, allowed to clot at room temperature (RT) for 2 h, and centrifuged at 700 *g* for 20 min. Separated serum was transferred to fresh plain Eppendorf tubes and immediately frozen.

2.2.3.2 Nasopharyngeal wash

To assess nasopharyngeal colonisation density, trachea was exposed, catheterized with a sterile 20G cannula (BD) which was flushed towards the head with 200µl sterile PBS. Fluid exiting the nares was collected using a sterile Pasteur pipette and

cultured in serial dilution on blood agar plates supplemented with 5μg/ml gentamicin (Fluka) to prevent overgrowth of contaminants.

2.2.3.3 Bronchoalveolar lavage fluid (BALF)

BAL was performed by inserting the sterile cannula towards the lungs into the exposed trachea and lavaging three times with 1ml sterile PBS. BAL fluid (BALF) was used for several assays and processed as follows.

BALF was vortexted prior to reserving 100 μl for cytospin and a further 200 μl for bacterial culture. The remaining volume was recorded for use in calculating the absolute cell count (see below). Cells were harvested by centrifugation at 700 *g* for 3 min. The supernatant was immediately frozen at -80 °C for subsequent analysis. Following red blood cell (RBC) lysis, the pellet was used to determine the absolute cell count of the original BALF as described below.

2.2.3.4 Lungs

Following BAL, the thoracic cavity was opened. 2ml sterile PBS was used to gently perfuse the lungs via injection into the right ventricle. This was done to minimise intra-vascular blood content of lungs that may confound cellular and bacterial assessments. Lungs visibly blanched following this procedure. The left lung was removed and placed into 5ml of 4% paraformaldehyde (PFA) solution to fix for histological examination. The right lung was removed and placed into 5ml sterile PBS.

Lung samples collected into PBS were used to prepare single-cell suspensions for bacterial culture and flow cytometry analysis. Lungs were rinsed with sterile PBS to remove contaminating surface blood. They were chopped into 2mm cubes and homogenised by mashing through a single cell 70μm strainer (BD Falcon) into a 50ml

Falcon tube to a final volume of 4ml. 200 µl of this suspension was reserved for bacterial culture. Cells were harvested following centrifugation at 700 *g* for 7 min and the supernatant discarded. Following RBC lysis, the pellet was used to determine the absolute cell counts and for flow cytometry staining as described below.

All samples were maintained on ice throughout processing unless otherwise stated.

2.3 ANTIBODIES

All antibodies were used in experiments at optimal dilutions and temperature conditions. Details of antibodies are listed in Table 2.2.

Table 2.2. Antibodies used in this study[3].

Target[4]	Clone	Source species	Conjugate	Use[5]	Dilution	Supplier	Product code
CD3	145-2C11	Armenian hamster	PE	FC	1:100	eBioscience	12-0031
CD4	GK1.5	Rat	PECy7™	FC	1:200	eBioscience	25-0041
CD8	53-6.7	Rat	PerCP	FC	1:100	BD	553036
B220	RA3-6B2	Rat	APC	FC	1:100	eBioscience	17-0452
TCRγδ	GL3	Armenian hamster	PE	FC	1:50	eBioscience	12-5711
TCRβ	H57-597	Armenian hamster	FITC	FC	1:100	eBioscience	11-5961
Gr1	RB6-8C5	Rat	FITC	FC	1:100	eBioscience	11-5931
F4/80	BM8	Rat	PE	FC	1:50	Caltag	MF48004
IgG	Polyclonal	Goat	PE	FC	1:100	Jackson Labs	115-116-072
IgG	Polyclonal	Goat	PE	Luminex	1:50	Jackson Labs	115-116-072
IgG	Polyclonal	Goat	AP	ELISA	1:10,000	Sigma	A2429
IgA	Polyclonal	Goat	AP	ELISA	1:10,000	Sigma	A4937
IgM	Polyclonal	Goat	AP	ELISA	1:10,000	Sigma	A7784
IgG	Polyclonal	Goat	HRP	WB	1:1,000	Sigma	A0168
CD4	GK1.5	Rat	Nil	IVD	-	eBioscience	16-0041
FcγRIII/II	2.4G2	Rat	Nil	FC	1:200	BD	553141

[3] The further antibodies forming part of commercial kits for ELISA or Luminex cytokine measurement are described in the text.
[4] All targets are murine.
[5] FC, flow cytometry; WB, Western blotting; IVD, *in vivo* depletion.

2.4 HISTOPATHOLOGICAL ASSESSMENT

Sections of lung tissue were kindly prepared and stained by Mr Steve Bottoms (laboratory technician, UCL Centre for Respiratory Research, Rayne Building, 5 University Street, London, WC1E 6JF) using established techniques. Lungs were fixed in 4% paraformaldehyde in phosphate-buffered saline for a minimal period of 4 h. They were then rinsed in 50% ethanol/distilled water and stored in 70% ethanol/distilled water at 4 ºC. The lungs were processed to paraffin wax overnight using an automated tissue processor (Leica). Using a Shandon rotary microtome (Thermo Fisher Scientific) 3μm sections were prepared and dried overnight. The sections were stained with haematoxylin and eosin using a Sakura Diversified Stainer and coverslipped with a Coveraid Coverslipper (both Sakura-Finetek).

Lungs were scored for inflammatory change using an established scoring system (Yuste et al., 2007) by Mr Bottoms who was blinded as to which group of mice samples were derived. Sections were initially examined at x10 magnification to assess percentage pathological involvement. Six randomly selected fields were then examined at a magnification of x20. Typical appearances are shown in Figure 2.1. Fields were scored for degree of inflammatory change using an established scoring system (Yuste et al., 2007) as shown in Table 2.3. The mean field score was calculated and multiplied by overall percentage involvement to obtain a total score.

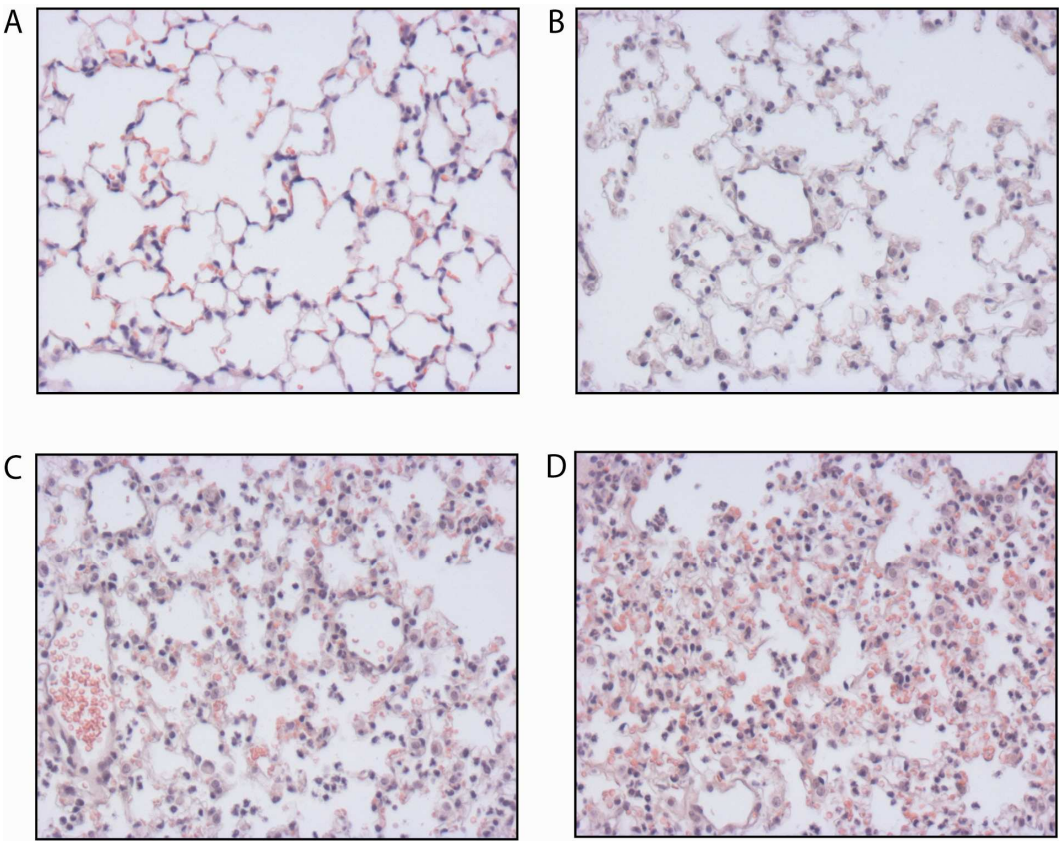

Figure 2.1. Histopathological appearances of *S. pneumoniae* infected mouse lungs.

Haematoxylin and eosin stained sections of lung tissue harvested from CBA/Ca mice 18 h following infection with 10^7 CFU *S. pneumoniae* D39 i.n. BAL was performed prior to harvesting of lungs. Images demonstrate varying amounts of inflammation corresponding to the stages of the scoring system used. (A) No visible inflammatory change. (B) Minimal swelling of alveolar walls with slight change in architecture. (C) Increased swelling with presence of erythrocytes and inflammatory cells and an increase in type II pneumocytes. (D) Considerable haemorrhage with inflammatory cell influx, widespread alveolar disorganisation with interstitial swelling and pneumocyte proliferation.

Table 2.3. Scoring system for assessment of inflammation in mouse lung.

Score	Features
1	No visible inflammatory change
2	Minimal swelling of alveolar walls with slight change in architecture
3	Increased swelling with presence of erythrocytes and inflammatory cells and an increase in type II pneumocytes
4	Considerable haemorrhage with inflammatory cell influx, widespread alveolar disorganisation with interstitial swelling and pneumocyte proliferation

Total score is determined by multiplying percentage of total area involved by mean score of six random fields assessed at x20 magnification (Yuste et al., 2007).

2.5 CELLULAR METHODS

2.5.1 Cell isolation and preparation

2.5.1.1 Broncho-aleveolar fluid (BALF) and lung red blood cell (RBC) lysis

To facilitate counting total leukocyte counts, RBCs were depleted from BALF and lung cell samples. Samples were resuspended (BALF to 100 µl, lung to 1ml) into red blood cell (RBC) lysis buffer (BioLegend, San Diego, USA) and incubated on ice for 5 min with occasional shaking. The reaction was stopped by adding a further PBS (900 µl for BALF, 10ml for lungs), the cells harvested by centrifugation and the supernatant discarded. The pellet was resuspended with PBS (to 0.5 ml for all samples). This preparation was kept on ice until used for absolute cell counting.

2.5.1.2 Human neutrophil isolation

Neutrophil isolation was kindly performed by Dr Catherine Hyams. Blood was donated by healthy human volunteers who had not received pneumococcal vaccination. Polymorphonuclear cells (PMNs) were extracted using an established technique (Segal et al., 1980). 100 ml of fresh blood was heparinised (300U / 50 ml) and carefully layered onto Lymphoprep (Axis Shield). This was centrifuged at 700 *g* for 30 min without brakes. This yielded three distinct cellular layers: an upper peripheral blood mononuclear cell (PBMC) cell layer; a middle layer containing PMNs suspended within Lymphoprep; and a lower layer of sedimented RBC. The PBMC interface was removed and the middle and lower layers pooled to create a final volume of 40 ml. To this, 10 ml of dextran (MW 200,000-300,000) (MP Biomedical) at 10% in normal saline was added. Following very gentle mixing, RBCs were allowed to sediment by leaving the preparation undisturbed at RT for one hour. The upper neutrophil-enriched layer was extracted into a fresh container, centrifuged at

700 *g* for 10 min and remaining RBC were removed by hypotonic lysis. Viable cells were counted and used within 4 h.

2.5.1.3 Cell counting

To determine viable cell counts, samples were diluted with an equal volume of 0.4% (w/v) Trypan blue (Sigma) and 10 µl placed into the counting chamber of a Neubauer haemocytometer (Camlab, Cambridge, UK). Cells were counted under microscopy at a magnification of x40. Only viable cells that excluded Trypan blue were counted. The viable cell count was determined by multiplying the number of counted cells by the dilution factor (x2) and the counting chamber volume factor ($x10^4$). This cell count represented the number of cells per ml of prepared cells.

2.5.2 Cellular analysis

2.5.2.1 Cytospin

For differential cell counting, 100µl BALF were spun onto glass slides at 700 revolutions per min (rpm) for 7 min using a Cytospin 3 instrument (Shandon). Air-dried slides were stained with rapid Romanowsky stain (Raymond A Lamb) and again allowed to dry. The slides were coverslipped with a Coveraid Coverslipper (Sakura-Finetek) and examined microscopically at x40 magnification. Leukocyte cell types were identified using standard morphological criteria. A total of 300 cells from random fields were counted, and numbers of neutrophils and monocyte/macrophages recorded. Thus, the proportion of neutrophils amongst BALF leukocytes was obtained. Using the absolute total leukocyte numbers obtained from haemocytometer counting, absolute neutrophil and macrophage numbers in BALF were determined. To confirm purity of human neutrophil extractions, cytospin preparations (100 µl at 1:100 dilution with PBS) were stained with Diff-quick (Merck) and examined

microscopically by Dr Hyams. 400 cells were morphologically assessed. Preparations used in phagocytosis assays consistently yielded over 95% neutrophils.

2.5.2.2 Flow cytometry: general methods

Cells harvested from BALF and lung preparations were stained prior to analysis by flow cytometry. Table 2.2 lists details of antibodies and the dilutions at which they were used. 120 µl aliquots of samples were loaded into wells of a 96-well U-bottomed plate and centrifuged at 700 *g* for 3 min and washed once with PBS + 1% BSA. This was used as the buffer throughout staining procedures. Cells were incubated with 10 µl of Fc-block at RT for 10 min prior to resuspension with 25 µl of antibody mix. Following 20 min incubation in the dark at 4 °C, cells were washed three times and resuspended in 200 µl 4% PFA. Samples were then kept at 4 °C in the dark until acquisition on a FACSCalibur instrument (BD Biosciences).

On every occasion, aliquots of freshly isolated splenocytes prepared as for lung cells above were also included. Aliquots of splenocytes were either unstained or stained with single antibody conjugates in addition to the combinations used within individual experiments. These samples were used to compensate during analysis of multi-colour stained samples.

2.5.2.3 Flow cytometry: gating

For lung samples, an initial gate was set according to forward scatter (reflecting cell size) and side scatter (reflecting cell granularity) to exclude any RBC and small debris present in the sample (Figure 2.2). This gate was used as the denominator in defining the proportion of lymphocytes present. A lymphocyte gate was set within this denominator gate on the tight FSC^{lo}/SSC^{lo} population. Backgating for CD3 (a pan T-cell marker) and B220 (B-cell marker) confirmed these cells as lymphocytes. Multi-parameter staining identified subsequent lymphocyte subsets within this population.

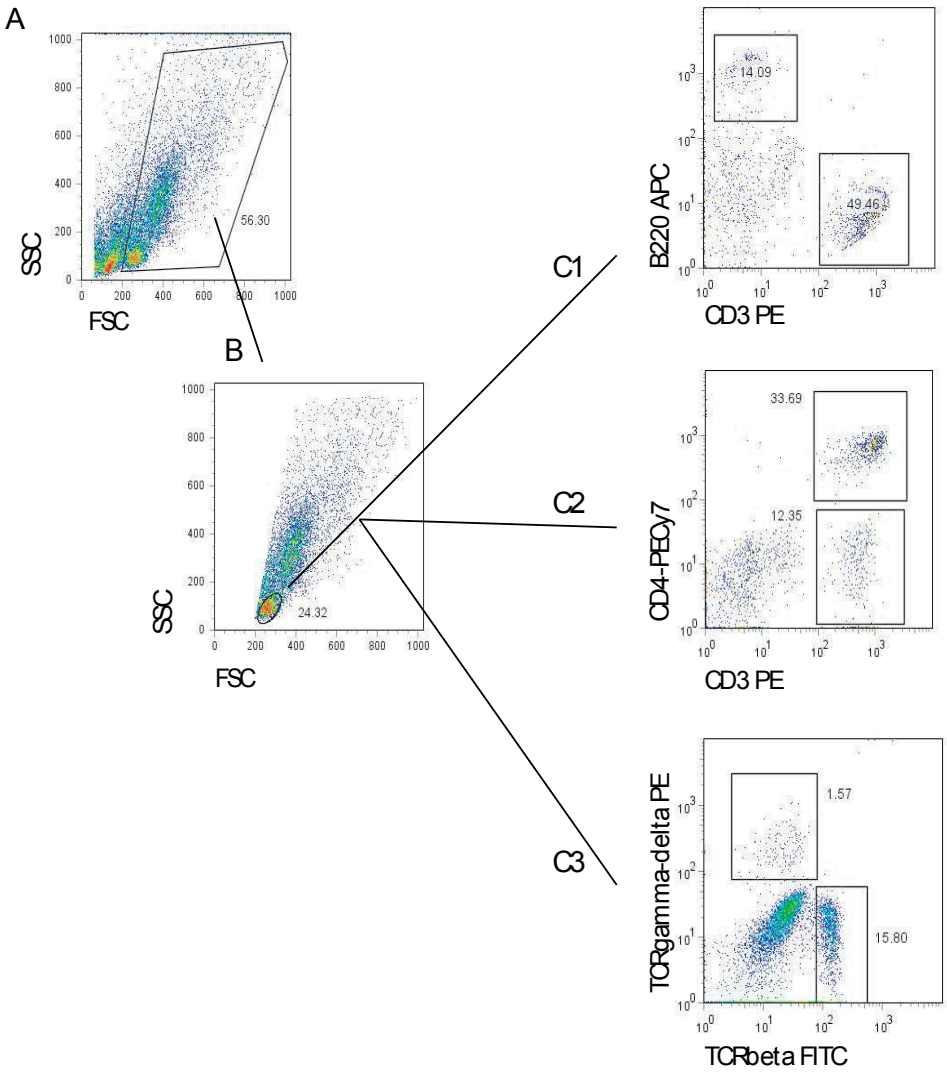

Figure 2.2. Representative flow cytometry plots showing lung lymphocyte subsets.

Single-cell preparation of lung cells (A) harvested from a CBA/Ca mouse 18 h after i.n. infection with 10^7 CFU *S. pneumoniae* D39. A gate (B) was set to include all leukocytes but exclude red blood cells and small debris and acted as a denominator gate. Lymphocytes were gated within this population and stained for surface markers. Backgating of CD3+ and B220+populations confirmed this population as lymphocytes. Following compensation for multi-colour staining, lymphocyte subsets were identified. Three representative subset plots are shown: C1 shows B-cells (B220+) and T-cells (CD3+); C2 shows CD4+ and CD4- T-cells; C3 shows TCRβ and TCRγδ T-cells.

For BALF cellular preparations, a gate was required to identify alveolar macrophages. Excluding debris/RBC and cells falling in the lymphocyte region, remaining cells were either clustered in the FSC^{mid}/SSC^{mid} region or distributed more widely into the FSC^{mid+hi}/SSC^{hi} region. Gates were set on these two regions and cells analysed according to staining with Gr1 (granulocyte marker) and F4/80 (macrophage marker) (Figure 2.3). The FSC^{mid+hi}/SSC^{hi} region contained predominantly $F4/80^{+}Gr\text{-}1^{-}$ cells, identifying them as predominantly macrophages. The FSC^{mid}/SSC^{mid} region was more heterogeneous, but relatively enriched with $Gr\text{-}1^{hi}$ granulocytes compared to other regions. The FSC^{mid+hi}/SSC^{hi} region was therefore used for analysis of alveolar macrophages in subsequent *in vivo* phagocytosis experiments.

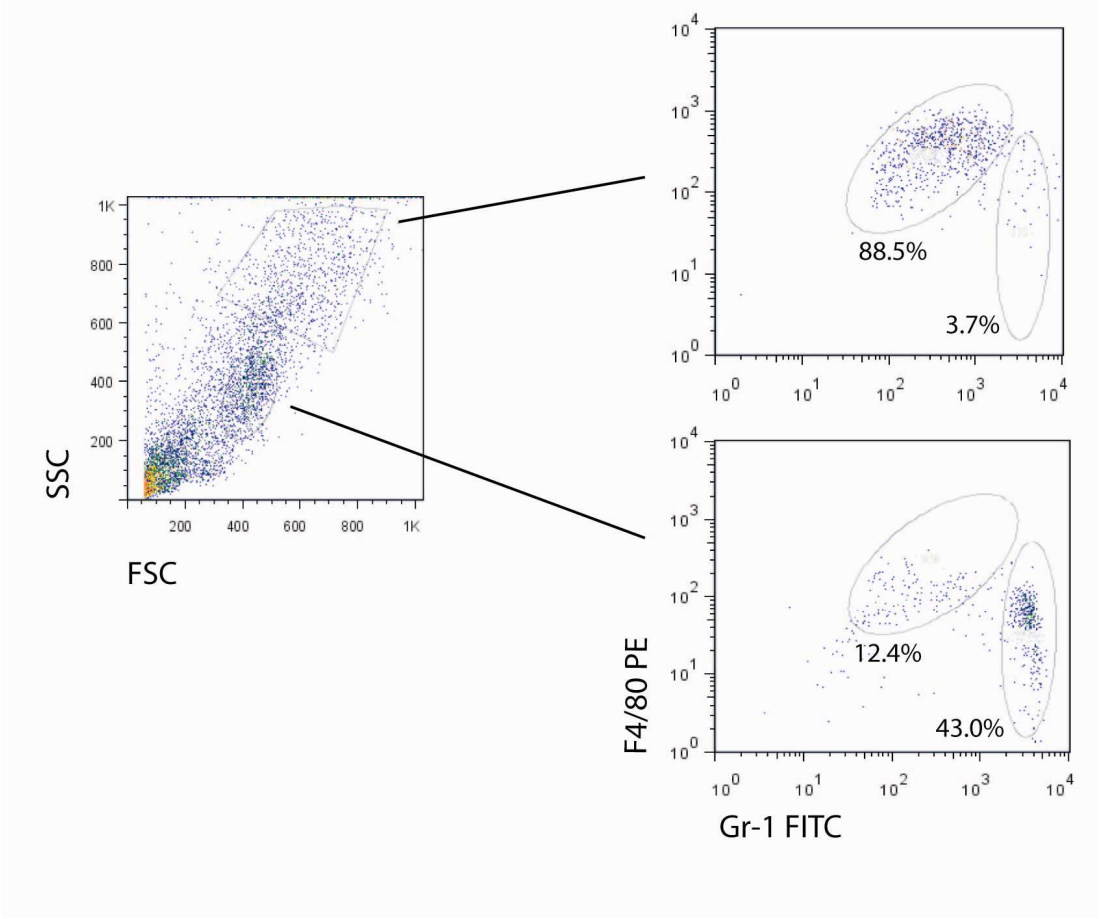

Figure 2.3. Representative flow cytometry plots showing macrophage gating of BALF cells.

Cells recovered by BALF 4 h following infection of a CBA/Ca mouse with 10^7 CFU *S. pneumoniae* D39 i.n., stained with Gr1 (granulocyte marker) and F4/80 (macrophage marker).

2.5.3 Phagocytosis assays

2.5.3.1 Alveolar macrophage *in vivo* phagocytosis assay

Following infection with FAM-SE labelled bacteria, phagocytosis by alveolar macrophages was assessed by flow cytometric examination of cells recovered from BALF of mice (Arredouani et al., 2006). In this assay, recovered cells were washed twice with PBS and fixed with 200 µl 4% PFA prior to flow cytometry. Macrophages were gated according FSC and SSC criteria as established above. All cells within the sample were analysed and the median fluorescence intensity (MFI) for channel FL-1 (in which FAM-SE emits) of this population determined. Previous experiments indicated that bacteria recovered from BALF remained fluorescently labelled by this technique at the timepoints used in this study.

2.5.3.2 Human neutrophil phagocytosis assay

This assay was kindly performed by Dr Catherine Hyams (UCL Centre for Respiratory Research, Rayne Building, 5 University Street, London, WC1E 6JF) as described (Hyams et al., 2010a). It measures the effect of serum pre-incubation of bacteria on subsequent association with freshly isolated human neutrophils. Aliquots of serum pooled from colonised or control mice were thawed and diluted with an equal volume of PBS. 10µl diluted serum was added per well in a 96-well plate. Wells containing PBS alone were used as controls. 5 x 10^6 CFU thawed, washed FAM-SE labelled *S. pneumoniae* in 10 µl PBS were added to each well. Bacteria were incubated for 30 min at 37 °C on a horizontal shaker at 150 rpm. Freshly isolated human neutrophils were washed and resuspended in HBSS with divalent cations. 5 x 10^5 cells were added per well resulting in a pre-optimised multiplicity of infection (MOI) of 10. Neutrophils were incubated with bacteria for 30 min at 37 °C

shaking at 150rpm, and then fixed with 50µl 3% PFA. Cells were analysed by flow cytometry on a FACScalibur instrument. The MFI of 15,000 cells per well was determined.

2.6 PNEUMOCOCCAL SPECIFIC IMMUNOASSAYS

2.6.1 Pneumococcal ELISAs

2.6.1.1 Whole cell ELISA

S. pneumoniae were grown in THY to late-log phase (OD_{580} 0.7-0.8) was washed twice in PBS and resuspended in PBS to OD_{580} 1.0. 50 µl aliquots were placed into wells of 96-well Maxisorp (Nunc) plates and incubated overnight at 4 °C. The following day, the plates were washed with PBS + 0.05% Tween-20 (Sigma) (PBS/Tw), blocked with 200 µl PBS+1% BSA and incubated for one hour at 37 °C. 50 µl samples were added in duplicate. Starting dilution of serum was 1:20 in PBS + 1% BSA. For titrations, serial 5-fold dilutions were used. Blank wells contained dilution buffer only. Following 2 h incubation at RT, plates were washed and 100 µl alkaline phosphatase conjugated secondary antibody added, diluted 1:10,000 in PBS/Tw (see Table 2.2). After a further 2 h incubation at RT, plates were washed and developed with 100 µl N-nitrophenylphosphate disodium (pNPP) at 1mg/ml in diethanolamine (DEA) buffer (see Appendix). After a further 30 min incubation in the dark at RT, absorbance was read at OD_{405}, with correction for absorbance at OD_{620}. These values were further corrected by subtraction of blanks.

Where required, end-point titres were defined as the dilution of serum giving a corrected OD_{405} of 0.1. This was extrapolated from results from dilutions resulting in OD in the linear range (Figure 2.4). Samples with $OD_{405} < 0.1$ at 1:20 dilution were assigned a titre of 1:10 (i.e. half the lower limit of detection).

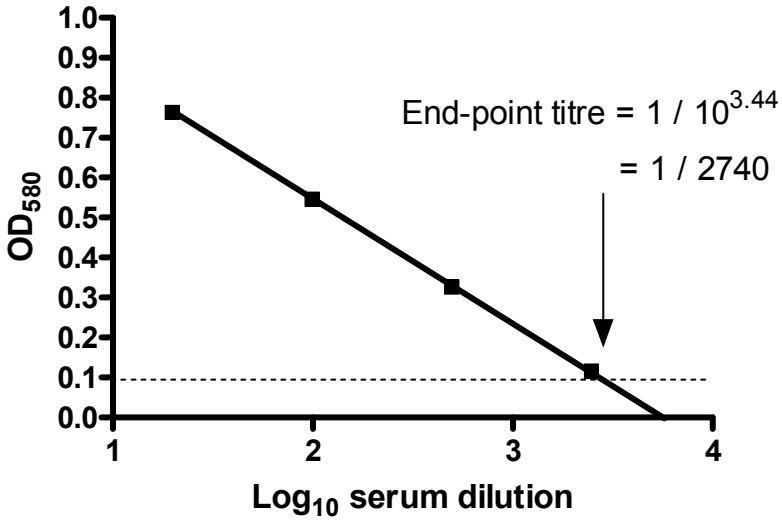

Figure 2.4. Representative end-point titration of pneumococcal whole cell ELISA.

The OD_{580} obtained is plotted against log_{10} dilution of the sample in serial dilution. A line is plotted based on values falling in the linear portion, from which the sample dilution corresponding to an OD of 0.1 is obtained. This is defined as the titre for that sample.

2.6.1.2 Capsular polysaccharide ELISA

Antibodies to CPS were measured following the established protocol (Wernette et al., 2003). Briefly, medium-absorbance plates (Greiner) were coated with 100 µl CPS at pre-optimised concentrations (type 2 CPS, 100 µg/ml; type 4 CPS, 1 µg/ml) and incubated at 4 °C overnight. Samples were pre-incubated in PBS/Tw supplemented with CWPS (Statens Serum Institut) 10 µg/ml and type 22F CPS (5 µg/ml) (ATCC) for 1 h prior to addition of 50 µl in duplicate to the washed plates. This pre-incubation stage competes out non-specific binding caused by contaminating CWPS and pneumococcal surface proteins present in the CPS preparation, increasing the specificity of anti-CPS antibody measurement. Following 2 h incubation at RT, plates were developed as for the whole cell ELISA.

2.6.1.3 Cell wall polysaccharide ELISA

To measure anti-CWPS antibodies, plates were coated with CWPS at (10 µg/ml) and refrigerated overnight. Samples were not pre-incubated prior to addition to the coated plates. Otherwise, the assay was the same as for measurement of anti-CPS antibodies.

2.6.2 Flow cytometry detection of bacterial IgG binding

To quantitate binding of serum IgG to the surface of bacteria an established assay was used (Yuste et al., 2008). Aliquots of 5 x 10^5 CFU *S. pneumoniae* were placed in wells of a U-bottomed 96-well plate, centrifuged at 700 *g* for 3 min and washed twice with PBS. Serum was diluted with an equal volume of PBS and 10 µl added per well. Control wells contained no serum. Bacteria were resuspended and plates incubated for 30 min at RT. After three washes, binding was detected by incubating with anti-mouse IgG PE conjugate (see Table 2.2) at 1:100 dilution in PBS/Tw for 20 min at 4 °C. Following three further washes, bacteria were fixed in 3% PFA. Fluoresence of

bacteria was measured on a FACScalibur instrument. Data from 25,000 cells was collected for analysis.

2.6.3 Pneumococcal protein immunoblotting

2.6.3.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Recombinant pneumococcal proteins expressed from the TIGR4 genome were kindly supplied by Dr Carmen Giefing (Intercell AG, Campus Vienna Biocenter 3, 1030 Vienna, Austria) (Giefing et al., 2008). Recombinant prolipoproteins expressed from the ST3 genome were prepared by Dr Suneeta Khandavilli (Brown et al., 2001a, Brown et al., 2001b). The preparation of bacterial lysates is described above. Aliquots of bacterial lysate or recombinant proteins were treated with NuPAGE™ reducing agent (Invitrogen). 10µl aliquots of reduced samples and 5µl of PageRuler Plus™ (Fermentas) protein marker ladder were loaded into lanes of NuPAGE™ 4-12% gradient bis-tris gels (Invitrogen). Proteins separated with 120V in NuPAGE™ MOPS running buffer (Invitrogen) until the ladder was well resolved. For protein identification, gels were stained with Coomassie brilliant blue (see Appendix) until bands appeared, then destained with Destain Solution (Appendix).

2.6.3.2 Western blotting

Separated proteins were transferred to nitrocellulose membrane (GE Healthcare) by applying 20V for 50 min in the presence of Transfer Buffer (Appendix). Successful transfer was confirmed by Ponceau S (Sigma) staining to membranes. Membranes were blocked overnight with tris-buffered saline (TBS, see Appendix) + 0.1% Tween-20 (TBST) plus 5% milk at 4 °C. Membranes were washed three times with TBST, and incubated with the serum to be tested diluted to 1% in TBST + 5% milk for 2 h at RT whilst rolling. Following three further washes, membranes were incubated with

anti-mouse IgG – horseradish peroxidase (HRP) conjugate (see Table 2.2) in TBST + 5% milk. Membranes were then washed three more times. Serum IgG binding was detected using Amersham ECL detection kit (GE Healthcare) following manufacturer's instructions. Developed membranes were exposed to photographic film (Amersham) and developed using a Photon Imaging System developer (Nikon).

2.6.4 Pneumococcal protein Luminex™ bead immunoassay

Luminex™ is a recently introduced multiplex bead immunoassay system where multiple target molecules are conjugated to microspheres of multiple colours. These conjugated beads provide the surface for an immunoassay reaction. Binding is measured on a flow cytometric platform. Beads conjugated to pneumococcal proteins were kindly provided by Prof Alex van Belkum (Department of Microbiology and Infectious Disease, Erasmus MC, Ziekenhuis, 's-Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands). Recombinant pneumococcal protein antigens used in the reactions were from a range of strain backgrounds (see Table 7.1). The purified proteins were coupled to SeroMAP beads, a carboxylated bead type developed for serological applications. The coupling reaction was been described (Verkaik et al., 2009). Briefly, 25 μg recombinant protein was covalently attached to activated carboxyl groups on the surface of 5.0 x 10^6 activated microspheres. Using positive control sera, the multiplex system was validated by comparing binding on a singleplex basis to the full set of multiplexed antigens. Conjugated beads were kept refrigerated until use.

To measure anti-pneumococcal protein IgG levels in test samples, a mixture was prepared of equal numbers of each bead type, suspended in PBS + 1% BSA. Beads that had undergone the chemical conjugation protein in the absence of a specific pneumococcal protein were included as controls. Prior to mixing, beads were

sonicated in a waterbath and vortexed. 96-well filter membrane plates (Multiscreen HTS™, Millipore) were used to hold the beads during the reactions. Wells were wet with PBS + 1% BSA which was then aspirated from below using a vacuum manifold (Millipore). 50 µl mixed beads was added to each well. This resulted in each well containing 3000 beads of each bead type. Beads were washed with PBS + 1% BSA and 50 µl serial dilutions of serum added in duplicate. Plates were incubated for 35 min at RT protected from light shaking at 600 rpm. Following two washes with PBS + 1% BSA, beads were resuspended in anti-mouse IgG PE conjugate diluted 1:50 in PBS + 1% BSA (see Table 2.2). Plates were incubated for a further 35 min as previously. Beads were washed two further times and beads resuspended thoroughly in 100 µl PBS + 1% BSA. The fluoresence of beads of each antigen type was measured using a Bio-Plex (Bio-Rad) machine. IgG binding at a given serum dilution to a specific protein was defined as the mean MFI of replicate wells.

2.7 CYTOKINE MEASUREMENT

2.7.1 Cytokine ELISAs

2.7.1.1 IL-17 ELISA

IL-17 was measured using the Mouse IL-17 Quantikine™ ELISA kit (R&D Systems, Minneapolis, MN, USA). Briefly, 50 µl sample or standard was added to 50 µl of Assay Diluent in wells of a 96-well plate pre-coated with IL-17 capture antibody. Plates were incubated for 2 h at RT, then washed five times. 100 µl diluted detection antibody conjugated to HRP was added to each well, and plates incubated for a further 2 h at RT. Following five further washes, 100 µl tetramethylbenzadine (TMB) Substrate Solution was added and plates incubated 30 min at RT protected from light. Finally 100 µl Stop Solution was added. Plates were read at OD_{450} corrected for absorption at OD_{570}. Concentrations of IL-17 in unknown samples were obtained from a standard curve, generated by log-log curve fit using GraphPad 4.0 (Prism), as illustrated in Figure 2.5.

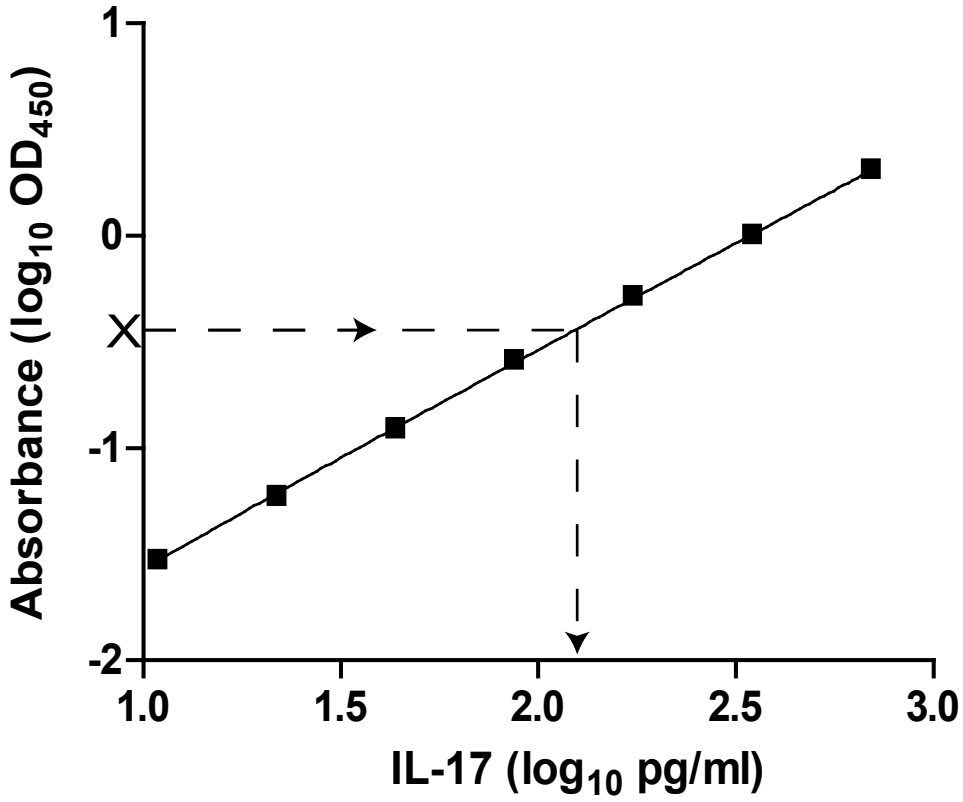

Figure 2.5. Derivation of unknown cytokine concentrations from ELISA standard curve.

Log_{10} absorption (as corrected OD_{405}) was plotted against log_{10} concentration for dilutions of a standard of known concentration. A log-log curve was fitted using GraphPad 4.0 (Prism), from which the concentration of samples (X) could be determined.

2.7.1.2 IFN-γ ELISA

IFN-γ was measured using a Ready-SET-Go!™ IFN-γ ELISA kit (eBioscience). Briefly, 100 µl sample, control or standard was added to wells of a 96-well plate pre-coated with capture antibody. Plates were incubated for 2 h at RT, then washed five times. 100 µl diluted biotinylated detection antibody was added to each well, and plates incubated for a further hour at RT. Following five further washes, 100 µl Avidin-HRP conjugate was added, and plates incubated a further 30 min. 100 µl TMB Substrate Solution was added and plates incubated 15 min at RT protected from light. Finally 50 µl Stop Solution was added. Plates were read and data analysed as for the IL-17 ELISA.

2.7.1.3 IL-22 ELISA

IL-22 was measured using the Mouse IL-22 Quantikine™ ELISA kit (R&D Systems). Briefly, 50 µl sample or standard was added to 100 µl of Assay Diluent in wells of a 96-well plate pre-coated with IL-17 capture antibody. Plates were incubated for 2 h at RT, then washed five times. 200 µl diluted detection antibody conjugated to HRP was added to each well, and plates incubated for a further 2 h at RT. Following five further washes, 120 µl TMB Substrate Solution was added and plates incubated 30 min at RT protected from light. Finally 120 µl Stop Solution was added. Plates were read and data analysed as for the IL-17 ELISA.

2.7.1.4 IL-23 ELISA

IL-23 was measured using the Mouse IL-23 Cytoset™ system (Invitrogen). Briefly, plates were coated with 100 µl capture antibody (1.25 µg/ml) and incubated overnight at 4 °C. Following washing, plates were blocked with PBS/Tw + 0.5% BSA for 1 h at RT. Following further washing, 100 µl sample or standard was added to wells and

plates incubated for 2 h at RT. Following further washing, 100 µl diluted biotinylated detection antibody was added to each well, and plates incubated for a further hour at RT. Following washing, 100 µl streptavidin-HRP conjugate was added and plates incubated 30 min at RT. Following five further washes, 120 µl TMB Substrate Solution was added and plates incubated 30 min at RT protected from light. Following washing, 100 µl Stop Solution was added. Plates were read and data analysed as for the IL-17 ELISA.

2.7.2 Cytokine Luminex™ assay

Mouse IL-1β, IL-6, TNFα, KC, IL-12p70 and GM-CSF were measured by Luminex™ bead immunoassay (Invitrogen) according to manufacturer's instructions. Briefly, wells of a 96-well filter membrane plate (Multiscreen HTS™, Millipore) were wetted with Assay Buffer. Equal quantities of each bead type were added to 6 ml Assay Buffer and 50 µl added to each well. Beads were sonicated and vortexed prior to mixing and again prior to adding to wells and washing. 50 µl of standard or samples were added to wells in duplicate, and the plate incubated for 1 h at RT shaking at 300 rpm. Beads were washed three times and 50 µl anti-mouse IgG PE conjugate (see Table 2.2) added at 1:50 dilution. Following a further 30 min incubation shaking at RT, beads were washed again and resuspended in 130 µl Assay Buffer. Fluoresence of beads was measured using a Bio-Plex (Bio-Rad) machine. Cytokine levels were obtained from standard curves generated using Bio-Plex Manager v3.0 (Bio-Rad). Typical standard curves are shown in Figure 2.6.

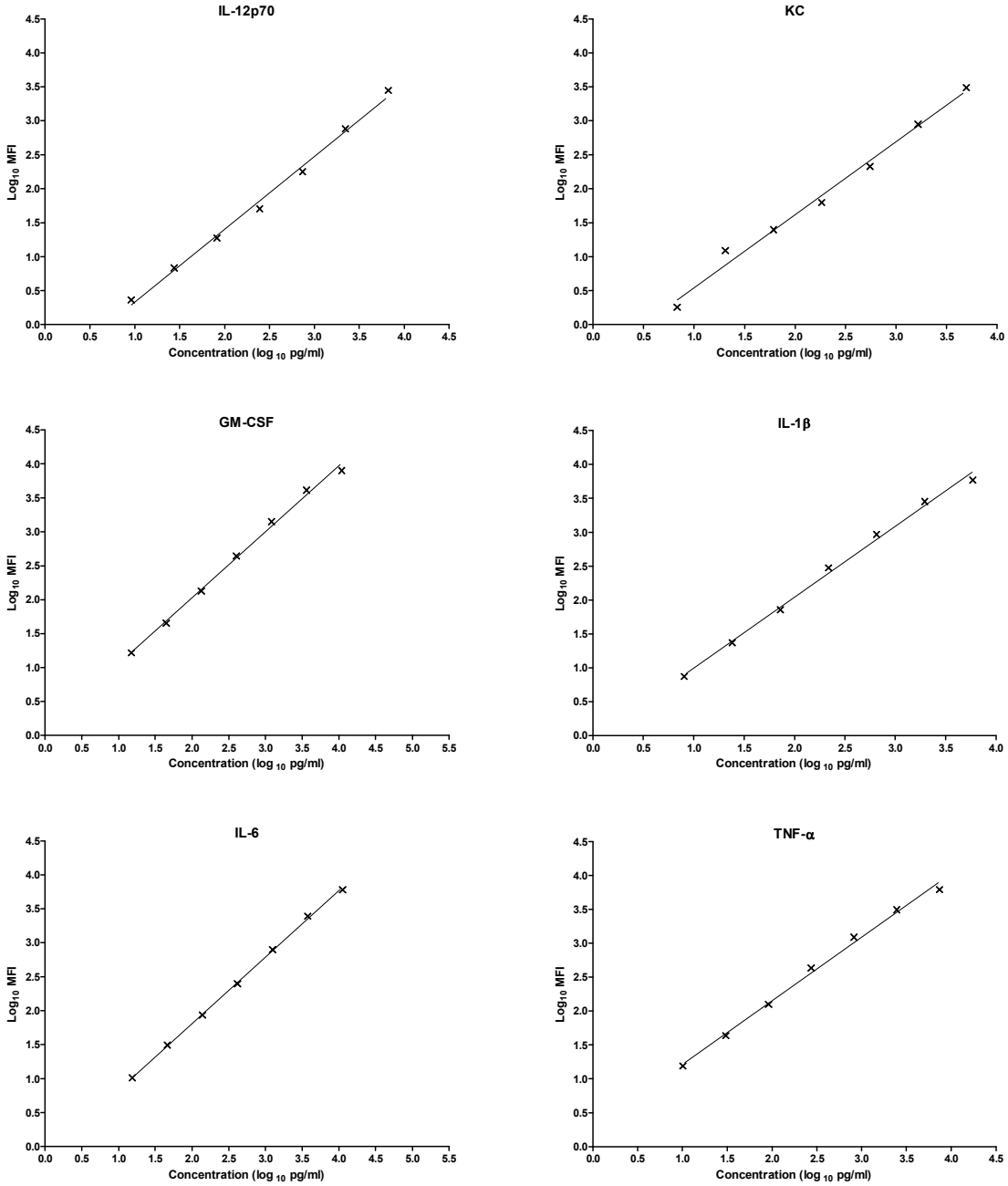

Figure 2.6. Standard curves for cytokine measurement using Luminex™ bead assay.

Log_{10} MFI of serial dilutions of a standard containing known concentrations of multiple cytokines measured in parallel using Luminex™. A log-log curve was fitted using GraphPad 4.0 (Prism), from which the concentration of samples (X) could be determined.

2.8 BIOINFORMATICS

Pneumococcal proteins sequences were obtained from the NCBI (National Library of Medicine, USA) Entrez-Protein Database accessed at http://www.ncbi.nlm.nih.gov/protein. Percentage identity of the amino acid sequences of *S. pneumoniae* proteins from strains D39 and TIGR4 were assessed using the NCBI Basic Local Alignment Search Tool (BLAST) accessed at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

2.9 STATISTICS

Non-parametric data are presented as median with interquartile range and compared by Mann-Whitney U-test or Kruskal-Wallis test. Parametric data are presented as either mean ± standard deviation (SD) to represent the spread of values from a group of mice, or mean ± standard error of the mean (SEM) to represent the spread of data from *in vitro* replicates. Parametric data was compared by Student t-test or analysis of variance (ANOVA) with post-hoc tests. Survival data is presented as Kaplan-Meier survival curves and compared by log rank test. In general, P values <0.05 were considered significant. Statistical tests used are stated in text or in figure legends.

3 D39 WT and MUTANT COLONISATION-INDUCED PROTECTION IN CD1 MICE

3.1 INTRODUCTION

Limited data suggests that colonisation with live attenuated mutants lacking either capsule (Roche), Pspa/Ply (Roche) or Ply alone (Richards) can protect against lethal disease in mice. The mechanisms of protection remain unclear. Furthermore, the protection that follows WT colonisation, reflecting natural immunity in humans, has not been explored.

In this chapter, the effects of nasopharyngeal colonisation of CD1 mice with WT and mutant strains of D39 and TIGR4 *S. pneumoniae* were examined as follows. Models of colonisation with D39 and TIGR4 WT were established. The time course of D39 colonisation was defined, and the immunogenicity and protection induced by colonisation was studied. The time course, immunogenicity and protection following colonisation of the mutant strains D39-DΔ, D39Δ*lgt* and D39Δ*pab* was then compared to that of the parent WT strain. Finally, the ability of D39 colonisation to cross-protect against serotypes TIGR4 and ST3 was also tested.

These mutants were chosen as they have not been previously studied in such a system. All the mutations lead to attenuated virulence (unpublished data, see Introduction) and are potential candidates for inclusion in a live attenuated nasal vaccine. Perhaps most importantly, comparison of each mutant with the WT enabled a set of specific questions to be asked regarding the induction of protective responses during colonisation.

Limited data suggests that despite more rapid clearance from the nasopharynx, colonisation with unencapsulated strains of *S. pneumoniae* can still protect against subsequent pneumonia (Roche et al., 2007). Colonisation with unencapsulated strains of *S. pneumoniae* may in fact be more immunogenic than with WT, since lack of capsule can lead to enhanced pro-inflammatory responses (Hyams et al., 2010a).

Direct comparison of D39 WT with D39-DΔ explored how the balance between the inflammatory responses and rapid clearance influence immunogenicity and protection against pneumonia.

D39Δ*lgt* lacks surface lipoproteins (see Introduction) which may affect responses to colonisation in several ways. Lipoproteins have important nutrient transporter functions (e.g. PiaA and PiuA (Brown et al., 2001a, Basavanna et al., 2009), LivJ (Basavanna et al., 2009)). Poor *in vivo* growth may therefore limit the ability of Δ*lgt* to establish colonisation. Furthermore, several lipoproteins specifically mediate adherence to the nasopharynx (e.g. PsaA (Tong et al., 2002), PpmA (Cron et al., 2009), SlrA (Hermans et al., 2006) and the Ami-AliA/AliB complex (Kerr et al., 2004)). If density and duration of colonisation are important determinants of immunogenicity, these factors could affect the ability of Δ*lgt* to induce protective immunity. Colonisation can also induce serum antibodies to certain lipoproteins, (e.g. PpmA and SlrA (Adrian et al., 2004) and PiuA and PiaA (Whalan et al., 2005)). Thus, loss of these antigens from Δ*lgt* could impact on protective immunogenicity in a more specific way. Finally, lipoproteins are important TLR2 agonists (Travassos et al., 2004). Th17 responses to colonisation that protect against re-colonisation are induced in a TLR2-dependent manner (Zhang et al., 2009), and antibody responses to *S. pneumoniae* antigens are affected by TLR2 signalling *in vitro*. The role of TLR2 signaling in the humoral response to colonisation has not been reported. Inclusion of a Δ*lgt* mutation in this study allowed the importance of these interactions to be investigated.

To investigate the role of duration of colonization in inducing protective immune responses, the auxotrophic D39Δ*pab* was used. It does not grow *in vivo* without PABA supplementation. It was anticipated that it would be rapidly cleared from the nasopharynx. This avoided the possible inflammatory response to active bacterial

killing associated with antibiotic use to terminate colonization. By controlling for rapid clearance, data generated using this mutant strain may aid in the interpretation of the data generated by the other mutants.

In this chapter, we addressed the following specific questions:

- Is WT colonisation immunogenic?
- Is WT colonisation protective against lethal pneumonia?
- Does the loss of capsule, surface lipoprotein or the ability to replicate affect the ability of these mutant strains to:
    - colonise the nasopharynx?
    - induce immune responses against bacterial antigens?
    - protect against lethal pneumonia?
- Is the protection induced through colonization serotype-specific?

3.2 RESULTS

3.2.1 Establishing a nasopharyngeal colonisation model

3.2.1.1 Identification of *S. pneumoniae* recovered from nasopharyngeal wash

To determine the inoculum of the D39 *S. pneumoniae* strain required to establish nasopharyngeal colonisation in CD1 mice, groups of mice were inoculated i.n. with bacteria in a volume of 10µl PBS. This volume was delivered as a single drop to the nares of a mouse held vertically without anaesthesia. Blood agar plates containing gentamicin were used to exclude contaminants but this did not prevent growth of viridans streptococci recovered on occasion. Sensitivity to optochin, which selectively inhibits growth of *S. pneumoniae*, was therefore used to confirm that recovered bacteria were *S. pneumoniae*.

3.2.1.2 Establishing inoculum sufficient to colonise nasopharynx

Mice received light halothane prior to inoculation as this allowed more consistent application of inocula. Pilot experiments explored whether introducing a target inoculum of 0.5-1 x 10^6 CFU was sufficient to colonise the nasopharynx of CD1 mice. There was inconsistent recovery of pneumococci from the nasopharynx of mice during the seven days following colonisation, with no bacteria recovered from 50% of mice at several timepoints (Figure 3.1A).

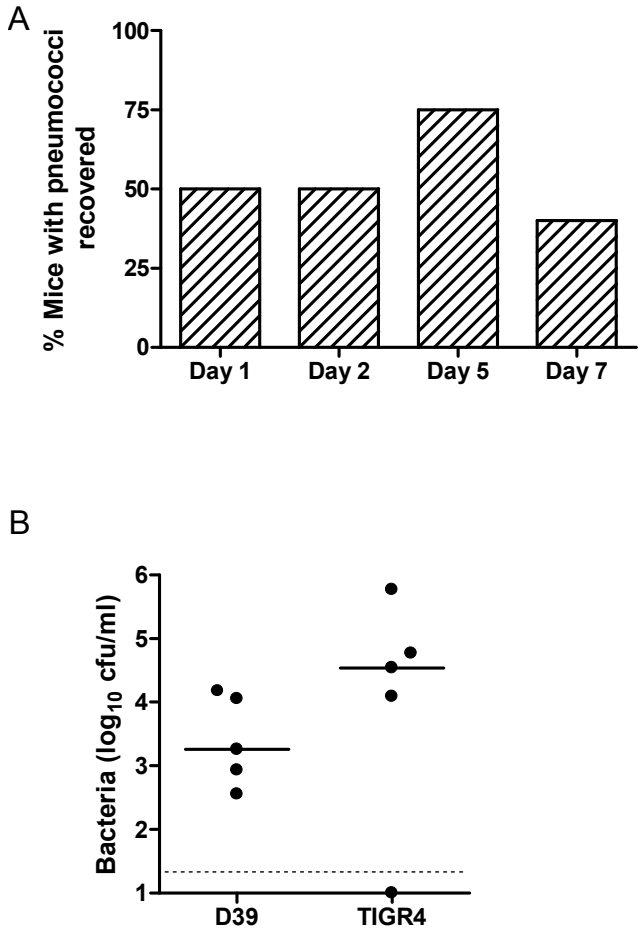

Figure 3.1. Optimisation of colonising inoculum in CD1 mice.

(A) Groups of mice (n=4) were nasally inoculated with 0.5-1x10^6CFU *S. pneumoniae* D39. Nasopharygeal wash was collected from culled mice at fixed timepoints for bacterial culture. (B) Bacterial load in day 2 nasopharyngeal wash of mice inoculated with 0.4x10^7 CFU of D39 or TIGR4. Dotted line indicates limit of detection.

Using anaesthesia and a higher inoculum of 0.4 x 10^7 CFU, pneumococci were recovered from all mice inoculated with *S. pneumoniae* D39, and 80% of mice inoculated with *S. pneumoniae* TIGR4 (Figure 3.1B) on day 2 post-colonisation. Despite introducing a similar number of bacteria (0.5 x 10^7 CFU), approximately 10-fold more TIGR4 bacteria were recovered than D39, suggesting that this strain colonises the nasopharynx at a higher density. No bacteria were recovered from the BALF, lungs or blood of mice and all animals remained well despite this high nasopharyngeal load.

3.2.2 Time course of nasopharyngeal colonisation

3.2.2.1 Time course of colonisation with wild-type D39

Further groups of CD1 mice (n=4 to 10) were colonised with *S. pneumoniae* D39 using an inoculum of 1.0 x 10^7 CFU. They were culled at fixed timepoints for bacterial culture of nasopharyngeal wash to determine the stability and duration of colonisation with the wild-type strain (Figure 3.2 A). There was a large initial reduction in numbers of CFU recovered from a median of 1.74 x 10^4 CFU/ml (interquartile range (IQR) 1.1–4.7) on day 1 to 4.00 x 10^3 CFU/ml (1.20–11.2) on day 2. Similar numbers of bacteria were recovered on day 5, suggesting stable colonisation from day 2. There was a further reduction by day 10 to a median of 5.05 x 10^2 CFU/ml (1.70–9.59), and by day 17, bacteria were no longer recovered.

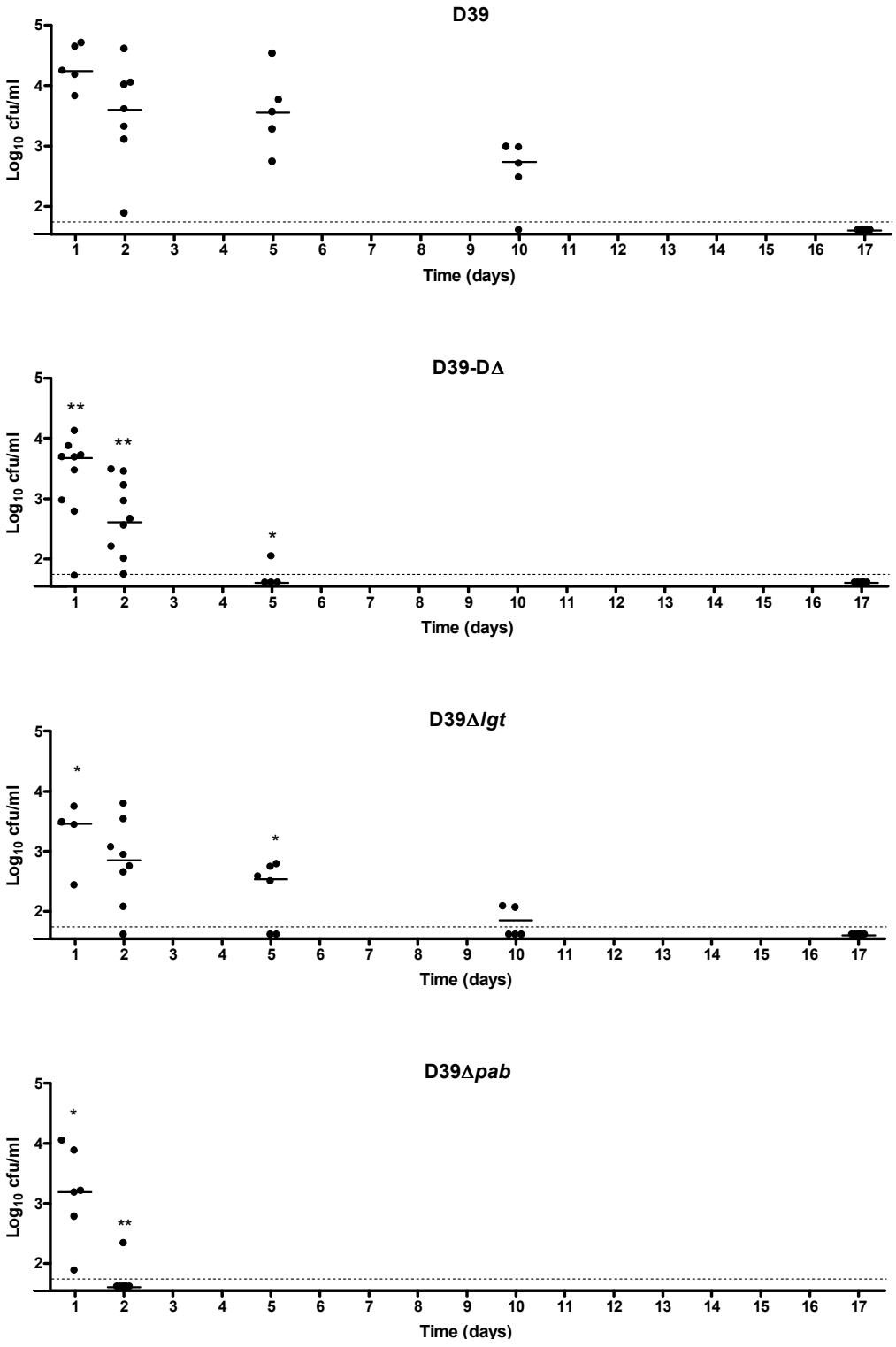

Figure 3.2. Time course of *S.pneumoniae* D39 WT and mutant nasopharyngeal colonisation in CD1 mice.

Groups of mice (n=4-10) were colonised with 1 x 10^7 CFU D39 WT, D39-DΔ, D39Δ*lgt* or D39Δ*pab* , and culled at fixed timepoints for culture of nasopharyngeal wash. Dots represent individual mice, bars indicate group median. Comparison to D39WT at each timepoint by Mann-Whitney U-test (*P<0.05, **P<0.01).

3.2.2.2 Time course of colonisation with D39-DΔ, Δ*lgt* and Δ*pab* mutants

To assess the ability of the mutant strains to colonise the nasopharynx, groups of CD1 mice (n=4-9) were inoculated i.n. with 1 x 10^7 CFU of either D39-DΔ, D39Δ*lgt* or D39Δ*pab* suspended in 10µl PBS. Mice were culled at fixed timepoints for assessment of nasopharyngeal colonisation density as shown in Figure 3.2 (B-D). D39-DΔ was recovered from the nasopharynx from 9/10 mice on the second day following colonisation. The median colonisation density was 4.77 x 10^3 CFU/ml (IQR 0.76–6.27), significantly less than that of D39 WT (*P*=0.004). Colonisation density fell to 4.02 x 10^2 CFU/ml by day 2 (IQR 0.77–22.0). Unlike the D39WT colonised mice, all of which remained colonised, only 1 of 5 mice colonised with D39-DΔ remained colonised on day 5.

By day 1, D39Δ*lgt* was recovered from all inoculated mice, with median colonisation density 2.85 x 10^3 (IQR 1.49–4.20), significantly less than D39WT (*P*=0.016). 2 of 6 mice had cleared colonisation by day 5, by which time the median density of colonisation had fallen to 3.43 x 10^2 (IQR 0.30–5.72). Only 2 of 5 mice remained colonised at day 10, and all had cleared colonisation by day 17.

D39Δ*pab* was recovered from all mice on the first day following colonisation with median 1.54 x 10^3 CFU/ml (IQR 0.33–9.22). In contrast to D39WT and the other mutants studied, D39*Δpab* was recovered from only 1 of 7 inoculated mice by day 2, at a low density of only 2.14 x 10^2 CFU/ml.

Thus by one day following inoculation, all three mutants were recovered from the nasopharynx at significantly lower colonisation densities than for D39WT. In the majority of mice, all three mutants were more rapidly cleared than D39WT. The auxotrophic strain D39Δ*pab* was least able to establish nasopharyngeal colonisation.

3.2.2.3 Effect of lack of capsule and Δpab mutation on TIGR4 colonisation density

To confirm that these patterns were not specific to the strain background of the mutants, the colonisation density of genetic mutants of *S. pneumoniae* TIGR4 were compared with their WT parent strain on day 2 following colonisation (n=4-5 per group). Inoculation with TIGR4 WT led to recovery from all mice on day two of a median of 4.68 x 10^4 CFU/ml (2.36– 32.3), as shown in Figure 3.3. However, when inoculated with either TIGR4Δ*cps* or TIGR4Δ*pab*, no bacteria were recovered (*P*=0.0169 compared to wild-type for both mutant strains). This suggested that the faster clearance of *S. pneumoniae* acapsular and Δ*pab* mutants was not specific to the strain background. Unlike D39-DΔ, TIGR4Δ*cps* was entirely cleared by two days post-colonisation, despite greater numbers of WT TIGR4 than D39 at this time. This would suggest that capsule exerts stronger effect in supporting colonisation in the TIGR4 strain than it does in D39.

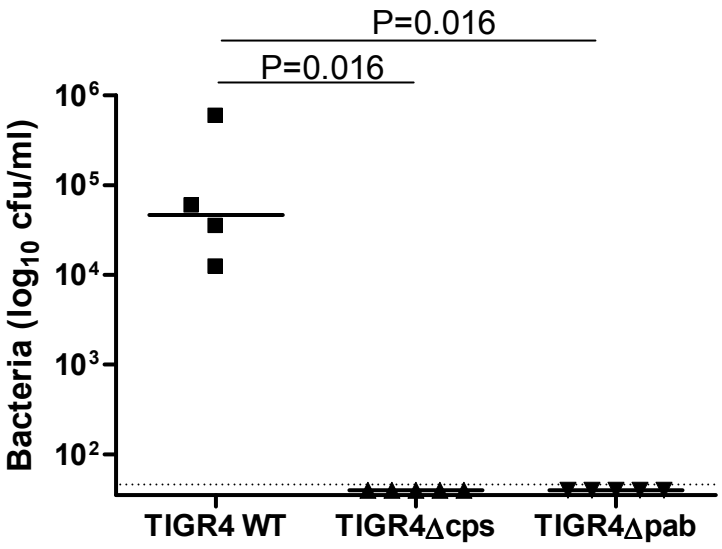

Figure 3.3. Recovery of TIGR4 strains from nasopharynx of CD1 mice.

Median and IQR of bacterial load in nasal wash collected two days following nasal inoculation with 0.5 – 1 x 10^7 CFU of either TIGR4 WT, TIGR4Δ*cps* (acapsular mutant) or TIGR4Δ*pab* (n=4 per group). Dotted line, limit of detection. Group medians compared by Mann-Whitney U-test.

3.2.3 Immunogenicity of nasopharyngeal colonisation

Having demonstrated that the mutant strains of D39 were cleared more rapidly from the nasopharynx than the parent WT strain, the next questions to be addressed were whether colonisation was immunogenic and whether it was protective against pneumonia. For these experiments, groups of 20 mice were colonised with *S. pneumoniae* strains as described below. Serum was collected from 10 mice per group for immunogenicity studies. Mice were subsequently challenged as described in Section 3.2.4.

3.2.3.1 Serum anti-whole cell IgG responses to colonisation with D39 strains

To assess the immunogenicity and protective efficacy of nasopharyngeal colonisation, further groups of CD1 mice (n=20) were colonised with either D39WT (1.23×10^7 CFU), D39-DΔ (0.92×10^7 CFU), D39Δ*lgt* (1.56×10^7 CFU) or D39Δ*pab* (1.69×10^7 CFU). A further group of control mice received PBS without bacteria. 10 mice per group were bled 28 days following colonisation and serum IgG specific to D39WT antigens was measured using a whole bacterial cell ELISA. To increase the likelihood that all mice became colonised, and to investigate whether repeat exposure boosts antibody responses, a further group of mice (n=10) received two 'colonising' inoculations 14 days apart. Serum was collected prior to each inoculation, and then 28 days following the second inoculation. The mice were age matched such that the second dose in the two-dose groups corresponded to the single colonising dose in the single-dose groups. All mice were subsequently challenged as described in Section 3.2.4.2 below.

70% of mice colonised with D39 WT had an IgG titre greater than the range found in control sera, with an overall median of 1/2640 (IQR 1/10 – 1/5730, P=0.052 compared to controls [Mann-Whitney U-test]) (Figure 3.4A). Fewer mice developed

a serum anti-D39 IgG response to colonisation with the mutants which were not significantly different to controls. Only 22% of mice seroconverted following colonisation with D39-DΔ with a median titre of 1/36 (IQR 1/10 – 1/321). 30% of mice seroconverted following colonisation with D39Δ*lgt*, with a median titre of 1/114 (IQR 1/10 – 1460). 30% of mice seroconverted following colonisation with D39Δ*pab*, with a median titre of 1/21 (IQR 1/10 – 1/656). In the group of mice scheduled to receive 2 colonising doses of D39 WT, all mice seroconverted 14 days following the first dose (Figure 3.4B), with a median titre of 1/1340 (IQR 1/810 – 1/1510). This was increased to 1/3960 (IQR 1/2870 – 1/14,000) by a second dose ($P<0.0001$ compared to PBS controls). Thus, repeated exposure boosted whole cell antibody responses.

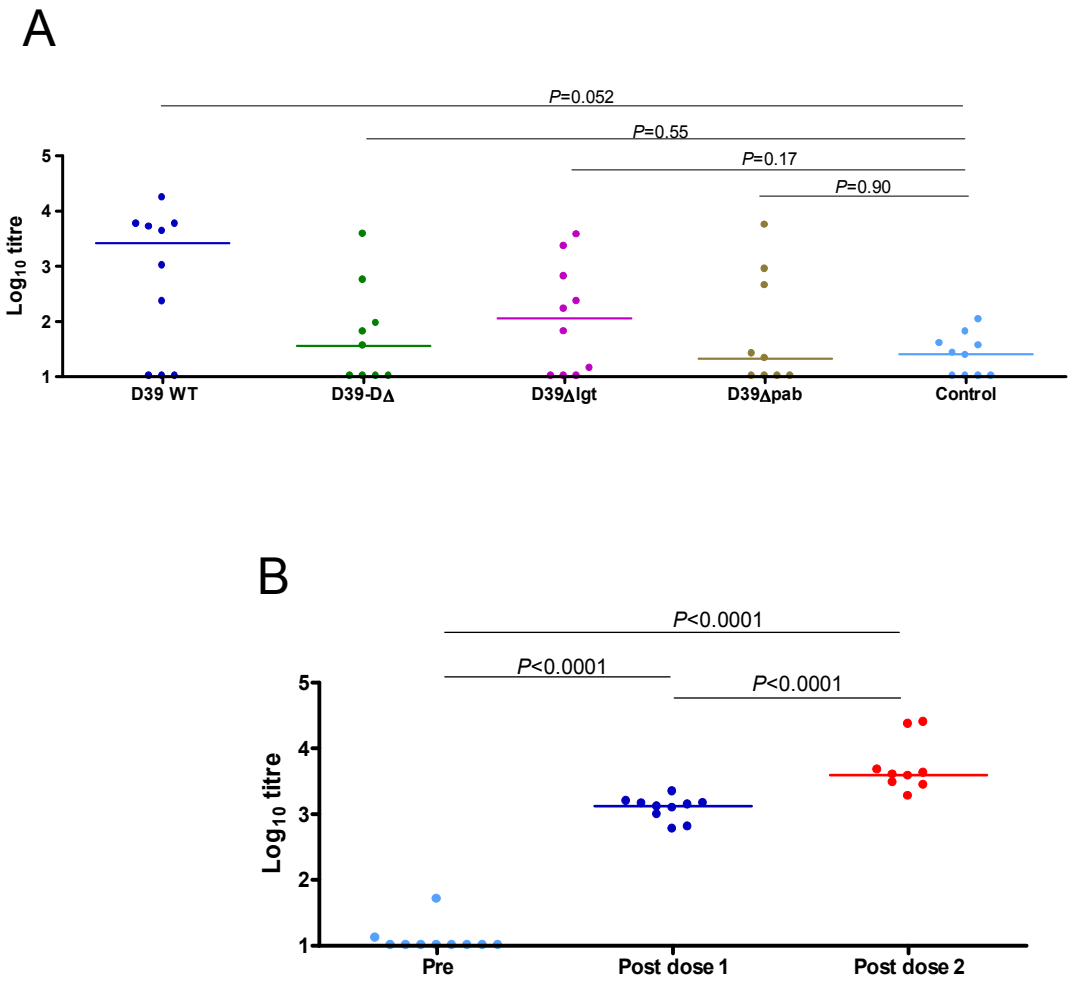

Figure 3.4. Serum anti-D39 IgG following colonisation with D39 strains in CD1 mice.

(A) Serum anti-D39 IgG titres 28 days following colonisation with D39 WT, or mutants or controls (PBS) measured by whole cell ELISA. (B) Anti-D39 serum IgG titre prior to or following each of two nasal colonisation inocula given 14 days apart. Dots represent titre of individual mice, bars represent median of group. Group medians compared by Mann-Whitney U-test.

3.2.3.2 Serum anti-capsular polysaccharide IgG responses to colonisation with D39 strains

In is not clear whether nasopharyngeal colonisation with *S. pneumoniae* is sufficient to induce a systemic IgG response against CPS. Serum IgG responses to type 2 capsular polysaccharide (PPS2) were therefore measured by ELISA in the sera of mice colonised with either D39 WT or mutants (Figure 3.5A). Only one of ten mice colonised with D39 WT developed an anti-capsule response, and this was of low level (OD_{405} 0.5 at 1:20 dilution). No mice colonised with the mutants studied developed anti-capsule IgG. It appeared therefore that the antigens in the whole cell ELISA that were recognised by the positive sera were not capsular polysaccharide.

In the absence of a positive control serum for PPS2, to further confirm that the reactive antigens in the whole cell ELISA were not PPS2, four sera of mice colonised with D39 WT with high anti-D39 whole cell titres were tested by competitive inhibition ELISA. In this assay, binding of serum IgG to solid-phase antigens was in competition with increasing concentrations of soluble PPS2 (Figure 3.5B). Even at 100 μg/ml, soluble PPS2 was unable to inhibit binding, evidence that this antigen was not binding IgG in these positive samples.

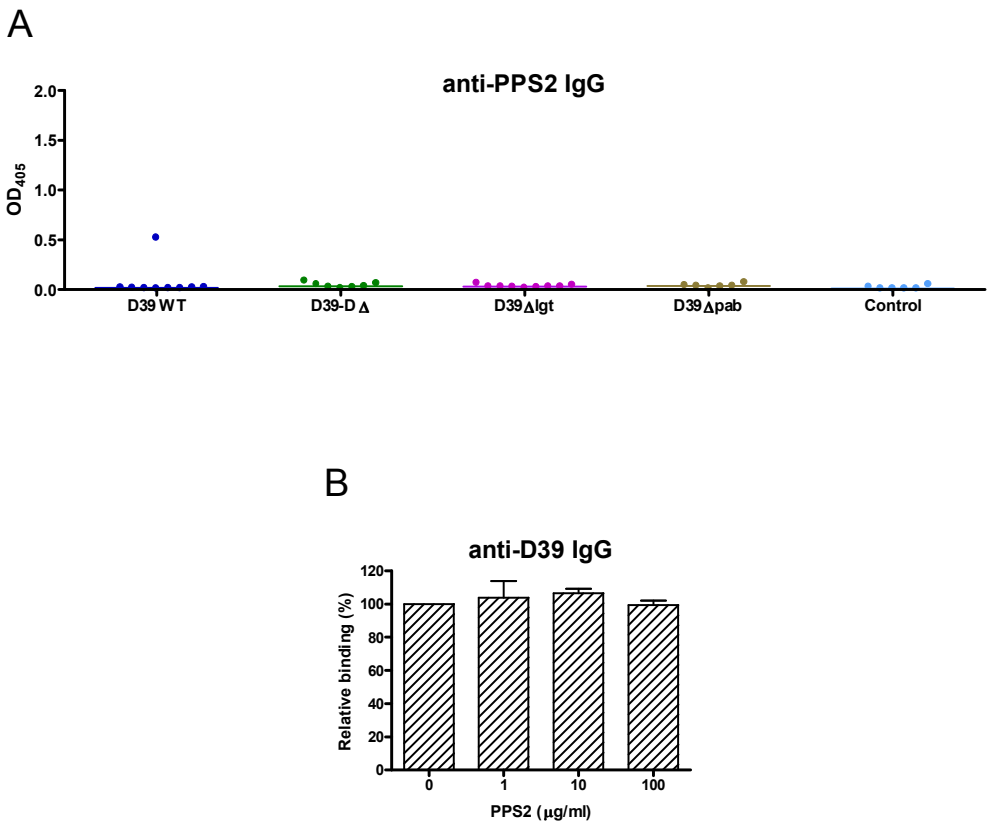

Figure 3.5. Serum anti-capsular IgG following colonisation with D39 strains in CD1 mice.

(A) Serum IgG against type 2 capsular polysaccharide (PPS2) 28 days following colonisation with D39 WT, or mutants or controls (PBS) measured by ELISA. Dots represent OD_{405} at 1:20 serum dilution for individual mice, bars represent median of group. (B) Competitive inhibition ELISA. Effect of increasing concentration of soluble PPS2 on binding of IgG from sera of mice colonised with D39 to D39 coated-ELISA plates (Mean +/- SD, 100% represents binding in absence of inhibitor, n=4).

3.2.3.3 Serum IgG responses to colonisation with TIGR4

To investigate whether the serum response to whole cell antigens was strain specific and whether the lack of PPS2 specific antibody was serotype specific, a further group of mice (n=20) were colonised with 0.52 x 10^7 CFU *S. pneumoniae* TIGR4. 10 mice were bled on day 28 for serum IgG against whole cell TIGR4. All mice were subsequently challenged as described in Section 3.2.4.5 below. Titres up to 1/400 were detected in sera from naïve control mice (Figure 3.6A). 60% of mice colonised with TIGR4 WT developed an IgG titre outside this control range, with a median titre of 1/1080 (IQR 1/129 – 1/1430) versus a median for controls of 1/87 (IQR 1/13 – 1/387, *P*=0.029). TIGR4 colonisation also induced cross-reactive IgG against D39 antigens with a titre of 1/1760.

Anti-type 4 capsular polysaccharide (PPS4) IgG was also measured in the serum of mice colonised with TIGR4 WT (Figure 3.6B). In this assay, pooled serum from mice that had received systemic immunisation with the 7-valent pneumococcal conjugate vaccine Prevenar™ was used as a positive control. These positive control mice received 2 intraperitoneal injections of 100 µl (=0.2 x standard human dose) vaccine 14 days apart and were bled 28 days following the second vaccination. Whilst systemic immunisation with protein conjugated CPS induced systemic anti-PPS4 IgG (titre = 1/29,000), colonisation with TIGR4, an encapsulated strain of this serotype, did not, similar to colonisation with serotype 2 strain D39.

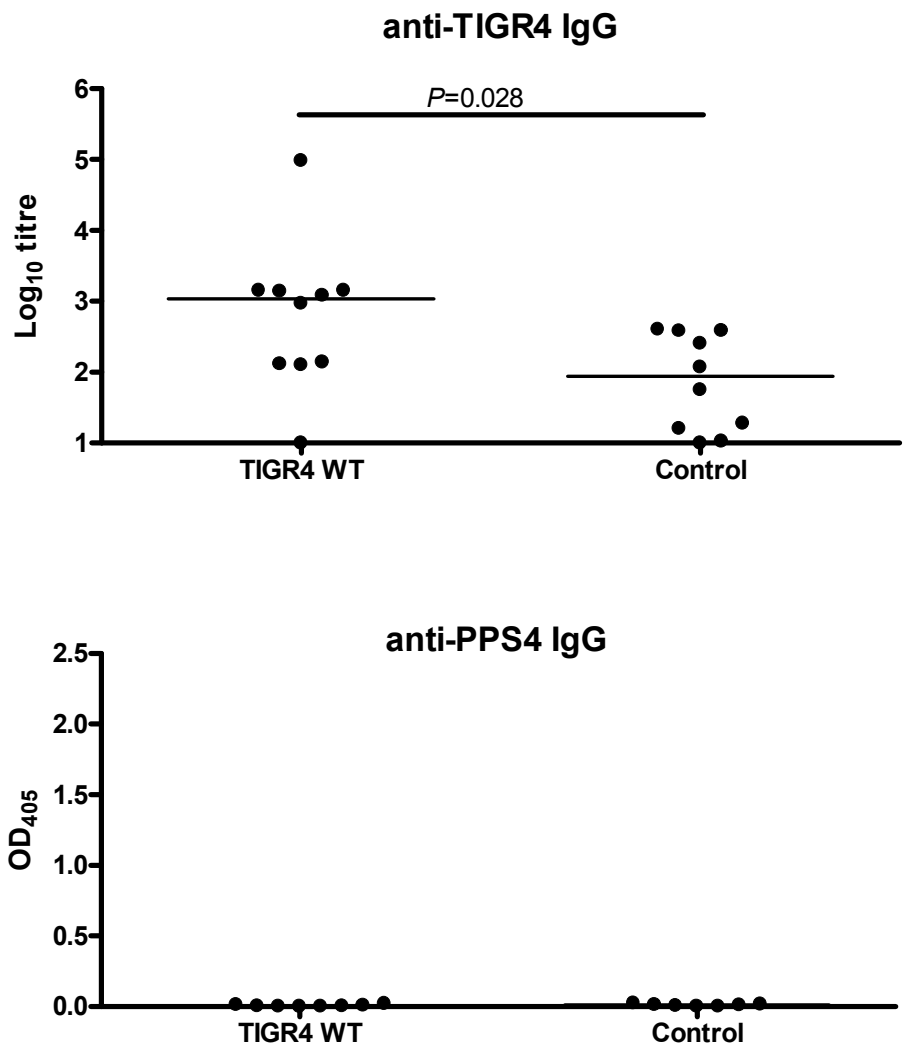

Figure 3.6. Immunogenicity of colonisation with TIGR4 WT in CD1 mice.

Serum IgG anti-TIGR4 WT whole cell (A, as titre) and anti-PPS4 (B, as OD405 at serum 1:20 dilution) on day 28 following colonisation with TIGR4 WT or controls (PBS) measured by ELISA. Dots represent individual results, bars represent median of group. Medians compared by Mann-Whitney U-test.

3.2.4 Protection against lethal pneumonia

3.2.4.1 Pilot studies of protection against D39 pneumonia

Pilot experiments were performed to assess the potential magnitude of the protective effect of colonisation with WT and mutant strains of *S. pneumoniae* against lethal D39 pneumonia. In the first pilot, groups of 6-week old CD1 mice (n=10 per group) were colonised with 0.1 x 10^7 CFU of D39 WT, D39Δ*lgt* or D39 –DΔ. Controls received PBS. After 28 days, all mice were challenged with 0.75 x 10^7 CFU D39 i.n., which led to 60% survival in the control group (Figure 3.7). There was a trend towards greater survival in mice that had been colonised with D39 WT (90%, *P*=0.14), but no suggestion of protection with either D39Δ*lgt* or D39 –DΔ. This pilot experiment indicated that a higher challenge inoculum was required to ensure the development of lethal infection in the majority of control mice to power the study for testing of protection.

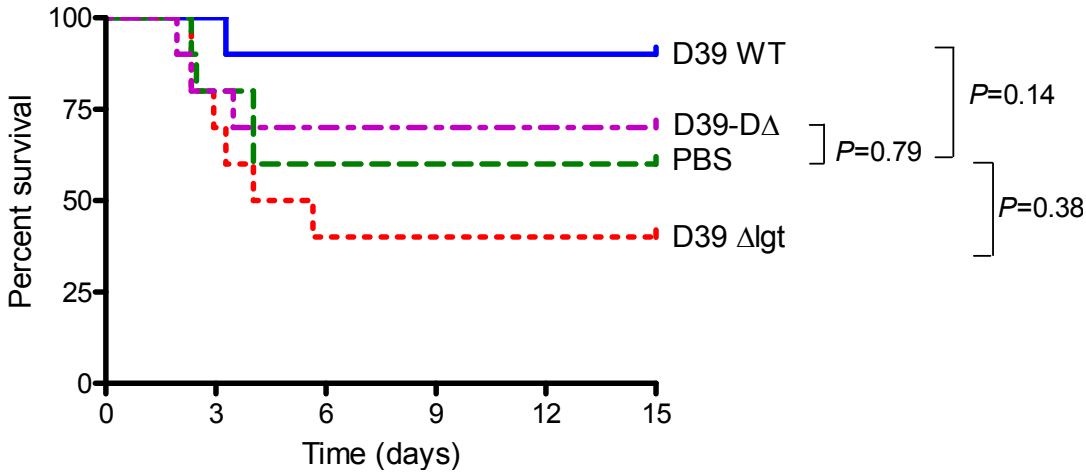

Figure 3.7. Protective efficacy of colonisation-induced protection against D39 pneumonia in CD1 mice (Pilot 1).

Kaplan-Meier plots showing survival of mice previously colonised with 1 x 10^6CFU D39 WT, D39-DΔ, D39Δlgt or PBS (controls) and challenged 28 days later with 0.75 x 10^7 CFU D39 WT (n=10 per group, comparisons to control group by log rank test).

Colonisation with both D39 and TIGR4 induced serum anti-D39 IgG. A second pilot experiment was designed to estimate the protective effects of colonisation with these strains against D39 pneumonia. Further groups of mice (n=10 per group) were colonised with wild-type strains of D39 and TIGR4 at 0.3 x 10^7 CFU and challenged after 28 days with 1.5 x 10^7 CFU D39. The median anti-D39 serum IgG titre at the time of challenge was 1/2560 (IQR 1/1500 – 1/3320) in mice colonised with D39, and was 1/3770 (IQR 1/2070 – 1/5200) in mice colonised with TIGR4. Again, there was a trend towards a protective effective in mice colonised with D39 (50% survival versus 30% in controls, P=0.14) (Figure 3.8). No protection was observed in mice that had been colonised with TIGR4 (20% survival).

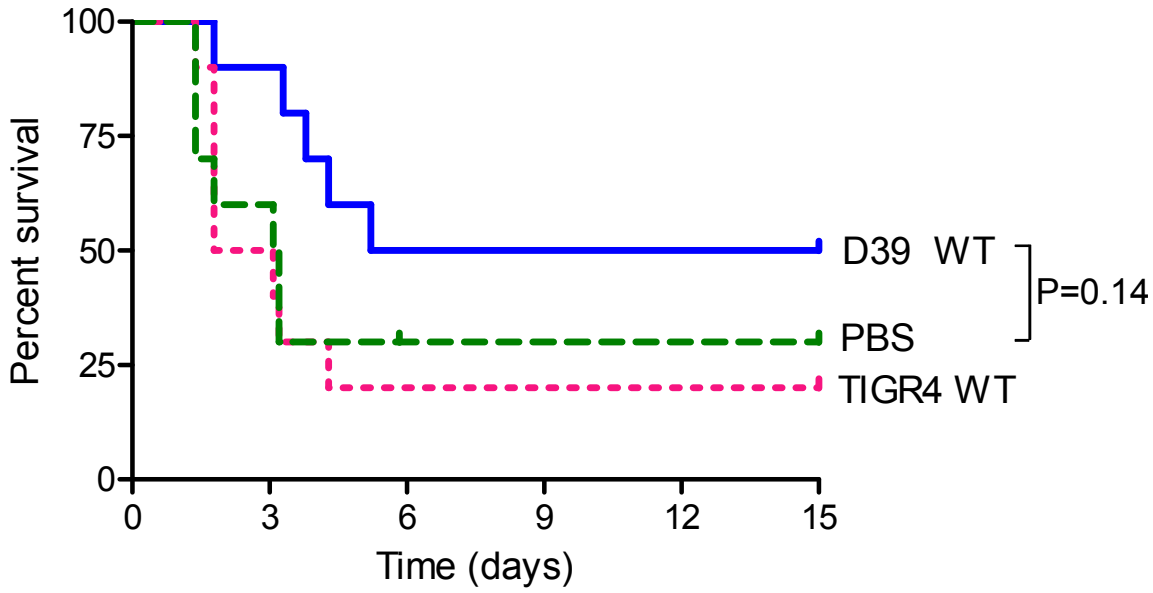

Figure 3.8. Protective efficacy of colonisation-induced protection against D39 pneumonia in CD1 mice (Pilot 2).

Kaplan-Meier plots showing survival of mice previously colonised with 0.3 x 10^7CFU D39 WT, TIGR4 WT or PBS (controls) and challenged 28 days later with 1.5 x 10^7 CFU D39 WT (n=10 per group, comparisons to control group by log rank test).

Thus, a challenge inoculum of 1.5 x 10^7 CFU D39 was appropriate to test protection in CD1 mice. However, if the protective effect of a single colonising dose of D39 was real, the effect was relatively modest, and larger groups of mice would be required to test this. A power calculation indicated that a group size of 20 mice would be required. This pilot study also suggested that TIGR4 colonisation was unlikely to protect against D39 challenge, and this element of cross-protection was not pursued further.

3.2.4.2 Effect of colonisation with D39 strains on lethal D39 pneumonia

Based on the data obtained from pilot experiments, a protection experiment was performed to test the ability of colonisation with WT or mutant strains of D39 to protect against lethal D39 pneumonia. Groups of mice (n=20) that had been colonised with either D39WT, D39-DΔ, D39Δ*lgt* or D39Δ*pab*, were challenged 28 days post-colonisation with 1.6 x 10^7 CFU *S. pneumoniae* D39 WT i.n. A further group of mice that had received two colonising inocula of D39 WT, 14 days apart, were challenged 28 days following the second colonising dose. The timing was such that the second colonising dose and challenge were at the same time as for the single dose groups. The inocula and immune responses elicited by colonisation of these mice have already been described in Section 3.2.3.1 above.

Only those groups that had been colonised with D39 WT were significantly protected against pneumonia (Figure 3.9). 40% of D39 WT colonised mice survived compared to 15% of controls (P=0.03). Survival was greater still (55%, P=0.001) in those mice who received two colonising doses. None of the three D39 mutant strains tested elicited significant protection against challenge.

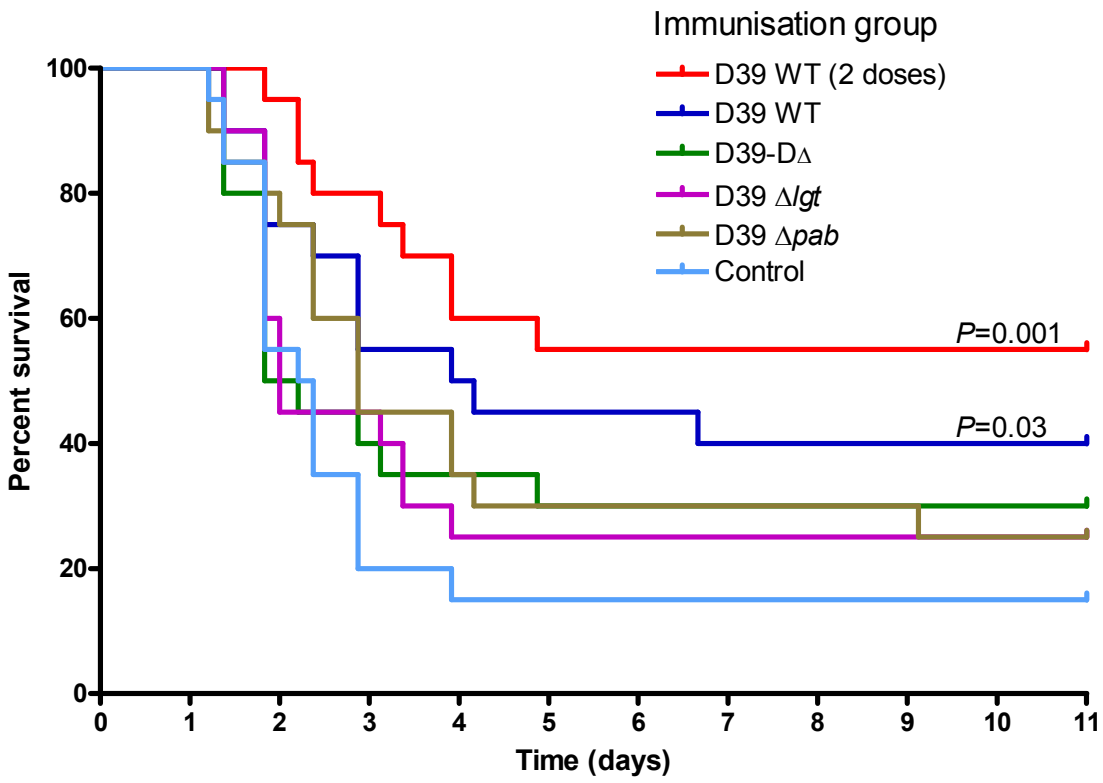

Figure 3.9. Protective efficacy of previous colonisation with D39 strains on subsequent lethal D39 WT pneumonia in CD1 mice.

Kaplan-Meier plots showing survival of mice previously colonised with 1 or 2 doses of D39 WT, or 1 dose of D39-DΔ, D39Δlgt, D39Δpab or PBS (controls) and challenged 28 days later with 1.6 x 10^7 CFU D39 WT (n=20 per group, comparisons to control group by log rank test).

3.2.4.3 Correlation between serum anti-D39 IgG responses to D39 colonisation and protection against D39 pneumonia

To explore the relationship between immunogenicity and protection, the correlation between immunogenicity (as geomean titre of anti-D39 whole cell IgG for the group) and protection (as percentage survival of the group) for the different colonisation regimes was examined (Figure 3.10). Protection was found to correlate very closely with immunogenicity on this basis (P=0.001, r^2=0.94). Although this does not imply that serum IgG is the mechanism of protection, it suggests that this assay of immunogenicity is likely to be a predictive correlate of protection for the testing of other colonisation regimens that elicit protection through similar mechanisms.

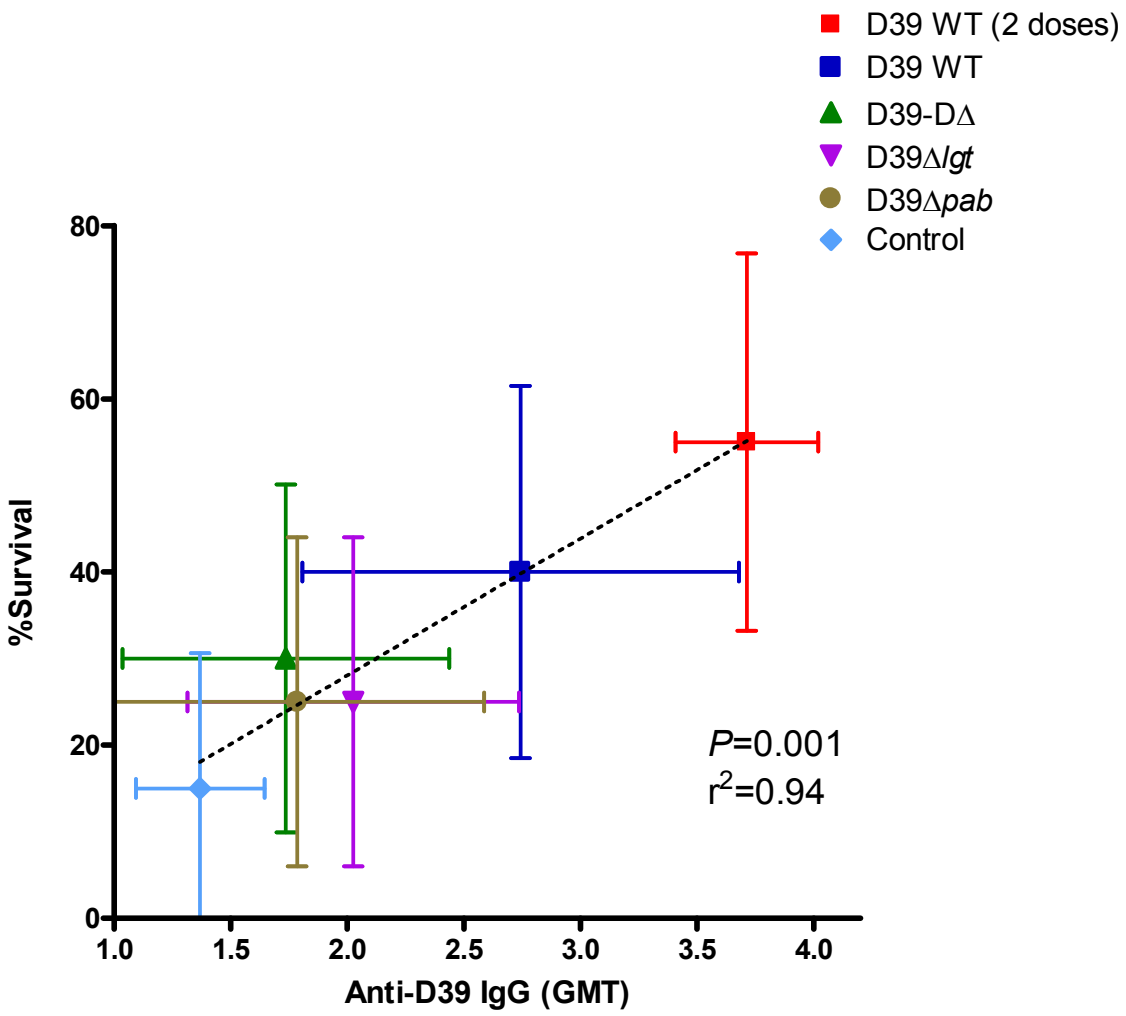

Figure 3.10. Immunogenicity of colonisation with D39 strains correlates with their protective efficacy against D39 pneumonia.

Groups of CD1 mice (n=20) were colonised with either D39WT, D39-DΔ, D39Δ*lgt* or D39Δ*pab*. Coloured dots and bars represent immunogenicity as geomean anti-D39WT whole cell ELISA titre of the group (+/- 95% CI) versus percentage survival (+/- 95% CI) of the group against day 28 D39WT pneumonia challenge. Dotted line shows correlation between immunogenicity and protection by linear regression.

3.2.4.4 Effect of D39 pneumonia on serum anti-D39 IgG

Exposure to antigens during the process of infection has a variable effect on induction of antibody responses. To explore whether *S. pneumoniae* D39 pneumonia is an immunogenic event in CD1 mice, the anti-D39 whole cell titres of serum pre- and 28 days post-infection were compared in surviving naïve control and D39WT colonised mice (Figure 3.11). There was an 5-fold increase in the serum anti-D39 IgG titre following infection in previously colonised mice from a median titre of 1/4,270 (IQR 1/618 – 1/11,200) to 1/19,500 (IGR 1/8,900 – 1/37,600, P=0.03). Although there were only three surviving control mice, all three developed a serum anti-D39 IgG response to infection and, the median titre post-infection of control mice was of a similar order to that achieved by colonisation without pneumonia challenge (Figure 3.4).

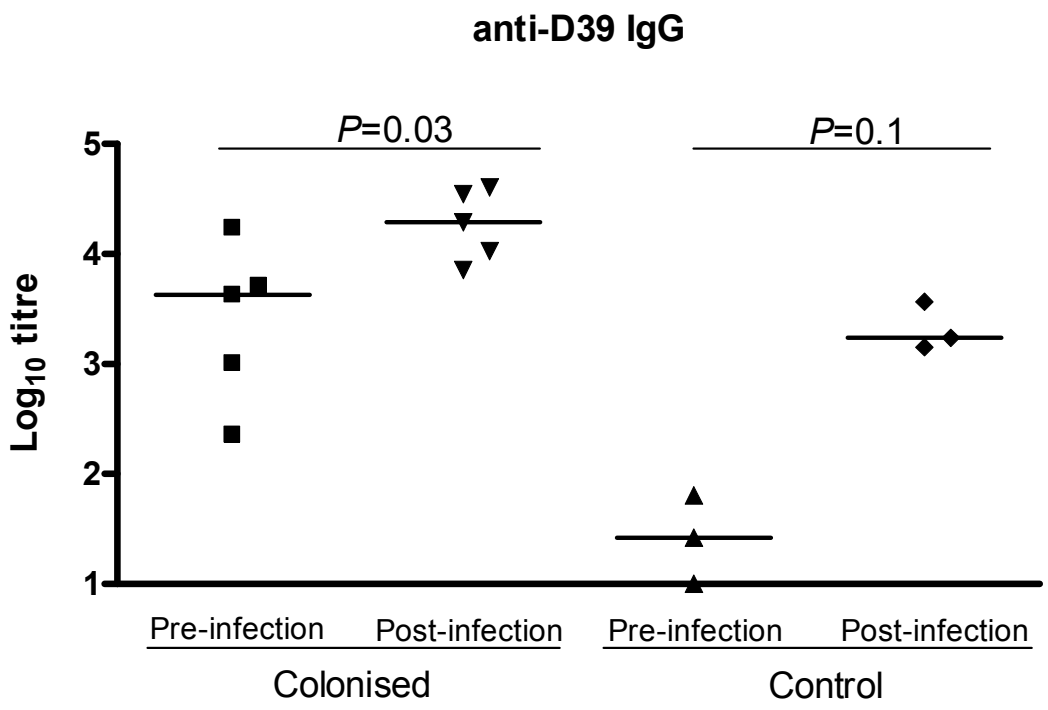

Figure 3.11. Effect of D39 pneumonia on anti-D39 serum IgG titres in previously colonised or control CD1 mice surviving D39 pneumonia.

Mice previously colonised with D39WT or controls were challenged with D39WT pneumonia on day 28. Serum was collected pre-infection and on day 28 post-infection and survivor sera analysed by anti-D39 whole cell ELISA. Dots represent individual mice, bars represent median of group. Medians compared by Mann-Whitney U-test.

3.2.4.5 Effect of TIGR4 colonisation on lethal TIGR4 pneumonia

To investigate whether colonisation with TIGR4 WT was able to induce protective immunity against subsequent TIGR4 WT pneumonia challenge, groups (n=20) of TIGR4 WT colonised and control mice were challenged with 5 x 10^7 CFU TIGR4 WT i.n (Figure 3.12) at 28 days post-colonisation. There was a trend towards protection with 20% of TIGR4 colonised mice surviving compared to 0% of controls (*P*=0.3 by log rank test). It is possible that significant protection was not observed in this experiment because the challenge inoculum was excessive, as reflected in 0% survival of controls.

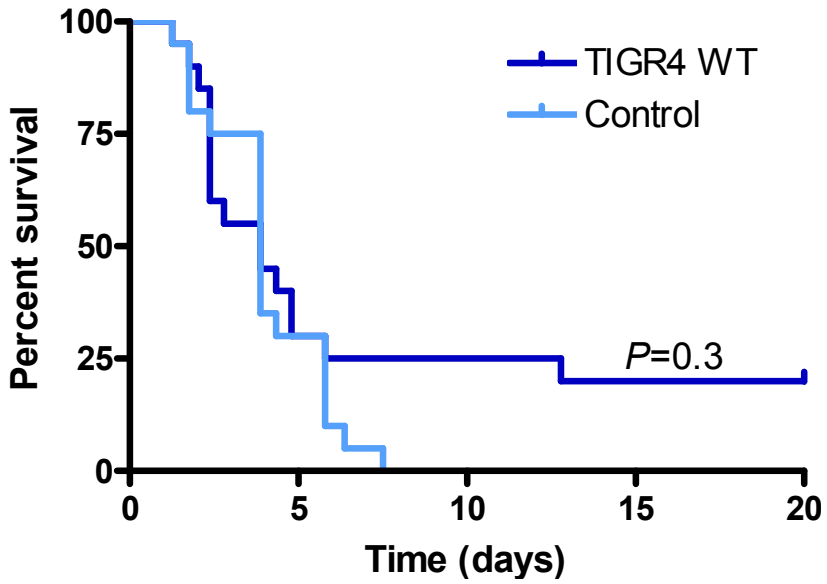

Figure 3.12. Protective efficacy of previous colonisation with TIGR4 WT on subsequent lethal TIGR4 WT pneumonia in CD1 mice.

Kaplan-Meier plots showing survival of mice previously colonised with 0.52 x 10^7 CFU TIGR4 WT and challenged 28 days later with 5 x 10^7 CFU TIGR4 WT (n=20 per group, comparisons to control group by log rank test).

3.2.5 Effect of duration of nasopharyngeal colonisation on immunogenicity and protection

3.2.5.1 Introduction

Both D39-DΔ, D39Δ*lgt* and D39Δ*pab* were all cleared from the nasopharynx more rapidly than D39WT, albeit at different rates (Figure 3.2). All were found to be less immunogenic than D39WT. Unlike D39WT, none protected against lethal pneumonia. This raises the possibility that the duration of exposure of the immune system to bacteria during nasopharyngeal carriage is a critical factor for the induction of protective immunity. The replicative capacity of an auxotroph can be switched on and off by the supplementation and withdrawal of a specific factor. It was hypothesised that the addition and withdrawal of PABA supplementation to mice colonised with D39Δ*pab* could be used as a tool to control the duration of colonisation, and thereby investigate the impact of duration of colonisation on immunogenicity and protection against pneumonia.

3.2.5.2 Effect of PABA supplementation on colonisation with D39Δpab

The inability of D39Δ*pab* to establish colonisation of the nasopharynx was demonstrated in Section 3.2.2.2 above. To assess the effect of PABA supplementation on D39Δ*pab* colonisation density, groups of CD1 mice were colonised with 0.8 x 10^7 CFU D39Δ*pab*. Drinking water was supplemented with PABA at 1 mg/ml for one day prior to colonisation. Fresh supplementation continued daily until 5 days following colonisation, at which point the supplementation was stopped. Mice were culled at fixed timepoints and colonisation density assessed by culture of nasopharyngeal wash (Figure 3.13). To assess whether PABA supplementation restored the ability of D39Δ*pab* to colonise, groups of mice (n=5-9

per group) were culled at 1, 2, and 5 days post-colonisation. To assess the effect of withdrawal of supplementation, a further group of mice were culled at day 7, two days after supplementation had ceased. This time was chosen as D39Δ*pab* had been cleared by two days in the majority of mice in the absence of supplementation as described above.

In the presence of PABA, D39Δ*pab* was recovered from all mice on day 1 and day 2 post-colonisation. Median colonisation density was 1.99 x 10^4 CFU/ml (IQR 0.41–5.28) on day 1 and 1.88 x 10^4 CFU/ml (IQR 0.51–5.14) on day 2. By day 5, 7 of 8 mice remained colonised at a high density with median 4.77 x 10^3 CFU/ml (IQR 1.84–6.25). The median bacterial CFU recovered following colonisation with D39Δ*pab* in the presence of PABA did not differ from D39 WT at any of these timepoints. However, on day 7, two days following cessation of supplementation, D39Δ*pab* was not recovered from any mouse.

To be certain that the effect of PABA was specific to strains containing the Δ*pab* mutation, the effect of PABA on colonisation with 0.54 x 10^7 CFU D39 WT was assessed. The median bacterial load after two days was 2.46 x 10^3 CFU/ml (IQR 1.8 –13.6), not significantly different to that previously obtained in the absence of PABA (*P*=1.00 by Mann-Whitney U-test, data not shown).

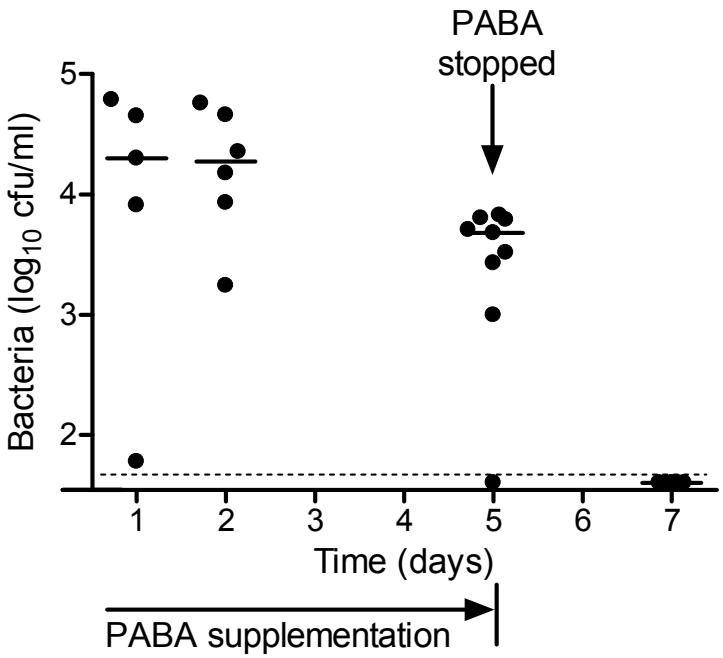

Figure 3.13. Effect of para-aminobenzoic acid (PABA) supplementation on colonisation density of D39WT and D39Δpab in CD1 mice.

Time course of bacterial load in nasopharyngeal wash following colonisation with D39Δ*pab* with PABA supplementation from day -1 to day +5. Dots represent individual mice, bars represent group median, dotted line represents limit of detection.

Thus, PABA supplementation restored the ability of D39Δ*pab* to colonise the nasopharynx with a similar density and time course to the wild-type D39 strain. This permits the technique to be used in future to explore in more detail how duration of colonisation affects immunogenicity of *S. pneumoniae*.

3.2.5.3 Effect of duration of PABA supplementation on immunogenicity

Using PABA supplementation as a tool to control duration of colonisation, immune responses to D39Δ*pab* colonisation were investigated to explore the relationship between colonisation duration and immunogenicity.

To test whether the duration of nasopharyngeal colonisation has an effect on immunogenicity, groups of CD1 mice (n=20) were colonised with D39Δ*pab* (0.55 x 10^7 CFU). One group (PABA+) received PABA supplementation until day 5 post-colonisation at which time it was stopped. The other group (PABA-) did not receive PABA. Serum was collected 14 and 28 days following colonisation from 10 mice per group and anti-D39WT IgG measured by whole cell ELISA, as shown in Figure 3.14. By day 14, only 60% of PABA- mice had developed detectable serum IgG to D39, whereas all PABA+ mice had seroconverted. The overall median (IQR) titre was 1/148 (1/30 – 1/543) for PABA+ mice versus 1/44 (1/10 – 1/86) for PABA- mice (*P*=0.018). By day 28, all mice had seroconverted, irrespective of whether supplemented with PABA or not. For the PABA+ group, the median titre was 1/2120 (1/1170 – 1.6500). For the PABA- group the median titre was 1/714 (1/107 – 1/2010), no longer significantly different to the PABA- group (P=0.16). Thus, supplementation with PABA for 5 days leads to earlier seroconversion in response to D39Δ*pab* colonisation, but does not affect overall anti-D39 IgG levels by day 28.

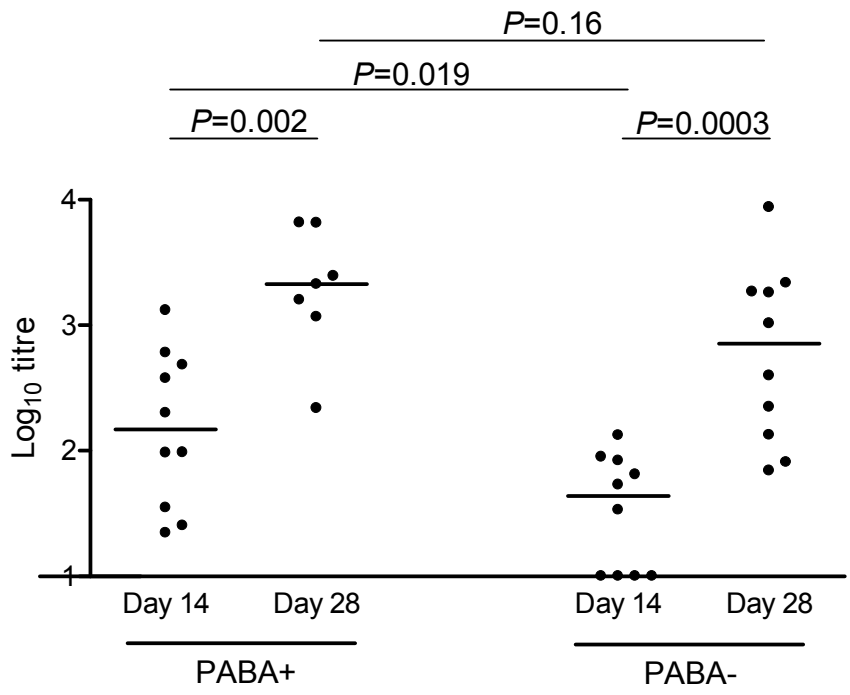

Figure 3.14. Effect of PABA supplementation on immunogenicity of nasopharyngeal colonisation with D39Δ*pab* in CD1 mice.

Serum anti-D39WT IgG measured by whole cell ELISA 14 and 28 days following colonisation with 0.55 x 10^7 CFU D39Δ*pab* in the presence (PABA+) or absence (PABA+) of PABA supplementation from day -1 to +5. Controls received PBS. Dots represent individual mice, bar represents median. Groups compared by Mann-Whitney U-test.

To test whether the duration of nasopharyngeal colonisation has an effect on protection against subsequent pneumonia, the groups of mice described above colonised with D39Δ*pab* (0.55 x 10^7 CFU) in the presence or absence of PABA supplementation until day 5 post-colonisation were subsequently given pneumonia challenge on day 28 with 1.2 x 10^7 CFU D39WT (Figure 3.15) . A further age-matched naïve control group (n=15) were also challenged at the same time. Although not statistically significant, there was a trend towards greater protection with D39Δ*pab* colonisation in the presence of PABA (35% survival with median survival time 6.9 days, versus 20% survival with median survival time 2.9 in controls,

P=0.088 by log rank test) than without PABA supplementation (30% survival with median survival time 3.0 days, P=0.49).

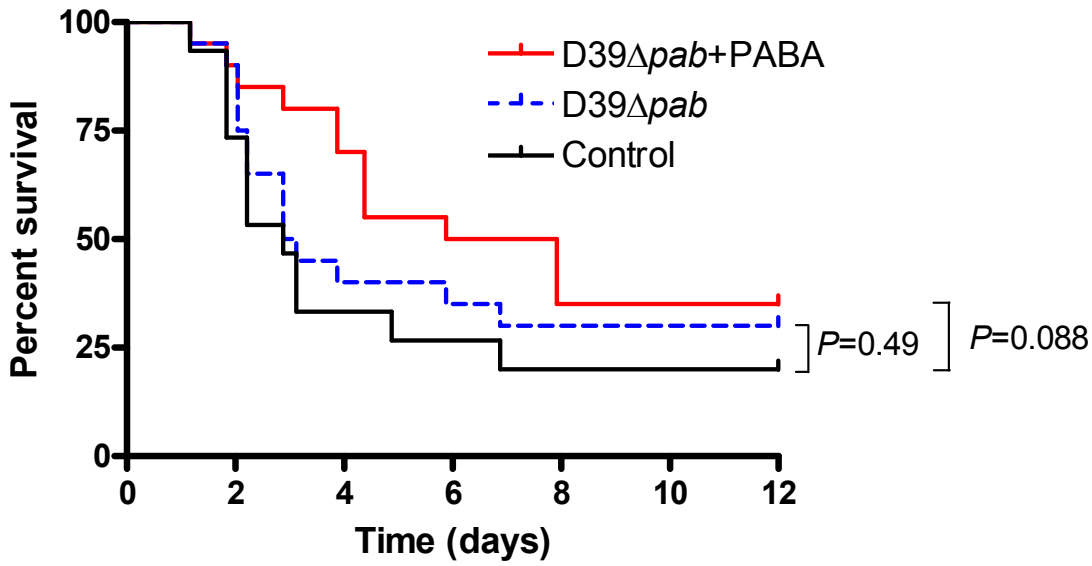

Figure 3.15. Protective efficacy of nasopharyngeal colonisation with D39Δ*pab* in presence of absence of PABA supplementation against D39WT pneumonia in CD1 mice.

Kaplan-Meier plots of survival of mice (n=15-18) colonised with 0.55 x 10^7 CFU D39Δ*pab* in the presence or absence of PABA supplementation from day -1 to +5 or naïve controls intra-nasally challenged on day 28 with 1.2 x 10^7 CFU D39WT. Groups compared by log rank test.

Supplementation with PABA enhanced the colonisation density and duration of D39Δ*pab* to levels similar to D39WT. Its cessation led to rapid loss of colonisation within two days. When mice were colonised with D39Δ*pab* in the presence of PABA, seroconversion was accelerated and there was a trend towards protection against D39WT pneumonia.

3.2.6 Efficacy of D39 colonisation in cross-protecting against TIGR4 and ST3 pneumonia

3.2.6.1 Experimental design and optimisation

D39WT colonisation induced non-CPS specific serum IgG as detected by whole cell ELISA. To explore whether the antibody response generated was not restricted to the D39 strain, an experiment was designed to test the ability of D39WT colonisation to protect against lethal pneumonia challenge with either the serotype 4 strain TIGR4 or the serotype 3 strain ST3. Prior to challenge, serum was collected for analysis of IgG binding to these different strains of *S. pneumoniae* in whole cell ELISA.

The experiment included six groups of mice (n=20 per group), of which three were colonised with two doses of D39WT (1.35×10^7 CFU) and three control groups received PBS, each 14 days apart. Ten mice per group were bled for serum collection immediately prior to challenge 28 days following the final colonising inoculation. The bacterial strain used to challenge groups varied, with one colonised group and one control group each receiving either D39WT, TIGR4 or ST3 challenge. D39WT challenge groups were included as a positive control where protection was anticipated based on previous data. The dose of TIGR4 for challenge was reduced 10-fold from that used previously to 5×10^6 CFU as disease progression was too rapid with the higher dose. To determine an appropriate challenge dose of ST3, a pilot experiment challenging naïve CD1 mice with 1.4×10^6 CFU led to death of all mice with median

survival time 2.67 days (see Figure 3.16). The ST3 challenge inoculum for the cross protection experiment was therefore reduced to 5 x 10^5 CFU.

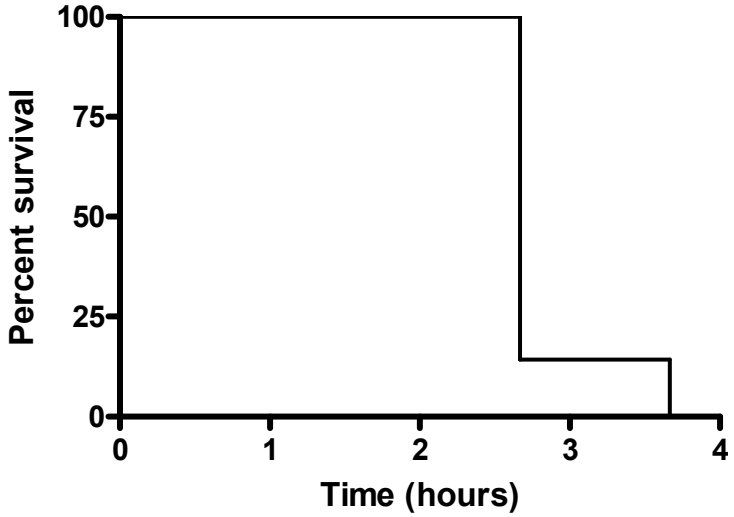

Figure 3.16. Pneumonia challenge of naïve CD1 mice with *S. pneumoniae* ST3.

Kaplan-Meier survival curve of mice (n=7) challenged with 1.4 x 10^6 CFU ST3.

3.2.6.2 Induction of cross-reactive anti-TIGR4 and anti-ST3 serum IgG by D39 colonisation

Sera collected from individual mice 28 days following D39WT colonisation were analysed by whole cell ELISAs to detect binding of IgG to either D39, TIGR4 or ST3 (Figure 3.17). D39 colonisation induced anti-D39 IgG in all mice with median titre 1/4420 (IQR 1/1960 - 1/91300, P<0.001 versus controls). D39 colonisation also induced anti-TIGR4 in all mice with median titre 1/2780 (IQR 1/1460 – 1/52000, P<0.001). 9 of 10 mice had detectable titres of anti-ST3 IgG with median titre 1/517 (IQR 1/257 – 1/1230). There was also low level binding from control serum with a median anti-ST3 IgG titre of 1/31 (IQR 1/10 – 1/216). However, titres in colonised mice were still significantly higher (P=0.043). Thus, colonisation with the strain

D39WT was able to induce serum IgG against from the heterologous TIGR4 and ST3 strains.

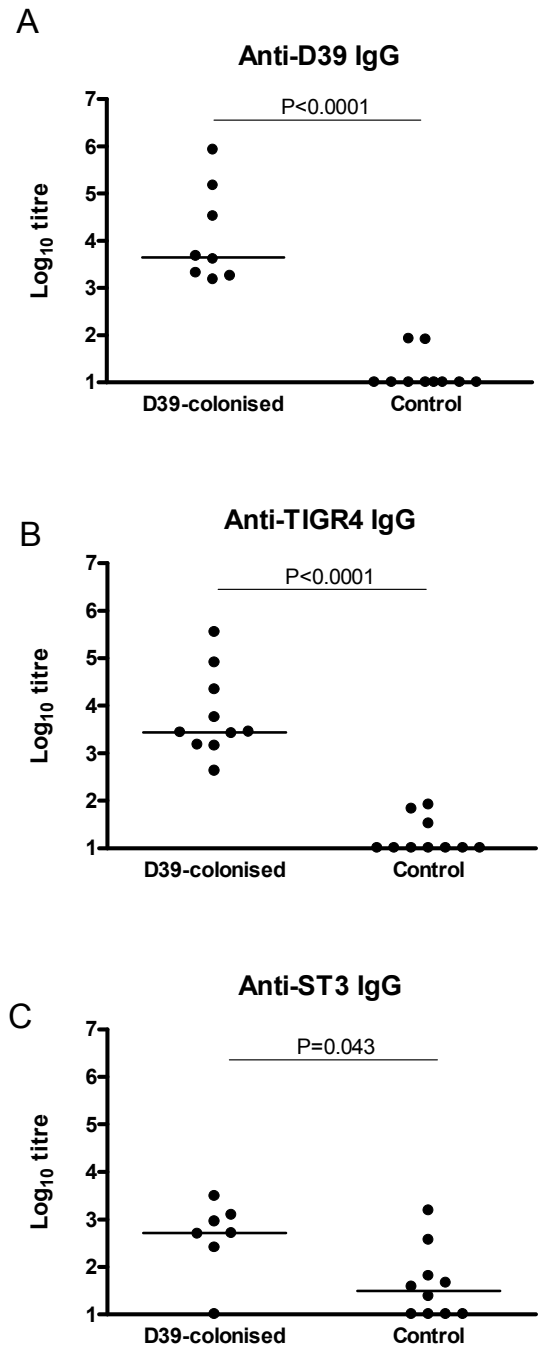

Figure 3.17. Cross-reactivity of serum induced by D39WT colonisation of CD1 mice.

Anti-D39 (A), anti-TIGR4 (B) and anti-ST3 (C) whole cell ELISA titres of day 28 serum collected from mice colonised by D39WT or controls. Dots represent individual mice, bars represent group medians, comparison by Mann-Whitney U-test.

3.2.6.3 Effect of prior D39 colonisation on subsequent TIGR4 and ST3 lethal pneumonia challenge

Similar to previous results, D39 colonised mice were strongly protected against homologous lethal pneumonia challenge with 75% survival versus 25% in controls (*P*=0.002 by log rank test) (Figure 3.18).

However, when challenged with TIGR4 pneumonia, there was no difference in overall survival between D39 colonised and control groups (25% survival each, P=0.61 by log rank test) and with similar median survival times (3.75 days in colonised versus 4.04 days in controls).

When challenged with ST3 pneumonia, there was a trend towards enhanced survival of colonised mice compared to controls. 35% of colonised survived compared to only 10% controls, with median survival times 4.03 days and 3.40 days respectively (P=0.057 by log rank test).

Thus although D39 colonisation established via two nasal inoculating doses induced IgG against both TIGR4 and ST3 antigens measured by whole cell ELISA, there was no significant protection against either TIGR4 or ST3 lethal pneumonia.

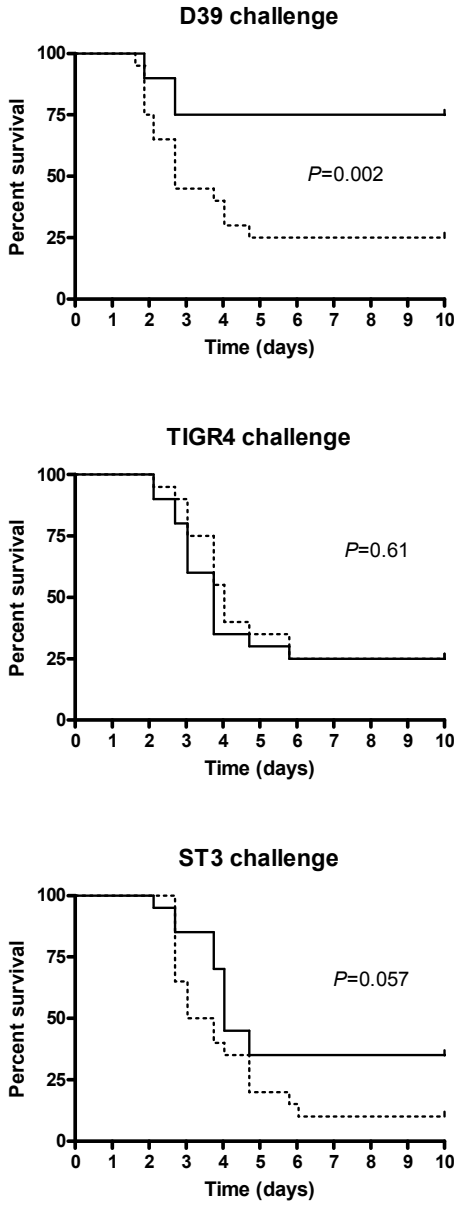

Figure 3.18. Cross-protective efficacy of D39WT colonisation against D39, TIGR4 and ST3 lethal pneumonia in CD1 mice.

Kaplan-Meier survival curves of D39WT colonised or control mice (n=20) challenged i.n. 28 days later wither either (A) 1.5 x 10^7 CFU D39, (B) 5 x 10^6 CFU TIGR4 or (C) 5 x 10^5 CFU ST3. Colonised group compared to control by log rank test.

3.3 SUMMARY OF RESULTS

Using an inoculum of 10^7 CFU, *S. pneumonia* strains D39 and TIGR4 were both able to colonise the nasopharynx of CD1 mice. D39 was cleared by 17 days. Both D39 and TIGR4 induced serum IgG against whole cell antigens but not against their respective capsules. Competitive inhibition confirmed the absence of anti-capsular IgG in the whole cell ELISA. Prior colonisation with D39 protected against subsequent lethal D39 pneumonia. However, prior colonisation with TIGR4 did not protect against TIGR4 pneumonia. Two colonising doses of D39 induced higher levels of serum anti-D39 IgG and a greater degree of protection than a single dose.

Unencapsulated D39-DΔ colonised the nasopharynx but with fewer bacterial CFU at all timepoints than its parent WT strain. It was cleared in nearly all mice by 5 days. Lack of capsule affected TIGR4 to a greater extent, with complete clearance of TIGR4Δ*cps* by 2 days post-colonisation. Whilst D39Δ*lgt* colonised with fewer bacterial CFU at most timepoints compared to its WT parent strain, some bacteria were still recovered in 40% of mice after 10 days. D39Δ*pab* was recovered from all mice after 1 day, but neither D39 Δ*pab* nor TIGR4Δ*pab* were recovered after 2 days. D39-DΔ, D39Δ*lgt* and D39Δ*pab* did not induce significant serum anti-D39 IgG in this model, and were not protective against D39 lethal pneumonia. The survival of groups of mice colonised with WT and mutant strains of D39 strongly correlated with levels of serum anti-D39 IgG.

Supplementation of mouse drinking water with PABA restored the ability of D39Δ*pab* to colonise the nasopharynx with similar numbers of bacteria recovered after 1, 2 and 5 days to those observed with D39 WT. Cessation of supplementation at day 5 led to rapid clearance of D39Δ*pab* by day 7. Colonisation with D39Δ*pab* in the presence of 5 days of PABA led to more rapid seroconversion with higher serum

anti-D39 IgG levels at 14 days but not at 28 days post-colonisation. There was a trend towards protection against lethal D39 pneumonia only observed when D39Δ*pab* colonisation was supported for five days with PABA.

Colonisation with D39 WT also induced serum IgG cross-reactive with TIGR4 and ST3 antigens. However, it did not cross-protect against lethal challenge with these strains in the challenge models used.

4 EFFECTS OF PRIOR COLONISATION ON PATHOGENESIS OF PNEUMONIA IN CBA/Ca MICE

4.1 INTRODUCTION

In the previous chapter, colonisation with D39 protected CD1 mice against subsequent lethal pneumonia. However, a significant proportion of colonised CD1 mice were unprotected despite seroconversion. The observation that prior colonisation can protect outbred mice is important in relating this model to naturally acquired protection in human populations. However, a more consistent model is required to characterise the mechanism of protection in detail. In an attempt to achieve consistency of results, an equivalent model of colonised-induced protection was established in CBA/Ca mice, an inbred mouse strain vulnerable to lethal pneumonia challenge with *S. pneumoniae* D39 (Gingles et al., 2001a). This CBA/Ca model is the basis of the remaining chapters of this thesis.

This chapter assessed the effect of prior colonisation on the pathogenesis of subsequent pneumonia. This will provide insight into how the adaptive immune response to colonisation may protect against subsequent disease. Richards studied the effects of prior colonisation with a Ply-deficient mutant on bacterial numbers in target organs following lethal pneumonia challenge (Richards et al., 2010). However, the earliest timepoint studied was 24 h following infection, but which time 50% of control mice had already died. It is not known how prior colonisation affects the progression of early pneumonia where its protective effect might be most important. It is also not clear whether the effects of previous colonisation on subsequent pneumonia pathogenesis are due to antibody or whether cell-mediated immunity including Th17-cell responses are important.

This chapter opens with experiments confirming that D39 colonised the nasopharynx of CBA/Ca mice without spreading to other organs. The immunogenicity and degree of protection against subsequent lethal pneumonia were then defined. The chapter

then detailed the pathophysiology of this lethal pneumonia model and described how this is affected by prior colonisation. In a series of experiments at timepoints, bacterial loads in target organs were assessed to identify how colonisation affects progression of disease. Cytokine responses at the mucosal and systemic level provide indication of the types of adaptive immune response induced by colonisation. Cellular changes were characterised by microscopic examination of broncheo-alveolar lavage fluid (BALF), and by flow cytometric and histological assessment of infected lungs. Finally, the effect of prior colonisation on alveolar macrophages phagocytosis of bacteria by was studied using fluorescently labeled *S. pneumoniae*.

In this chapter, we addressed the following specific questions:

- Does D39 colonisation protect CBA/Ca mice against lethal pneumonia?
- Does colonisation induce mucosal or systemic antibody responses?
- Does colonisation induce protection at the mucosal or systemic level?
- Does prior colonisation prime for enhanced cytokine or cellular responses during pneumonia?
- Do these observations suggest which immune effector mechanisms may be responsible for protection?

4.2 RESULTS

4.2.1 Colonisation and protection model in CBA/Ca mice

4.2.1.1 Time course of D39 nasopharyngeal colonisation

To confirm that nasal inoculation would establish nasopharyngeal colonisation without spread in CBA/Ca mice, groups (n=4-5) were colonised with 0.4-0.62 x 10^7 CFU *S. pneumoniae* D39 and culled at fixed timepoints. The amount of *S. pneumoniae* were measured in nasal wash fluid on day 2,8, and 11, and from BALF, lung homogenate and blood on days 2 and 11 (Figure 4.1). 5 of 5 mice were colonised on day 2, with median density 5.7 x 10^3 CFU/ml (IQR 2.9-12.0). By day 11, 4 of 5 mice remained colonised, at a median 1.4x10^3 CFU/ml (IQR 0.16-3.66). No bacteria were recovered from any of 4 mice at 28 days. Thus this inoculum established that colonisation persisted for at least 11 days but was cleared by 28 days. In 1 of 5 mice, a barely detectable number of *S. pneumoniae* were recovered from BALF (≤50 CFU/ml), but no *S. pneumoniae* were recovered from lung homogenate or blood at any timepoint.

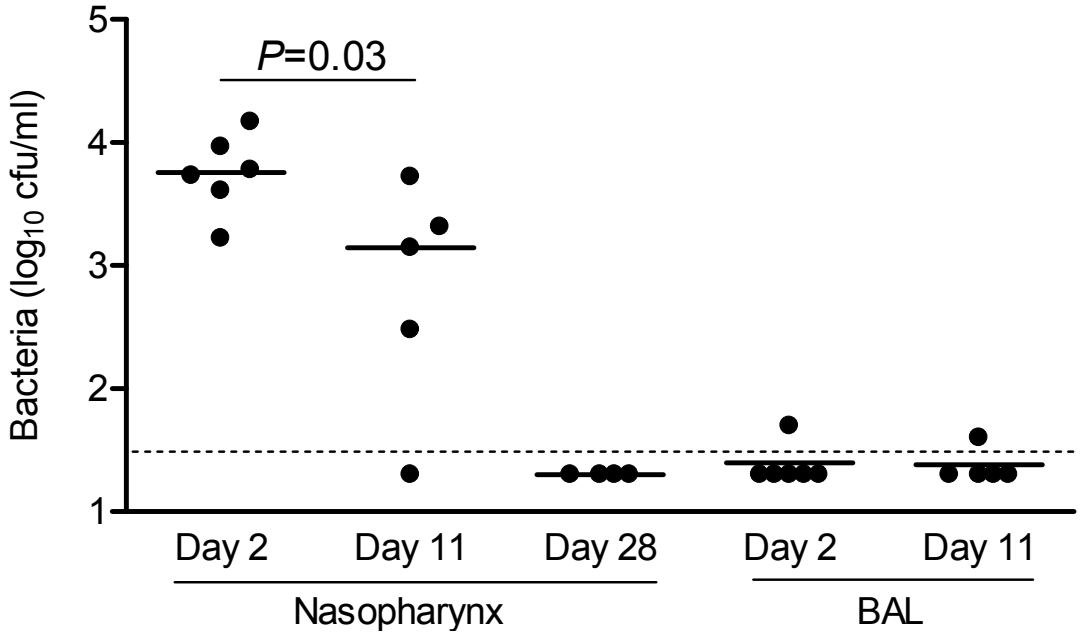

Figure 4.1. Nasopharyngeal model in CBA/Ca mice.

Groups of mice (n=5-6) were nasally inoculated with 0.4 - 0.62x10^7CFU *S. pneumoniae* D39. Nasopharygeal wash and broncheo-alveolar lavage (BAL) was collected from culled mice at fixed timepoints for bacterial culture. Dots represent data from individual mice, bars represent median for group. Dotted line indicates limit of detection. Nasopharyngeal bacterial load compared between day 2 and 11 by Mann-Whitney U-test.

4.2.1.2 Immunogenicity of nasopharyngeal colonisation

Immunogenicity of D39 colonisation in CBA/Ca mice was assessed by measuring anti-D39 IgG levels (Figure 4.2) and IgA and IgM levels (Figure 4.3) in both the BALF (n=5-6 colonised, n=3-6 controls) and serum (n=14 colonised, n=3-9 controls) by whole cell ELISA. BALF was assayed undiluted to maximise sensitivity. Serum was initially assayed at 1:20 dilution and then a titre was determined by limiting dilution.

Colonisation led to the generation of significantly higher levels of anti-D39 IgG (*P*=0.002) and IgA (*P*=0.009) in the BALF, with some mice showing small amounts of anti-D39 IgM in BALF. Anti-D39 IgG was also present in serum of colonised mice at a median titre of 1/273 (IQR 1/60-1/869), but undetectable in controls (*P*=0.002). Only 4 of 14 mice developed serum anti-D39 IgA. Whilst some mice developed a serum anti-D39 IgM response to colonisation, most did not and these mice had similar results to control mice. There was a very slight difference in median anti-D39 IgM levels between colonised and control mice (colonised median OD_{405} 0.66 (IQR 0.51-0.77) versus controls 0.46 (IQR 0.38-0.54), *P*=0.015), implying that colonisation led to less than a 50% increase in antibody level compared to that already existing in controls. This is in marked contrast to the effect of colonisation on serum anti-D39 IgG.

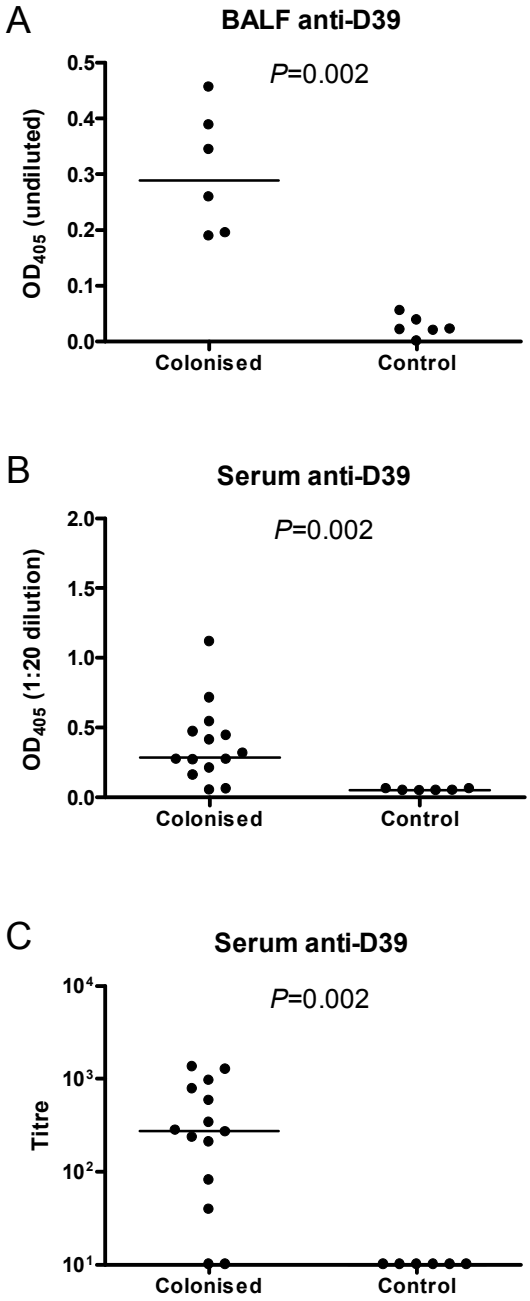

Figure 4.2. Anti-D39 IgG responses to D39 colonisation in CBA/Ca mice.

IgG responses to D39 in BALF (A) or serum (B and C) obtained from colonised or control mice measured using whole cell ELISAs. Responses are presented both as OD_{405} at 1:20 serum dilution (B) and as titres (C). All comparisons between colonised and control mice performed by Mann Whitney U-test.

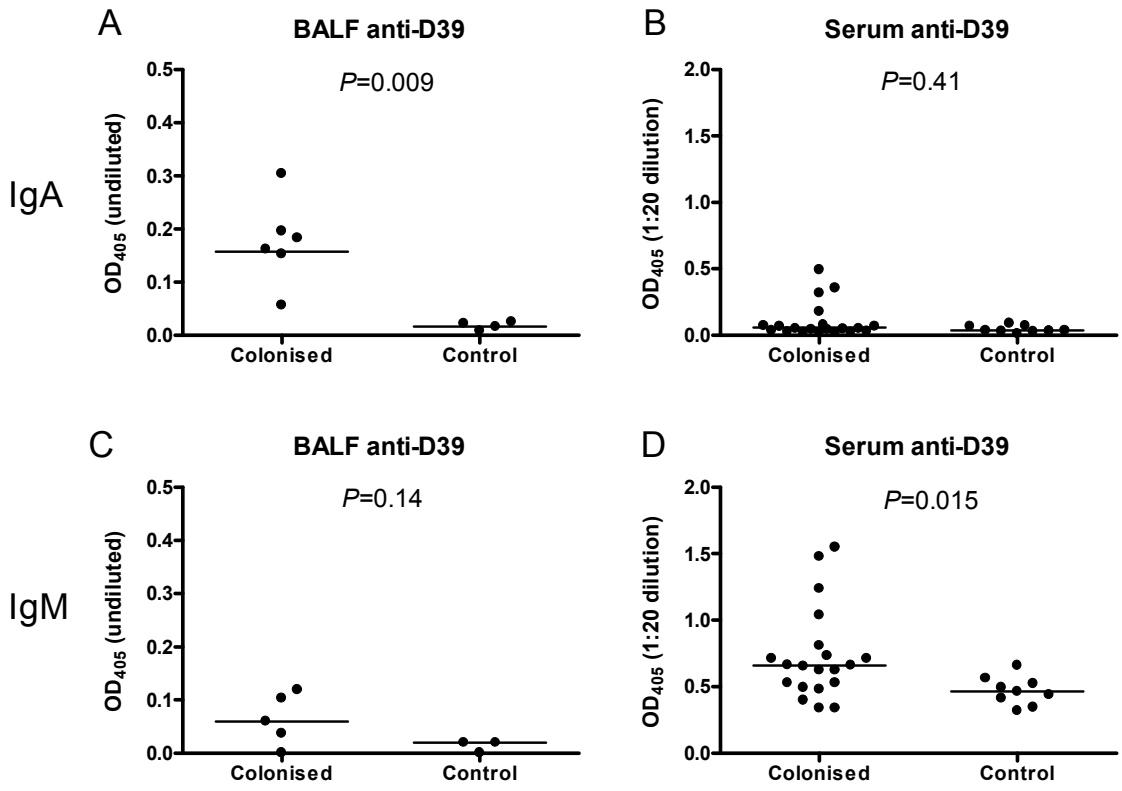

Figure 4.3. Anti-D39 IgA and IgM responses to D39 colonisation in CBA/Ca mice.

IgA (A and B) and IgM (C and D) responses to D39 in BALF (A and C) or serum (B and D) obtained from colonised or control mice measured using whole cell ELISAs. All comparisons between colonised and control mice performed by Mann Whitney U-test.

4.2.1.3 Protection against lethal pneumonia

To assess the protective efficacy of nasopharyngeal colonisation of inbred CBA/Ca against lethal *S. pneumoniae* D39 pneumonia, groups of mice (n=19 colonised, 18 controls) were colonised with 0.5 x 10^7 CFU D39 in 10µl PBS, or PBS alone (controls). 28 days later, mice received pneumonia challenge with 0.5 x 10^7CFU D39 i.n. Mice were monitored for signs of disease, and culled when terminally sick. Kaplan-Meier survival curves were generated as shown in Figure 4.4. All control mice developed fatal disease with a median survival of 30 h. However, 94% of previously colonised mice survived pneumonia challenge, a highly significant result (P<0.0001).

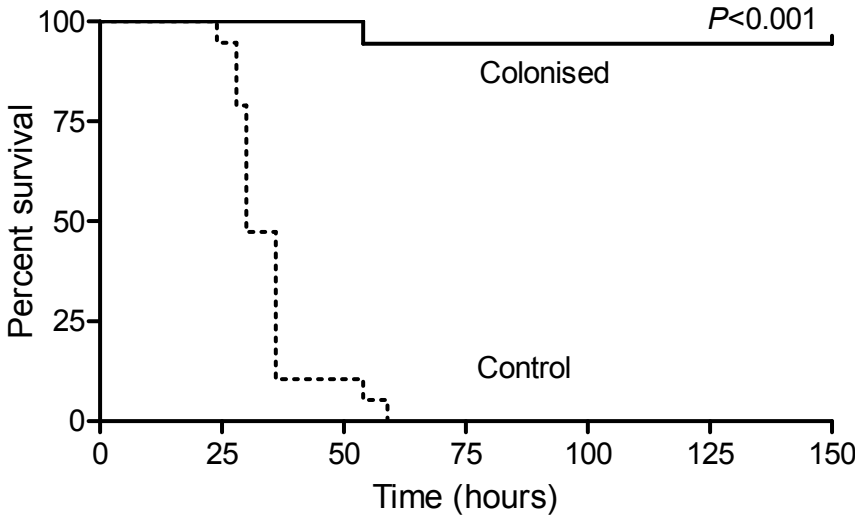

Figure 4.4. Effect of prior D39 colonisation on subsequent D39 pneumonia in CBA/Ca mice.

Kaplan-Meier survival curves of previously colonised or control CBA/Ca mice following pneumonia challenge with 5x10^6 CFU D39 *S. pneumoniae* in 50 µl PBS on day 28 post-colonisation (n=18 or 19). Survival compared by log rank test.

4.2.2 Overview of experiments to characterise pathophysiology

The effect of prior colonisation on disease progression appeared to operate within the first 24 h following infection. To understand the differences in disease process between colonised and control mice a series of experiments were designed to characterise the bacterial loads, the levels of inflammatory mediators and the cellular changes in various target organs at timepoints prior to 24 h.

Groups of mice (n=6-12) were colonised as above and controls received PBS. 28 days later, all mice were given pneumonia challenge with D39 as above. Mice were then culled at fixed timepoints (4, 9 and 18 h post-challenge) and whole blood, serum, BALF and lungs collected for analysis. Whole blood, BALF and lung homogenate were cultured to assess the burden of bacteria. Levels of innate inflammatory cytokines and chemokines were measured in BALF, and levels of T-helper cell cytokines were measured in BALF and serum. Neutrophil recruitment to alveolar spaces was assessed by examination of cytospin preparations of BALF, and lymphocyte and subset number in the lung measured by flow cytometry. In addition, lung inflammation was assessed histologically. In all experiments, the target inocula for both colonisation and infection was 1 x 10^7 CFU. Actual inocula were within the range 0.7-1.8 x 10^7 CFU for colonisation and 0.4-1.5x10^7 CFU for challenge.

4.2.3 Effect of prior colonisation on bacterial CFU in target organs during subsequent pneumonia

To further characterise how previous colonisation may protect against pneumonia, the bacterial load in blood, BALF and lung of previously colonised or control mice was measured 4, 9 and 18 h following infection. Figure 4.5 presents the time course of bacterial load in target organs in the colonised and control mice. The same data is

also presented in Figure 4.6 as a direct comparison between the colonised and control mice at each individual timepoint.

In control mice (Figure 4.5A), bacteria were recovered from BALF at all timepoints, with a median (IQR) of 8.6 x 10^5 CFU/ml (4.7-12.1) at 4 h. This did not change significantly by 9 or 18 h. In the lungs, there was a median of 1.2 x 10^4 CFU/ml (1.0-2.0) at 4 h. Whilst not significantly different at 9 h, this had fallen to 6.2 x 10^3 CFU/ml (0.7-292) by 18 h post-infection (*P*=0.009 vs 9 h, NS vs 4 h). Bacteraemia was assessed at 9 and 18 h. At 9 h post-infection, 4 of 6 mice were bacteraemic, with a broad range in the numbers of bacteria in the blood (median 1.3 x 10^3 CFU/ml (0.02-38)). The same proportion were bacteraemic at 18 h, but all mice that were bacteraemic had >10^5 CFU/ml of bacteria in the bloodstream.

In the BALF of previously colonised mice (Figure 4.5B), there was no change in numbers of bacteria recovered from BALF between 4 and 9 h. However, numbers fell by 18 h (9 hour median 1.0 x 10^6 CFU/ml (0.58-4.9) versus 18 hour median 3.5 x 10^5 CFU/ml (1.0-25.4), *P*=0.002 compared to 9hrs, *P*=0.001 compared to 4 h). In the lungs of previously colonised mice (Figure 4.5B), there was a median of 3.6 x 10^4 CFU/ml (1.9-4.2) at four h. This rose to 9.4 x 10^4 CFU/ml (3.9-25.2) (*P*=0.036) by 9 h, but then fell significantly to a median of 5.6 x 10^2 CFU/ml (1.4-40.0) at 18 h (*P*=0.002 vs 9 h, *P*=0.008 vs 4 hrs). No bacteria were recovered from the blood of previously colonised mice at either 9 or 18 h post-infection (Figure 4.5B).

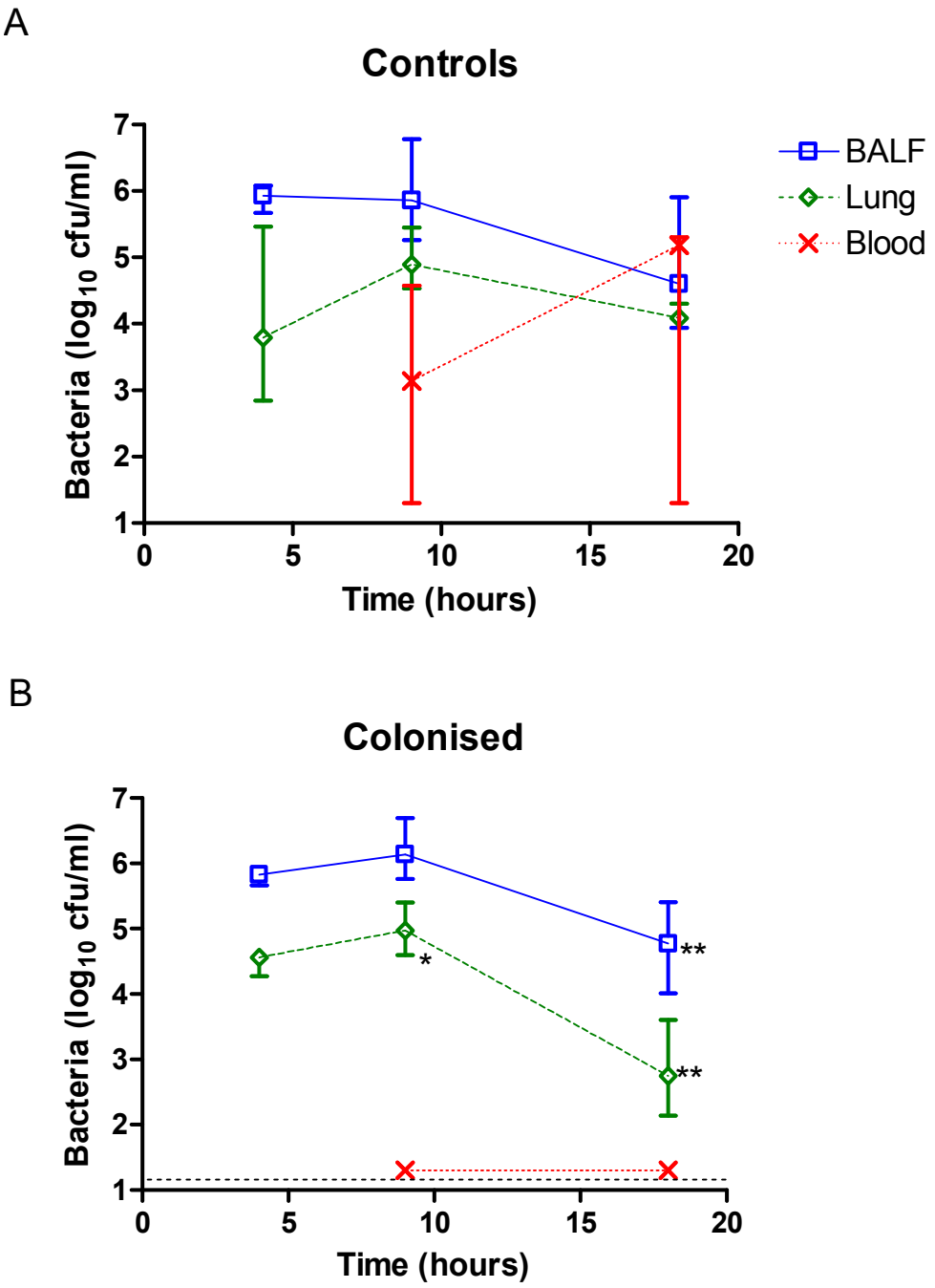

Figure 4.5. Bacterial load in target organs during D39 pneumonia in control or previously colonised mice.

Time course of recovered bacterial CFU in BALF, lung or blood of (A) control or (B) previously colonised CBA/Ca mice. Samples were harvested at 4h, 9h and 18h following challenge with 0.5-1.5x10^7 CFU D39 Pnc. Symbols indicate median and error bars indicate the IQR at that timepoint. *P<0.05, **P<0.01 compared to data at 4 h by Mann-Whitney U-test. Note: the same data is also presented in Figure 4.5 as a comparison of bacteria recovered from previously colonised or control mice. Data representative of two replicate experiments with similar results.

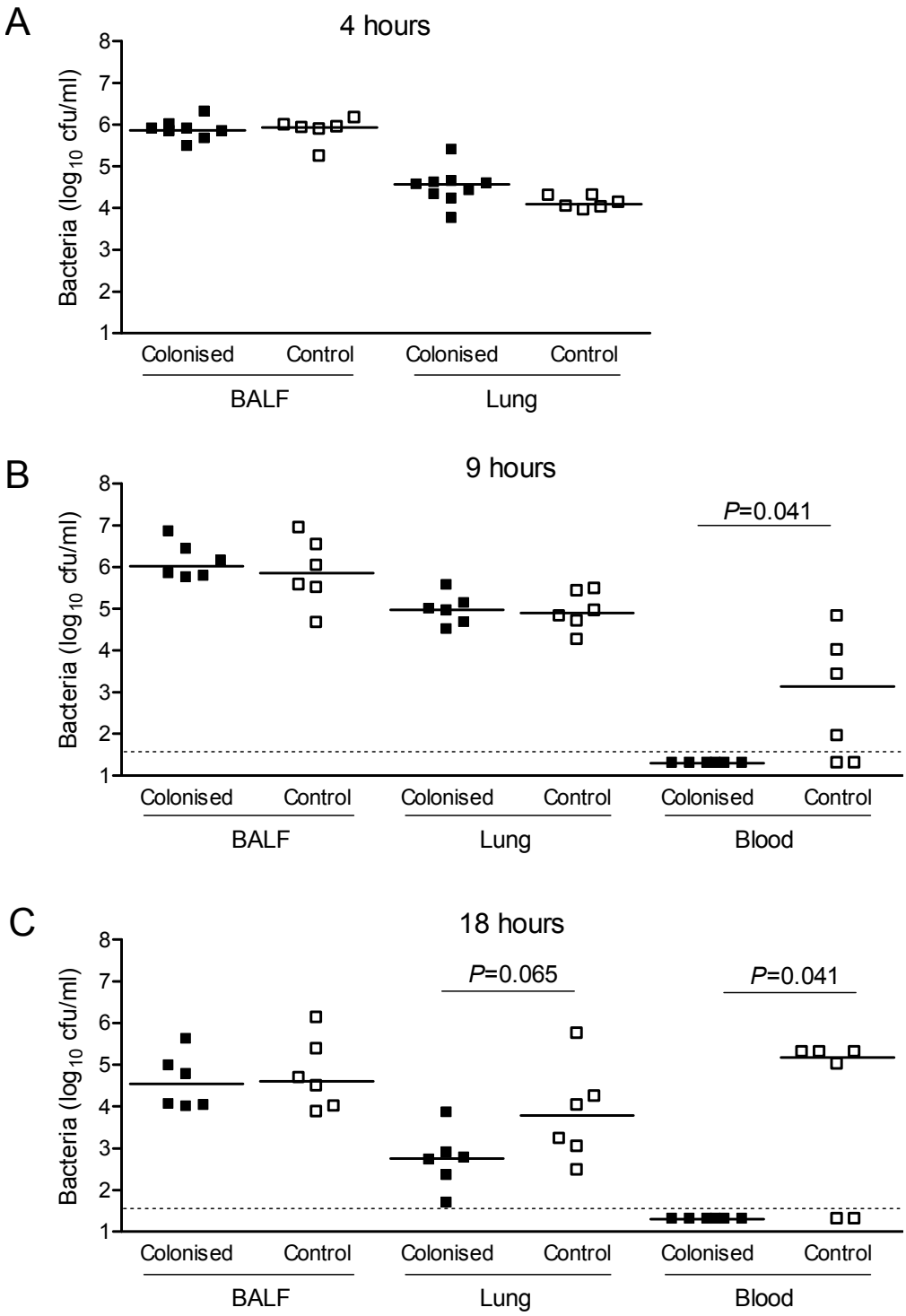

Figure 4.6. Effect of prior colonisation on bacterial load during D39 pneumonia.

Bacterial CFU in BALF, lung and blood of previously colonised or control CBA/Ca mice at 4h (A), 9h (B) and 18h (C) following challenge with 0.5-1.5x10^7 CFU D39 Pnc. Dots represent data from individual mice, bars represent group medians. Dotted line indicated limit of detection. Groups compared using the Mann-Whitney U-test. Note: the same data is also presented in Figure 4.4 as a separate time courses for colonised (Fig. 4.4a) and control (Fig. 4.4b) mice.

At no timepoint was there a statistically significant difference in numbers of bacteria in BALF or lung between colonised and control mice (Figure 4.6). However, there was a strong trend towards 10-fold fewer bacteria in the lungs of previously colonised mice at 18 h (P=0.064) (Figure 4.6), suggesting the possibility that colonisation was inducing some protection at this site and timepoint. Furthermore, at no point were any previously colonised mice bacteraemic, unlike the majority of controls at both 9 and 18 h.

Thus, previous colonisation strongly protected against the development of bacteraemia during subsequent pneumonia challenge. In addition there was a trend towards reduced lung bacterial numbers by 18 h post-infection.

4.2.4 Effect of prior colonisation on cytokine responses during subsequent pneumonia

To assess the inflammatory response to infection in previously colonised or control mice, levels of the innate cytokines KC, IL-6, TNF-α, GM-CSF, IL-1β and IL-12p70 were measured in BALF at 4 and 18 h post-infection, and are shown in Figure 4.7. KC, IL-6, TNF-α, GM-CSF and IL-1β were all detectable at 4 h post-infection in the BALF of control mice. Mean levels of all these inflammatory mediators appeared slightly higher at this timepoint in the BALF of colonised mice (Figure 4.7A). Levels were statistically significantly higher for IL-6 (mean 2860 pg/ml (SD 440) versus 1859 pg/ml (SD 681) controls, P=0.013) and TNF-α (mean 3312 pg/ml (SD 1565) versus 1698 pg/ml (SD 713) controls, P=0.044).

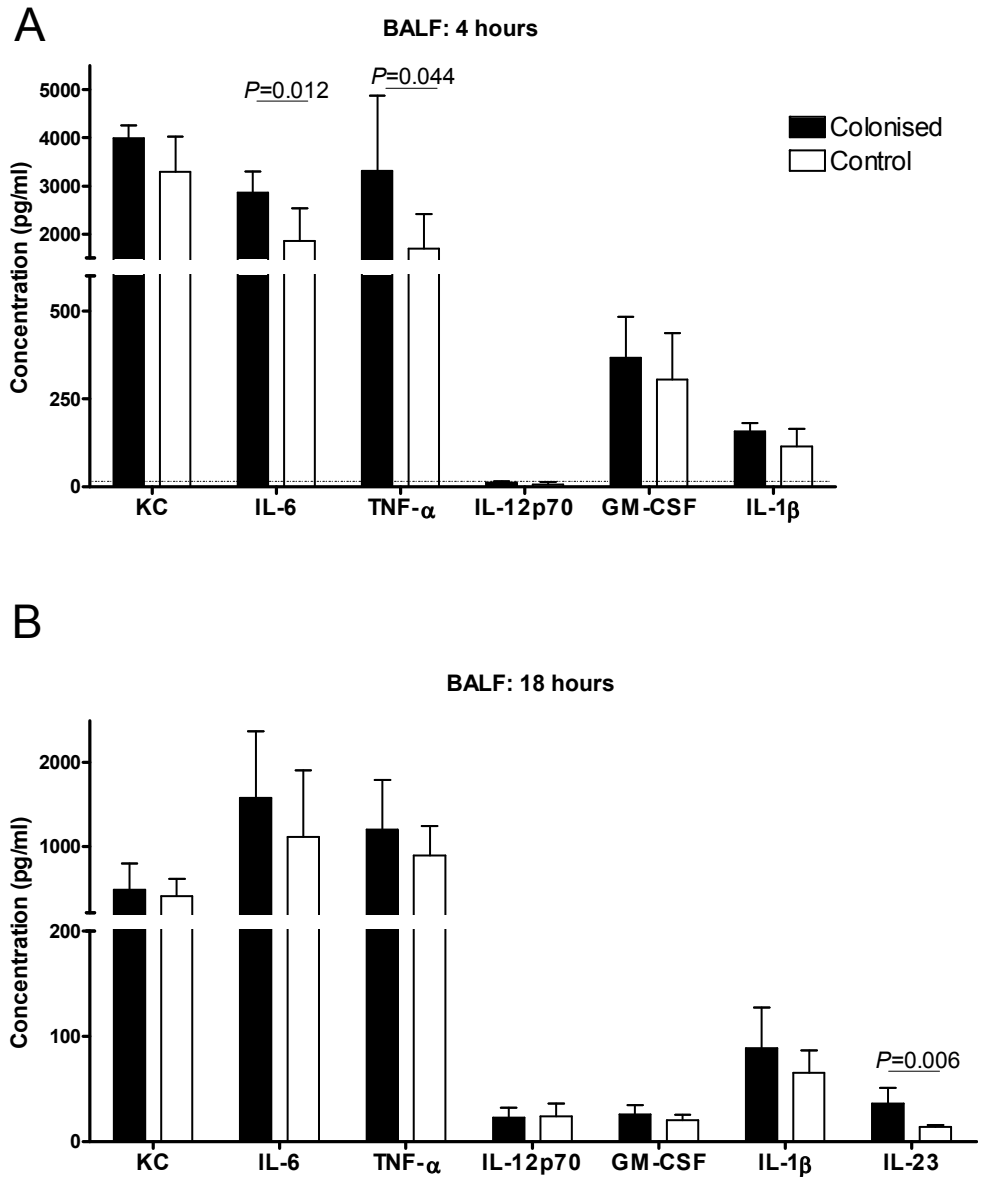

Figure 4.7. Effect of prior colonisation on levels of innate mediators in BALF during pneumonia.

BALF samples were collected either 4h (A) or 18h (B) following D39 *S. pneumoniae* pneumonia challenge with 0.5-0.9x10^7 CFU of previously colonised or control CBA/Ca mice (n=6 per group). Levels were measured by Luminex bead assay other than for IL-23 which was measured by ELISA. Group mean + SD are shown. Results compared using unpaired Student t-tests.

By 18 h post-infection (Figure 4.7B), levels of all these cytokines were lower than at 4 h with the exception of IL-12p70 which was only detectable at this timepoint. There were no significant differences between colonised and control mice for any of these mediators at 18 h. There was insufficient BALF available to test for levels of IL-23 at 4 h post-infection. However, levels of IL-23 were significantly higher in the BALF of colonised mice than controls (P=0.006) at 18 h. Thus, prior colonisation appeared to prime for higher BALF levels of IL-6 and TNF-α at 4 h and IL-23 at 18 h post-infection.

Although not the sole producers of these cytokines, differences in the levels of IL-17 and IFN-γ between colonised and control mice could be indicative of a colonisation-induced mucosal or systemic Th17 or Th1 cell responses. Levels were therefore measured in the BALF at 4 and 18 h post-infection and in serum after 18 h and are shown in Figure 4.8.

IL-17 was detectable in the BALF of all colonised mice at both timepoints (Figure 4.8A). IL-17 levels increased in colonised mice from 4 to 18 h post-infection (P=0.001), and by 18 h were higher than in control mice (P=0.004). Serum levels of IL-17 were also higher in colonised mice than controls, 18 h post-infection (P=0.003) (Figure 4.8B). No IFN-γ was detectable in the BALF or serum of either colonised or control mice at either timepoint (Figure 4.8B-C).

To identify further evidence of Th17 memory, levels of IL-22, another Th17-cell signature cytokine, were measured in BALF at 18 h post-infection (Figure 4.8D). Again, levels were significantly higher in BALF of colonised than control mice.

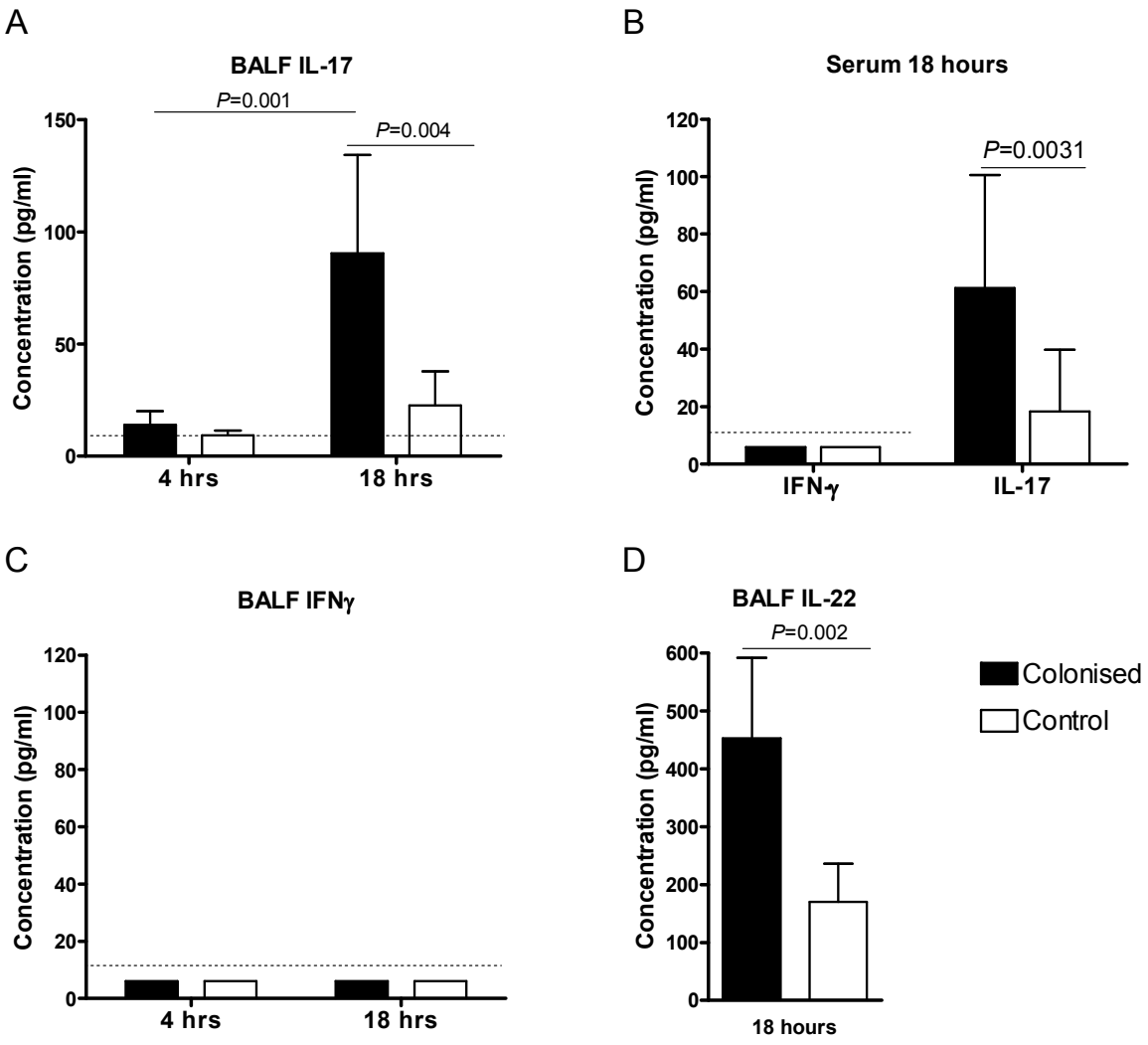

Figure 4.8. Effect of prior colonisation on BALF and serum levels of CD4 T-cell cytokines during pneumonia.

BALF and serum samples were collected either 4h or 18h following D39 Pnc pneumonia challenge with 5-9x10^6 CFU of previously colonised or control CBA/Ca mice (n=6 per group) and cytokine levels measured by ELISA. Mean (+SD) are presented for 4h and 18h BALF IL-17 (A), 18h serum IL-17 and IFN-γ (B), 4h and 18h BALF IFN-γ (C) and 18h BALF IL-22 (D). Dotted line indicates limit of detection. Colonised and control mice were compared by unpaired Student t-test. Data representative of two replicate experiments with similar results.

4.2.5 Effect of prior colonisation on cellular and inflammatory processes during subsequent pneumonia

4.2.5.1 Effect of prior colonisation on lung lymphocyte subsets

The effect of prior colonisation on the number and subsets of lymphocytes present in the lungs of infected mice was assessed at 18 h post-infection (Figure 4.9). Absolute numbers of total lymphocytes, B-cells, T-cells and CD4+ T-cells were measured. In addition, numbers of B- and T-cells as a proportion of all lymphocytes, and CD4+ T-cells as a percentage of all T-cells were calculated. There were no differences between previously colonised and control mice for any of these parameters.

In addition to Th17-cells, gamma-delta T-cells are recognized as a potential source of IL-17. Absolute numbers of total lymphocytes and gamma-delta T-cells were measured in the lungs 4 h post-infection. Again, there was no difference between colonised and control mice for either total lymphocytes or gamma-delta T-cells (Figure 4.10).

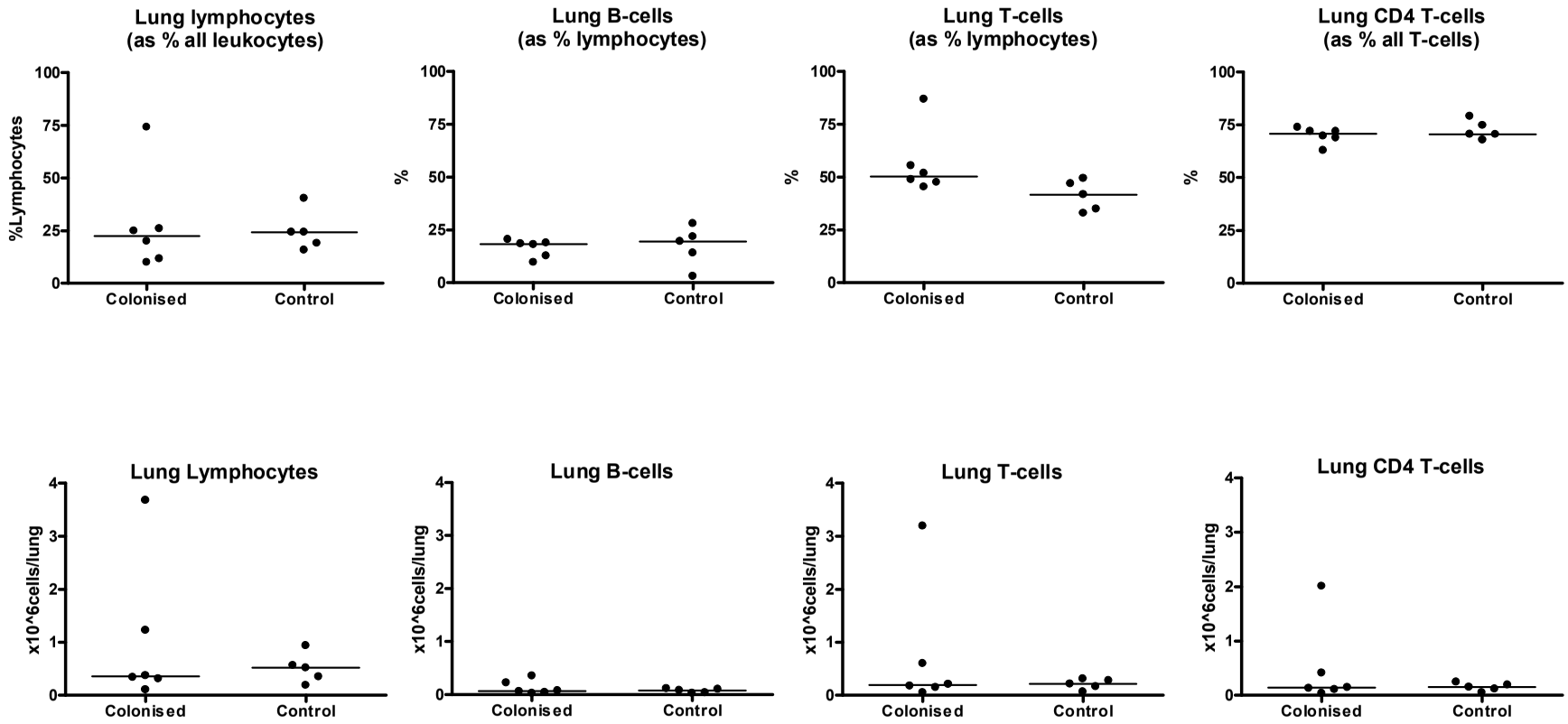

Figure 4.9. Effect of prior colonisation on subsets of lung lymphocytes and subsets 18 h following pneumonia challenge.

Total lung lymphocytes, B- and T-cells and CD4+ T-cells 18h following pneumonia challenge with $9x10^6$CFU D39 Pnc in previously colonised or control CBA/Ca mice. Proportions were obtained by flow cytometric assessment of lung homogenates. Data are presented both as proportions and as absolute cell numbers. Dots represent individual mice, bars represent group medians. There were no statistically significant differences between colonised and control mice for any subset measured ($P>0.05$ by unpaired Mann-Whitney U-test).

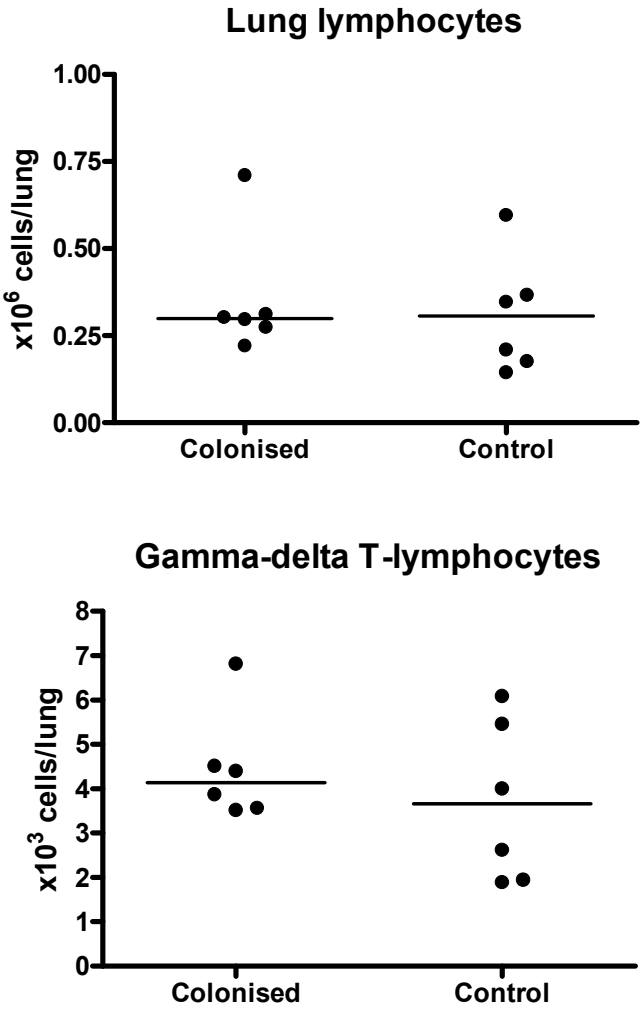

Figure 4.10. Effect of prior colonisation on subsets of lung lymphocytes and gamma-delta T-cell 4 h following pneumonia challenge.

Total lung lymphocytes, and gamma-delta T-cells measured by flow cytometry of lung homogenates collected 4h following pneumonia challenge with $0.5x10^7$CFU D39 Pnc in previously colonised or control CBA/Ca mice. Each dot represents an individual mouse, bars represent group medians. There were no statistically significant differences between colonised and control mice for any cell subset measured ($P>0.05$ by unpaired Student t-test).

4.2.5.2 Effect of prior colonisation on neutrophil recruitment to BALF

Although no differences were seen in lung lymphocyte numbers at either 4 or 18 h post-infection, there was evidence that colonisation primed for greater cytokine responses in the lung following infection. As neutrophils are the earliest cells recruited to alveolar spaces in *S. pneumoniae* pneumonia, numbers in BALF were assessed. To identify if this correlated with enhanced recruitment of neutrophils to alveolar spaces, cytospin preparations of BALF from infected mice were examined (Figure 4.11). The proportion of neutrophils amongst BALF leukocytes was measured prior to and at 4, 9 and 18 h post-infection (Figure 4.11A). The absolute numbers of neutrophils (Figure 4.11B) and monocytes/macrophages (Figure 4.11C) in BALF were also measured prior to and at 4 and 9 h post-infection.

Prior to infection, barely any neutrophils were found in the BALF from either groups of mice, with >99% monocytes/macrophages present. However, by 4 h following infection, there had been greater recruitment of neutrophils to the alveolar space in colonised mice than control mice (mean 2.2 x 10^5 (SD 0.4) cells/ml BALF versus 1.1 x 10^5 (0.7) respectively, P=0.002) (Figure 4.11B). There was no difference in the number of monocytes/macrophages present. This corresponded to a difference in percentage neutrophils at four h (79.4% neutrophils (SD 11.3) in the BALF of colonised mice, but only 58.3% (SD 13.9) in controls (P=0.0003) (Figure 4.11A). Absolute numbers of macrophages/monocytes rose in both groups of mice by nine h. However, neutrophil recruitment predominated such that the BALF from both colonised and control mice contained a mean of 93% neutrophils at both 9 and 18 h post-infection (Figure 4.11A). Thus, prior colonisation primed for greater neutrophil recruitment at 4 h, but there was no longer a difference by 9 h post-infection.

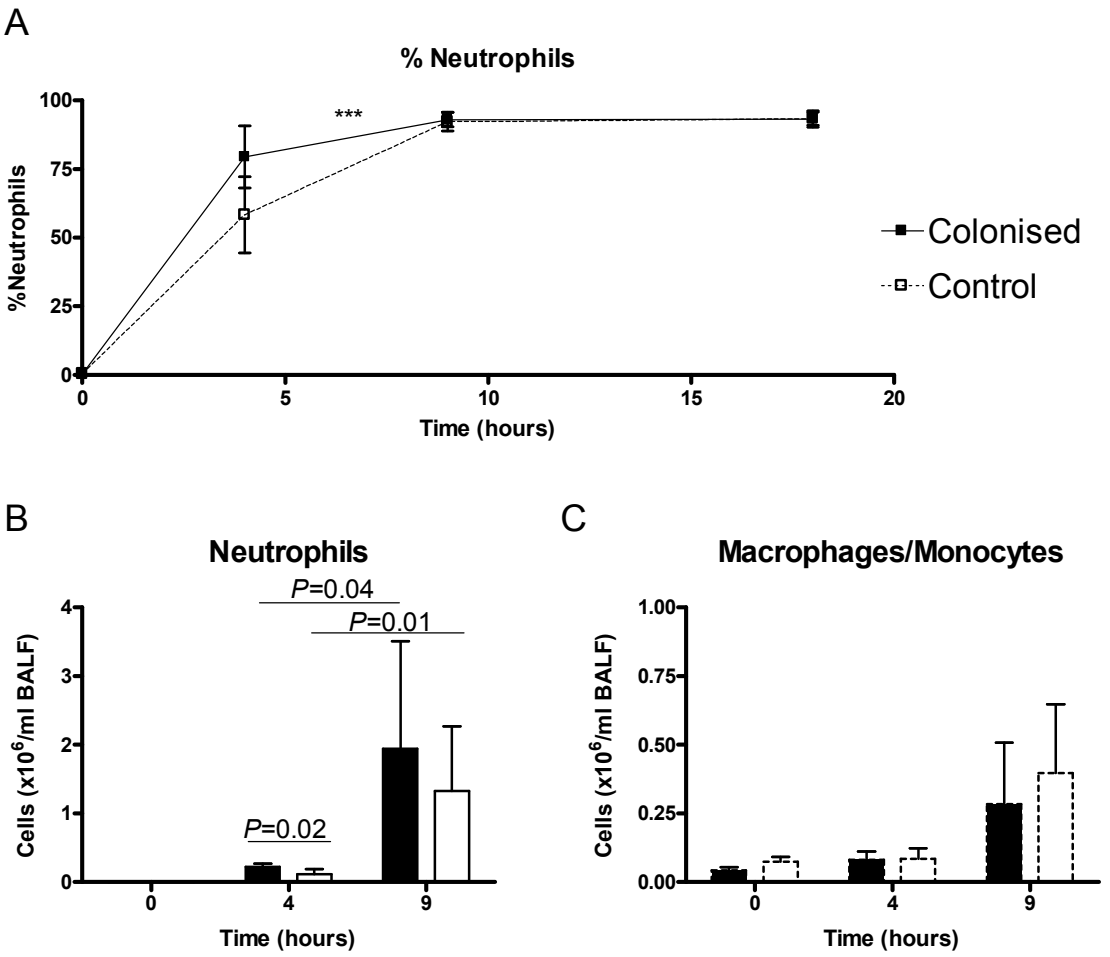

Figure 4.11. Effect of prior colonisation on leukocyte recruitment to alveolar spaces during subsequent pneumonia.

Numbers of neutrophils and monocyte/macrophages were assessed by haemocytometer count and staining of cytospin preparations of BALF collected from previously colonised or control CBA/Ca mice either prior to or at 4h, 9h or 18h following pneumonia challenge with 0.4-1.5x10^7 CFU D39 Pnc. (A) Neutrophil proportion of total BALF leukocytes. Data presented as mean ± SD (n=6 per group) throughout. Absolute cell counts were not available for 18h timepoint. Comparisons between groups at a timepoint and comparison for a group between two timepoints by unpaired Student t-test.

4.2.5.3 Effect of prior colonisation on pulmonary inflammation

To characterise the overall effect of prior colonisation on pulmonary inflammation during pneumonia, lungs were collected from mice 18 h post-infection. Haematoxylin and eosin-stained sections were examined and scored for degree of inflammatory change by Mr Steve Bottoms, an experienced histopathology research technician, who was blinded to which group the samples came from. This scoring system accounted for tissue swelling, cellular infiltrate, haemorrhage and disturbance of alveolar architecture. The lungs of all mice had evidence of inflammation. Whilst the previously colonised mice tended to have higher inflammation scores than non-colonised controls (median of colonised mice 1.9 (IQR 1.5-3.0) versus controls 1.6 (IQR 1.0-2.2)), this difference was not statistically significant (Figure 4.12).

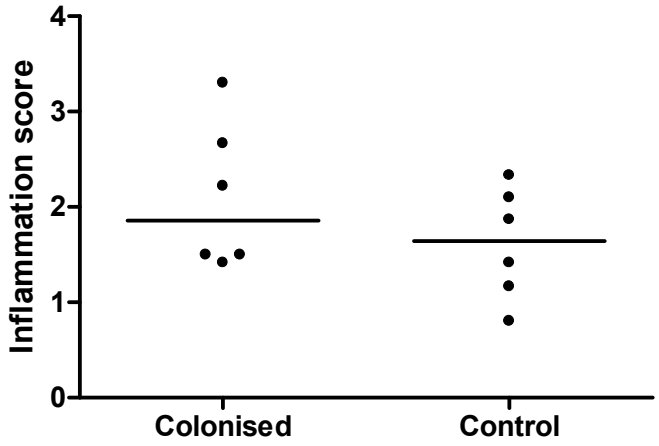

Figure 4.12. Effect of prior colonisation on lung inflammation during subsequent pneumonia in CBA/Ca mice.

Lung inflammation scores assessed by blinded examination of H&E-stained lung sections collected 18h following challenge with 5 x 10^7 CFU *S. pneumoniae* D39 in previously colonised or control mice. Dots represent individual mice, bars represent medians. P>0.05 by unpaired Student t-test. Sample processing, staining and inflammation scoring was kindly performed by Mr Steve Bottoms (UCL Centre for Respiratory Research).

4.2.6 Effect of prior colonisation on alveolar macrophage association during subsequent pneumonia

Prior colonisation with D39 induced detectable IgG and IgA against D39 antigens in BALF. It was possible that mucosal antibody could opsonise bacteria during infection enhancing phagocyte association and uptake. Phagocyte activity could also be greater due to higher TNFα or IL-6 levels in the BALF of previously colonised mice. To investigate the association of bacteria with macrophages within the alveolar spaces during infection, previously colonised or control mice were infected with $0.9x10^7$ CFU D39 fluorescently-labelled with FAM-SE. BALF was collected after 4 h, and the fluorescence of alveolar macrophages assessed as an indicator of their association with bacteria (Figure 4.13). The mean MFI of alveolar macrophages was not significantly different for the two groups (77.4 (SD 66.9) for colonised mice and 106.5 (SD 62.6) for controls). Thus, despite the presence of mucosal antibody and enhancement of cytokine responses, there appeared to be no difference in macrophage uptake of bacteria at 4 h following infection.

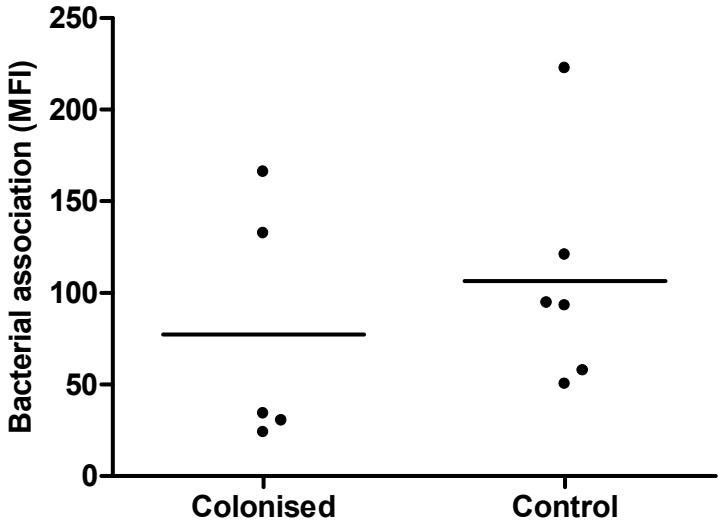

Figure 4.13. Effect of prior colonisation on alveolar macrophage bacterial association during subsequent pneumococcal pneumonia.

Previously colonised or control CBA/Ca mice were challenged with $0.9x10^7$ CFU FAM-SE-labelled D39 Pnc and BALF collected after 4h for flow cytometry. Macrophages were gated according to scatter characteristics and bacterial association assessed as MFI of this population. Dots represent individual mice, bars represent medians. P>0.05 by Mann-Whitney U-test.

4.3 SUMMARY OF RESULTS

S. pneumoniae D39 colonised the nasopharynx of CBA/Ca mice and was cleared by 28 days with a time course similar to that seen in CD1 mice. There was no significant spread to lungs or blood. Colonisation induced significant anti-D39 IgA and IgG in BALF and IgG in serum. A small number of mice had increased serum IgM to D39 antigens but this effect was modest. Colonised mice were strongly protected against lethal D39 pneumonia challenge. Despite similar levels of bacteria in BALF and lungs, colonised mice were completely protected against bacteraemia.

Previously colonised mice were primed for higher BALF levels of IL-6 and TNF-α at 4 h, and higher levels of IL-17, IL-22 and IL-23 at 18 h post-infection. In addition, prior colonisation led to higher levels of serum IL-17 at 18 h. No IFN-γ was detectable in BALF or serum at any timepoint. This suggested that previously colonised mice may be primed for a Th17 response elicited during pneumonia.

No differences were seen in any lung lymphocyte subset at 18 h post-infection, or in gamma-delta T-cells at 4 h post-infection. There were more neutrophils in BALF at 4 h than in control mice but not at 9 h. However, by 18 h no differences in lung inflammation could be observed histologically. Furthermore, prior colonisation did not affect the association of bacteria with alveolar macrophages 4 h post-infection.

Thus, prior colonisation protects against lethal pneumonia by preventing bacteraemia. Although cytokine levels suggest a Th17 response to colonisation, there was little evidence for mucosal protection in this model.

5 ROLE OF ANTIBODY AND CD4+ CELLS IN COLONISATION-INDUCED PROTECTION

5.1 INTRODUCTION

Data from the previous chapter demonstrated that CBA/Ca mice previously colonised with *S. pneumoniae* D39 survive subsequent lethal D39 pneumonia. They did not develop bacteraemia during pneumonia despite numbers of lung and BALF bacteria that are sufficient to cause bacteraemia in controls. This suggests that mice survive lethal challenge because they do not become bacteraemic. Colonisation led to both mucosal and systemic antibody responses against non-capsular antigens detectable in serum and BALF. Pathogenesis studies also revealed significantly higher levels of IL-17 and IL-22 in BALF and IL-17 in serum of previously colonised mice during subsequent pneumonia, but no detectable IFN-γ. This may be indicative of a Th17 response to colonisation. Thus, both antibody and CD4+ memory T-cells induced through colonisation could have roles in colonisation-induced protection.

Adaptive immune responses induced through colonisation may protect at the mucosal level by enhancing clearance of bacteria from alveolar spaces and lungs, and systemically by preventing bacteraemia. Both levels of protection could be mediated by antibody or adaptive cellular responses. Activation of CD4+ memory T-cells of the Th17 phenotype during pneumonia could contribute to protection in several ways. Th17 responses may affect the integrity of the mucosa and enhance the recruitment of phagocytic cells (Iwakura et al., 2008). They may also increase the killing capacity of phagocytes (Lu et al., 2008). Such responses appear to contribute to protection against re-colonisation (Zhang et al., 2009, Malley et al., 2005, Lu et al., 2008).

There are limited data regarding the relative contributions of these mechanisms to colonisation-induced protection. Colonisation with unencapsulated type 6A *S. pneumoniae*, which protected wild-type C57Bl/6 mice against wild-type type 6A challenge, did not protect μMT mice (Roche et al., 2007). μMT mice have a mutation

in the immunoglobulin μ-chain gene, resulting in an absence of mature B cells (Kitamura et al., 1991). Thus they cannot develop antibody responses. This suggests that antibody responses are important in colonisation-induced protection. Whether antibody is functioning at the mucosal or systemic level is not known, nor whether colonisation-induced antibody alone would be sufficient to protect. In this report, $MHCII^{-/-}$ mice, which congenitally lack CD4+ T-cells, were also not protected. CD4+ T-cells play a critical role in the development of antibody responses, facilitating B-cell somatic hypermutation and class switching in germinal centres (Heyzer-Williams et al., 2009). In addition to their inability to develop T-helper cell memory responses to colonisation, $MHCII^{-/-}$ mice will also be expected to have diminished antibody responses. Thus, it is not clear whether poor antibody responses rather than absence of memory T-cells is responsible for lack of protection in these mice.

This chapter attempts to define the specific roles of antibody and CD4+ cell responses during colonisation-induced protection in CBA/Ca mice. Numbers of bacteria in the lungs and blood of mice were assessed at 18 h following pneumonia challenge as indicators of protection. This timepoint was chosen at it is when the biggest differences between colonised and control mice were observed during pathogenesis studies, as described in the previous chapter. Experiments using *in vivo* CD4-depletion, μMT mice and passive transfer of serum help to define the relative contributions of antibody and CD4+ T-cell responses to colonisation-induced protection.

In this chapter, we addressed the following specific questions:

- Do the IL-17 responses to colonisation depend on the presence of CD4+ cells at the time of challenge?
- Are CD4+ cells required at the time of challenge to effect protection?

- Is antibody necessary for colonisation-induced protection?
- Is antibody sufficient for colonisation-induced protection?

5.2 RESULTS

5.2.1 Effect of CD4+ cell depletion on colonisation-induced protection

To test whether CD4+ T-cells are required to effect protection, a system was needed where they would be present at colonisation but absent at pneumonia challenge. *In vivo* cellular depletion by systemic administration of a monoclonal antibody (mAb) is an established technique for depleting specific cell types at particular times in animal models. The mAb GK1.5 was therefore used to deplete CD4+ cells specifically prior to challenge.

5.2.1.1 Optimisation of CD4+ cell depletion

Prior to administration of GK1.5 to colonised mice in a protection experiment, a pilot experiment was performed to optimize CD4+ cell depletion and ensure that systemic administration (i.p.) led to efficient depletion both in the spleen and in the lungs. Initially, 10-week old CBA/Ca mice (n=1 per group) were administered either 200µg GK1.5 i.p. or PBS. 24 h later, 2 mice (1 GK1.5 recipient and 1 control) were given pneumonia challenge with 8.5 x 10^6 CFU D39. Both challenged and unchallenged mice were culled at 18 h post-infection. In pilot experiments, a single dose of 200µg GK1.5 given 24 h pre-infection led to least 69% depletion of CD4+ T-cells from the spleen 18 h post-infection. There was 98% depletion of CD4+ T-cells from the lungs at this timepoint. Illustrative FACS plots showing the effect of GK1.5 are shown in Figure 5.1. To improve the splenic depletion, 2 doses of 250µg GK1.5 were administered, on days -2 and -1 prior to infection. This increased the depletion of CD4+ cells from the spleen to 91%. This 2-dose regimen was therefore used to deplete CD4+ cells from colonised and control mice prior to challenge.

5.2.1.2 Effect of prior colonisation on bacterial burden during pneumonia in the absence of CD4+ cells

To explore the effect of depletion of CD4+ cells prior to challenge, mice were colonised as previously described with 6.5 x 10^6 CFU D39 (n=6 per group). Controls received PBS. On days 26 and 27 post-colonisation, all mice received GK1.5 250μg i.p. On day 28, all mice were challenged with 4 x 10^6 CFU i.n. Mice were culled after 18 h, and blood, serum, BALF and lungs collected. Efficiency of CD4+ T-cell depletion from the spleens of mice included in the experiment was >90% as assessed by flow cytometry. Bacterial CFU recovered from target organs are shown in Figure 5.2. There were no significant differences in the number of bacteria recovered from BALF or lungs of colonised and control mice. 3 of 6 control mice were bacteraemic at 18 h. However, no previously colonised mice were bacteraemic (P=0.043 by Chi-squared test). Furthermore, there was a strong trend towards a lower median bacterial load in blood of previously colonised mice (P=0.09 by Mann-Whitney U-test). To confirm these findings, this experiment was repeated and similar results were obtained (data not shown). Thus, depletion of CD4+ cells prior to challenge did not affect the ability of previous colonisation to prevent bacteraemia secondary to pneumonia.

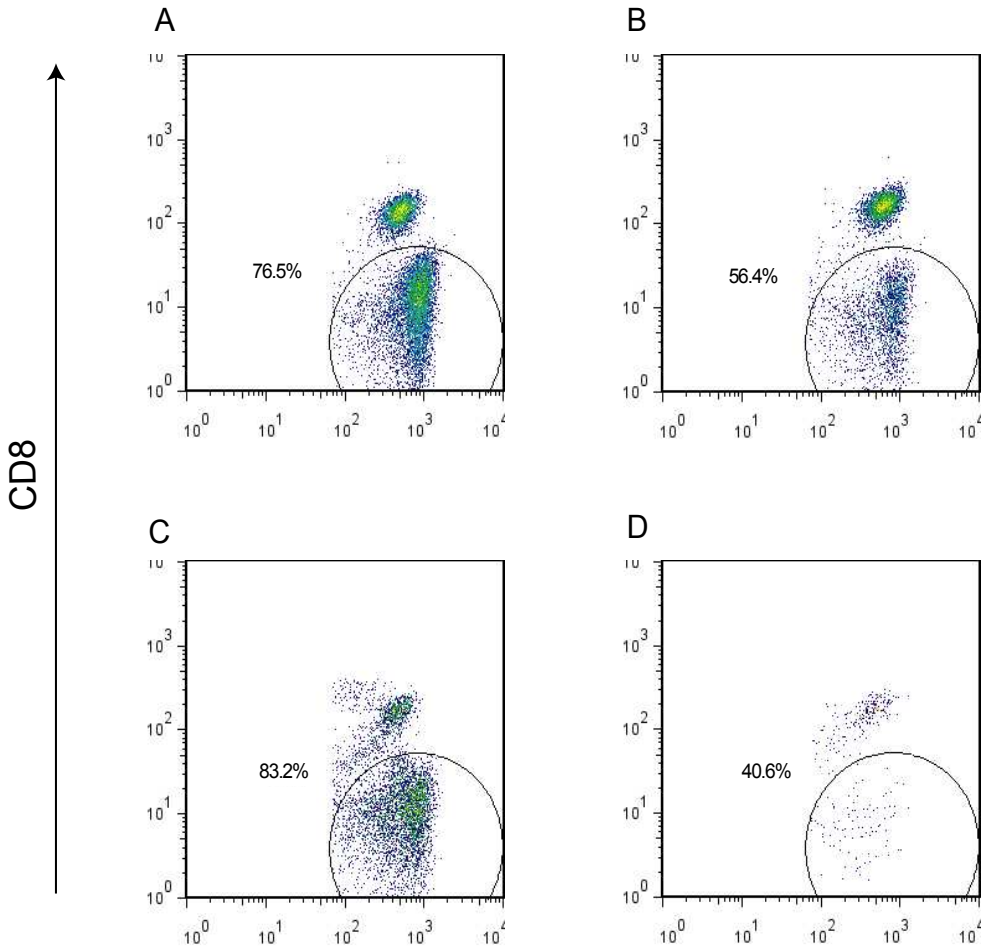

Figure 5.1. Depletion of CD4+ cells by mAb Gk1.5

Representative flow cytometry dot plots of splenocytes (A+B) and lung homogenates of CBA/Ca mice (C+D) collected 18h following pneumonia challenge with $8.5x10^6$ CFU D39 *S. pneumoniae* either without (A+C) or with (B+D) administration of 200µg GK1.5 i.p. 24h prior to infection. Cells were stained for CD3 and CD8, and percentage CD3+CD8- of total CD3+ is shown. Efficiency of depletion of the CD3+CD8- compartment was subsequently calculated by comparison to absolute CD3+ counts without GK1.5 treatment as described in Methods.

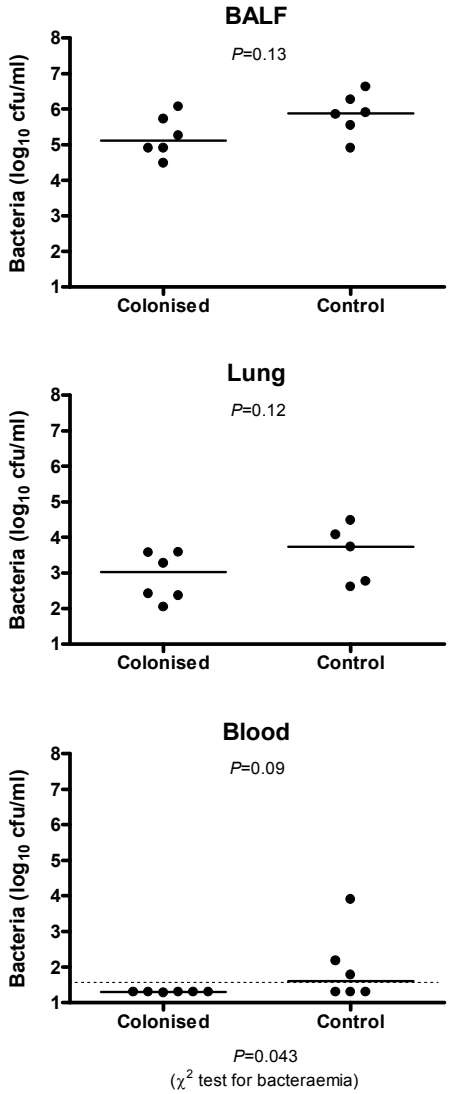

Figure 5.2. Effect of prior colonisation on bacterial loads in target organs following pneumonia challenge of mice depleted of CD4+ cells.

Previously colonised or control CBA/Ca mice were depleted of CD4+ cells by administration of mAb GK1.5 250µg i.p. 48h and 24h prior to i.n. challenge with 4.0×10^6 CFU D39 *S. pneumoniae*. Bacterial CFU were measured in BALF, lung and blood 18h following challenge. Dots represent data from individual mice, bars represent group medians. Dotted line indicated limit of detection. Comparison on numbers of CFU from colonised and control mice by Mann-Whitney U-test. Comparison for presence of bacteraemia by χ^2-test. Data representative of two experiments with similar results.

5.2.1.3 Effect of prior colonisation on cytokine responses during pneumonia in the absence of CD4+ cells

When CD4+ cells were present, no differences were found in the levels of KC, IL-6, TNF-α, GM-CSF, IL-1β and IL-12p70 between colonised and control mice at 18 h post-infection. However, levels of IL-17 were higher in both BALF and serum of previously colonised mice, suggestive of a Th17-cell response. Cytokine levels were therefore measured 18 h post-infection in the BALF and serum of the colonised and control mice that had been depleted of CD4+ cells prior to challenge, as shown in Figures 5.3 and 5.4. In the absence of CD4+ cells, there was no significant difference in BALF IL-17 levels between colonised and control mice (Figure 5.3), with mean 34.9 ± 26.2 pg/ml in colonised mice and 50.2 ± 14.5 pg/ml in controls (P=0.24). IL-17 levels in colonised mice depleted of CD4+ cells were similar to those previously obtained in undepleted controls (mean 27.6 ± 18.0 pg/ml (P=0.59). Interestingly, IL-17 was barely detectable in the serum of either colonised or control CD4+ cell depleted mice following challenge (Figure 5.3). No significant differences were observed between colonised and control CD4+ cell depleted mice in the levels of KC, IL-6, TNF-α, GM-CSF, IL-1β and IL-12p70 in BALF (Figure 5.4). Thus, the enhanced levels of IL-17 in BALF and serum during infection, attributable to prior colonisation, are dependant on the presence of CD4-cells at the time of challenge.

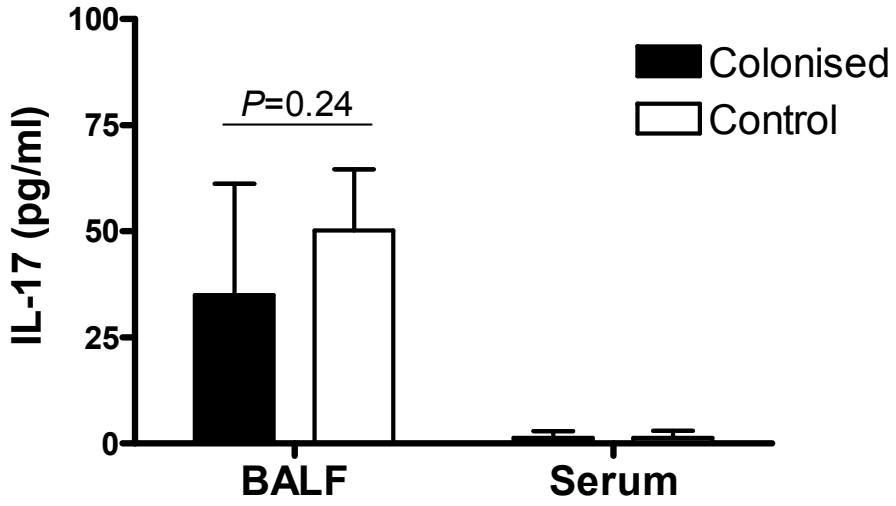

Figure 5.3. Effect of prior colonisation on BALF and serum IL-17 levels following pneumonia challenge of mice depleted of CD4+ cells.

Previously colonised or control CBA/Ca mice (n=6 per group) were depleted of CD4+ cells by administration of mAb GK1.5 250µg i.p. 48h and 24h prior to i.n. challenge with $4.0x10^6$ CFU D39 *S. pneumoniae*. Serum and BALF IL-17 collected 18h post-infection was measured by ELISA. Group means and SDs are shown, dotted line indicates limit of detection. Comparison between BALF IL-17 levels from colonised and control mice by unpaired Student t-test. Data representative of two experiments with similar results.

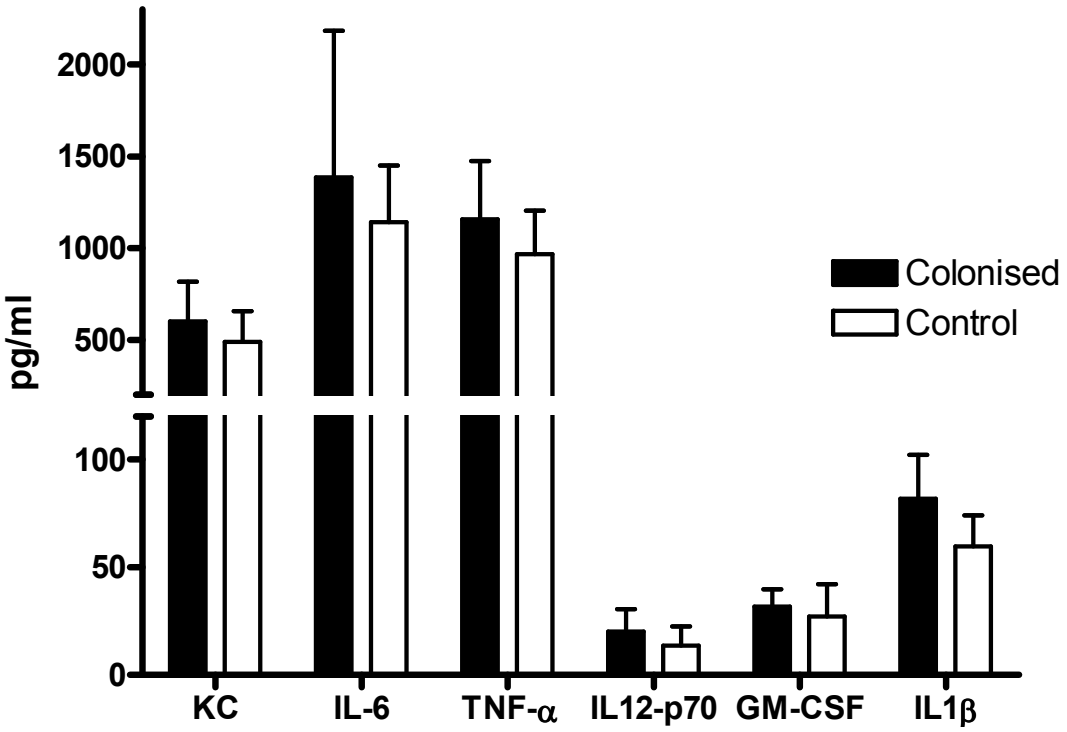

Figure 5.4. Effect of prior colonisation on levels of innate inflammatory mediators in BALF following pneumonia challenge of mice depleted of CD4+ cells.

Previously colonised or control CBA/Ca mice (n=6 per group) were depleted of CD4+ cells by administration of mAb GK1.5 250μg i.p. 48h and 24h prior to i.n. challenge with $4.0x10^6$ CFU D39 *S. pneumoniae*. BALF was collected 18h post-infection and levels of mediators measured by Luminex bead assay. Group means and SDs are shown. No significant differences were observed between colonised and control mice as assessed by unpaired Student t-test.

5.2.2 Effect of prior colonisation on subsequent pneumonia challenge in antibody deficient μMT mice

Given the redundancy of CD4+ cells in effecting protection against bacteraemia secondary to pneumonia, it was hypothesized that systemic antibody may play an essential role in mediating colonisation-induced protection. To test this hypothesis, experiments were performed involving the colonisation and infection of μMT mice, which congenitally lack antibody. C57/BL6 mice (the background strain from which μMT were produced) are known to be more resistant to *S. pneumoniae* pneumonia challenge than CBA/Ca mice (Gingles et al., 2001a). Therefore, prior to the definitive experiment, the pneumonia model in μMT mice was confirmed as equivalent to CBA/Ca mice as described below.

5.2.2.1 Establishing μMT mouse pneumonia model

To confirm that the pneumonia challenge model in μMT mice was equivalent to that in CBA/Ca mice, an initial experiment was undertaken to measure the bacterial loads in naïve μMT mice following pneumonia challenge. μMT mice (n=6) aged 10-12 weeks were infected with 9.2 x 10^6 CFU i.n. Mice were culled after 18 h and bacterial loads in target organs was assessed, as shown in Figure 5.5. For one mouse, bacteria were barely detectable in BALF and no bacteria were recovered from the lung or blood, suggesting that the mouse had not become infected. All other mice had bacteria recovered from both BALF and blood, and for 4 of 5 mice with lung infection, bacteraemia was also present. Median (IQR) CFU counts from BALF, lung and blood were 6.6 x 10^4 CFU/ml (0.15-34), 2.1 x 10^4 CFU/ml (0.008-2.8) and 1.52 x 10^5 CFU/ml (0.0002-17.5) respectively. These were similar to the bacterial loads in these organs in naïve CBA/Ca mice at 18 h following infection. Thus pneumonia

challenge of μMT mice provides an appropriate model for studying protective efficacy of colonisation in the absence of antibody.

5.2.2.2 Effect of prior colonisation on bacterial burden during pneumonia in μMT mice

To investigate the effect of prior colonisation on subsequent pneumonia in the absence of antibody, μMT mice (n=6) were colonised with 1.16 x 10^7 CFU D39. Control mice received PBS. After 28 days, all mice were challenged with 8.4 x 10^6 CFU i.n. Mice were culled at 18 h post-infection to assess bacterial load in BALF, lungs and blood (Figure 5.6). There were no significant differences in the number of bacteria recovered from either the BALF, lungs or blood between colonised and control μMT mice. Furthermore, all previously colonised μMT mice were bacteraemic at 18 h post-infection. This is in marked contrast to the strongly protective effect of prior colonisation in antibody-sufficient CBA/Ca mice. These data imply that colonised-induced antibody is required for limiting the lung bacterial load during pneumonia and in preventing bacteraemia.

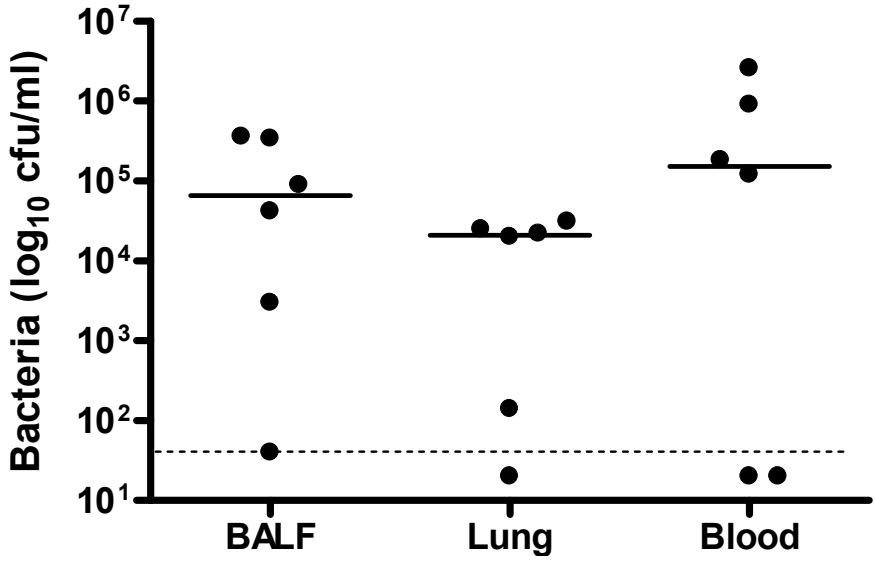

Figure 5.5. D39 S. pneumoniae pneumonia model in μMT mice.

Bacterial CFU recovered from BALF, lung and blood of μMT mice at 18h following challenge with $9.0x10^6$ CFU D39 *S. pneumoniae*. Dots represent data from individual mice, bars represent group medians. Dotted line indicated limit of detection.

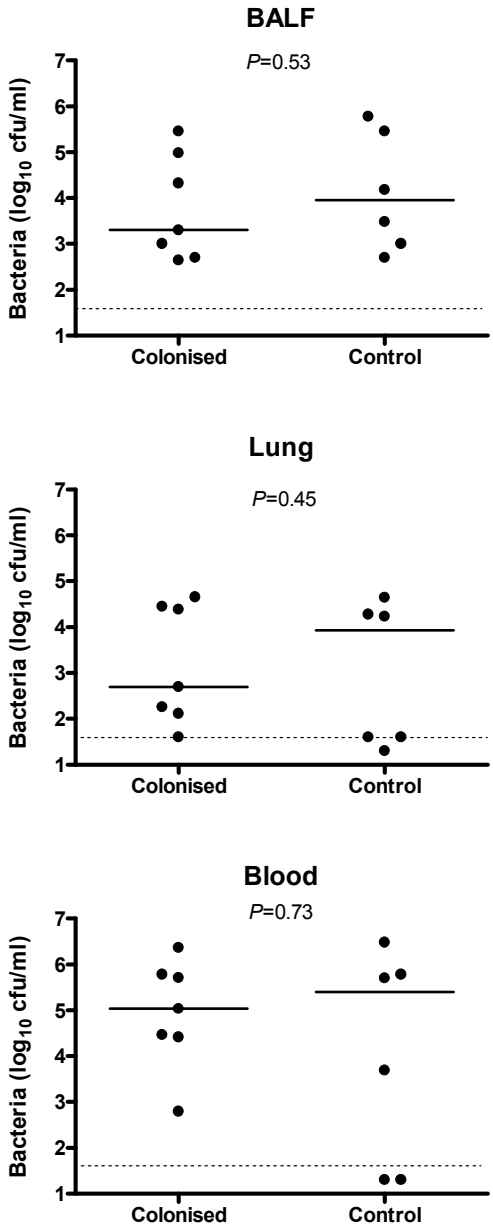

Figure 5.6. Effect of prior colonisation on bacterial load in target organs during D39 pneumonia in μMT mice.

Bacterial CFU recovered from BALF, lung and blood of previously colonised or control μMT mice at 18h following challenge with 8.1 x 10^6 CFU D39 *S. pneumoniae*. Dots represent data from individual mice, bars represent group medians. Dotted line indicated limit of detection. Comparison between colonised and controls by Mann-Whitney U-test.

5.2.3 Effect of passive transfer of serum from colonised mice on subsequent pneumonia challenge

To determine if systemic antibody alone was sufficient to protect against bacteraemia and whether it had any effect on lung bacterial burden passive serum transfer experiments were undertaken. In these experiments, groups of CBA/Ca mice were colonised with D39. Controls received PBS. Serum was collected from mice 28 days following colonisation. Groups of naïve recipient mice received pooled passive serum i.p. prior to challenge as described below. Mice were culled 18 h post-challenge and blood, lung and BALF collected. Bacterial loads in target organs were assessed.

5.2.3.1 Pilot experiment: effect of 'low dose' passive serum transfer on bacterial burden during pneumonia

In an initial pilot experiment, serum pools were prepared from three mice that had been colonised with 2.0 x 10^6 CFU i.n. and six control mice. The anti-D39 IgG titre for the pooled serum was 1/959. Naïve recipients (n=6 per group), aged 6-8 weeks, were given 100µl of either pooled colonised ('immune') or control serum i.p. 16 h later all mice were challenged with 2.0 x 10^6 CFU i.n. The bacterial loads in BALF, lung and blood at 18 h post-infection are shown in Figure 5.7. There were no significant differences in the amount of bacteria recovered from any organ between recipients of immune or control serum. It was noted that all recipients of control serum had high-level bacteraemia (>10^4 CFU/ml) at 18 h, despite the challenge inoculum not being high. This was possibly attributable to the recipient mice being aged 6-8 weeks old at challenge. In the experiments described previously where actively colonised mice were subsequently challenged, mice were 6-8 weeks old at colonisation and 10-12 weeks of age by the time of challenge. The relatively small

amount of serum passively transferred may have been insufficient to protect against challenge in more vulnerable younger mice. A further experiment was designed to overcome both of these issues.

5.2.3.2 Effect of 'high dose' passive serum transfer on bacterial burden during pneumonia

To increase the amount of passively transferred antibody, further groups of mice were colonised with D39 at 7.5 x 10^6 CFU i.n. Serum was pooled from 19 colonised donors ('immune serum') and 14 control donors. The anti-D39 IgG titre of the immune serum was 1/2260. Recipient mice were aged 10-12 weeks at the time of challenge in line with previous active colonisation experiments. Naive recipients (n=6 per group), received 2 doses of 225µl immune or control serum i.p. at 6 h and again 30 min prior to challenge. All recipient mice were challenged with 8.5 x 10^6 CFU i.n. Bacterial load in target organs was assessed 18 h post-infection (Figure 5.8). There was no difference in bacterial numbers in the BALF of recipients of immune or control serum. However, the median bacterial CFU was significantly lower (P=0.026) in the lungs of recipients of immune serum. Furthermore, only 1 of 6 recipients of immune serum was bacteraemic, compared to 5 of 6 controls (P=0.04).

To determine the efficiency of passive antibody transfer, the anti-D39 IgG levels in serum and BALF collected 18 h post-infection was measured by whole cell ELISA (Figure 5.9). All recipients of immune serum had detectable anti-D39 IgG in serum, with median titre 1/321 (IQR 1/194-1/411) (Figure 5.9A). Thus, the serum anti-D39 IgG level was approximately 7-fold less in the serum of recipients during infection than in actively colonised unchallenged mice. Anti-D39 IgG levels in the BALF of recipients of immune serum was also significantly higher than that of recipients of control serum (P=0.002) (Figure 5.9B). These data show that systemic antibody is sufficient to protect against bacteraemia and can limit bacterial load in the lungs.

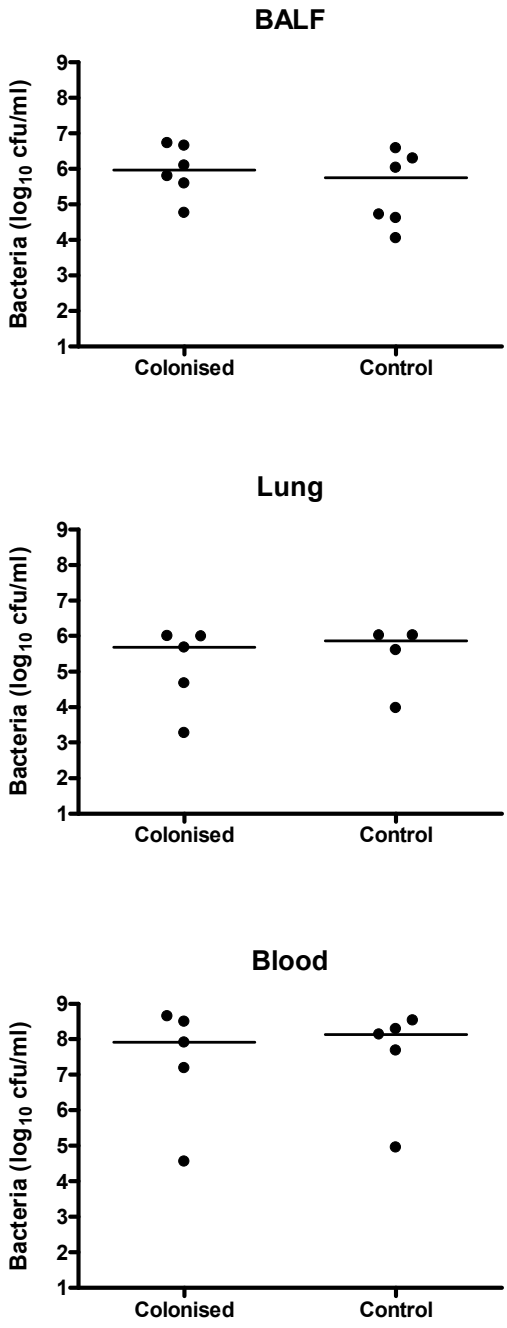

Figure 5.7. Effect of low dose passive transfer of serum from previously colonised mice on bacterial loads in target organs following pneumonia challenge of recipients.

100 µl of serum pooled from either previously colonised (n=3, pooled titre=1/959) or control (n=6) CBA/Ca mice was administered to naïve CBA/Ca recipients (n=6 per group) 16h prior to pneumonia challenge with $2x10^6$ CFU D39 *S. pneumoniae*. Bacterial CFU were measured in BALF, lung and blood 18h following challenge. Dots represent data from individual mice, bars represent group medians. Dotted line indicated limit of detection. Absent data points are due to contamination of cultures. No significant differences observed between colonised and control mice for any target organ by Mann-Whitney U-test.

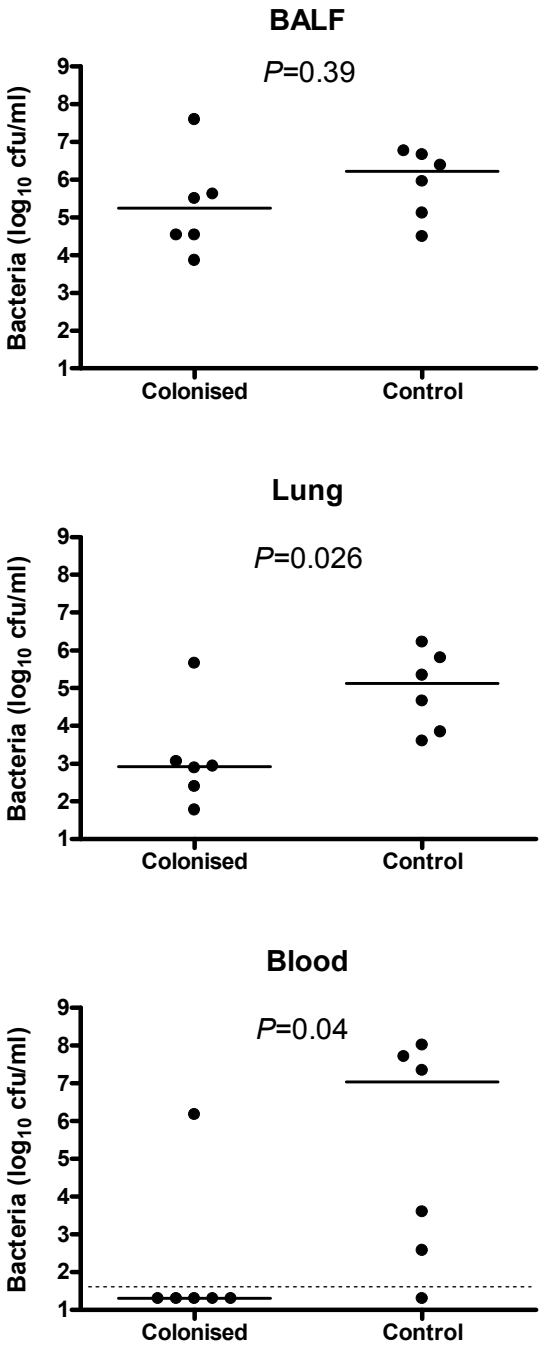

Figure 5.8. Effect of high dose passive transfer of serum from previously colonised mice on bacterial loads in target organs following pneumonia challenge of recipients.

225 µl of serum pooled from either previously colonised (n=19, pooled titre=1/2260) or control (n=14) CBA/Ca mice was administered to naïve CBA/Ca recipients (n=6 per group) 6h and 30min prior to pneumonia challenge with $8.5x10^6$ CFU D39 *S. pneumoniae*. Bacterial CFU were measured in BALF, lung and blood 18h following challenge. Dots represent data from individual mice, bars represent group medians. Dotted line indicated limit of detection. Groups compared by Mann-Whitney U-test.

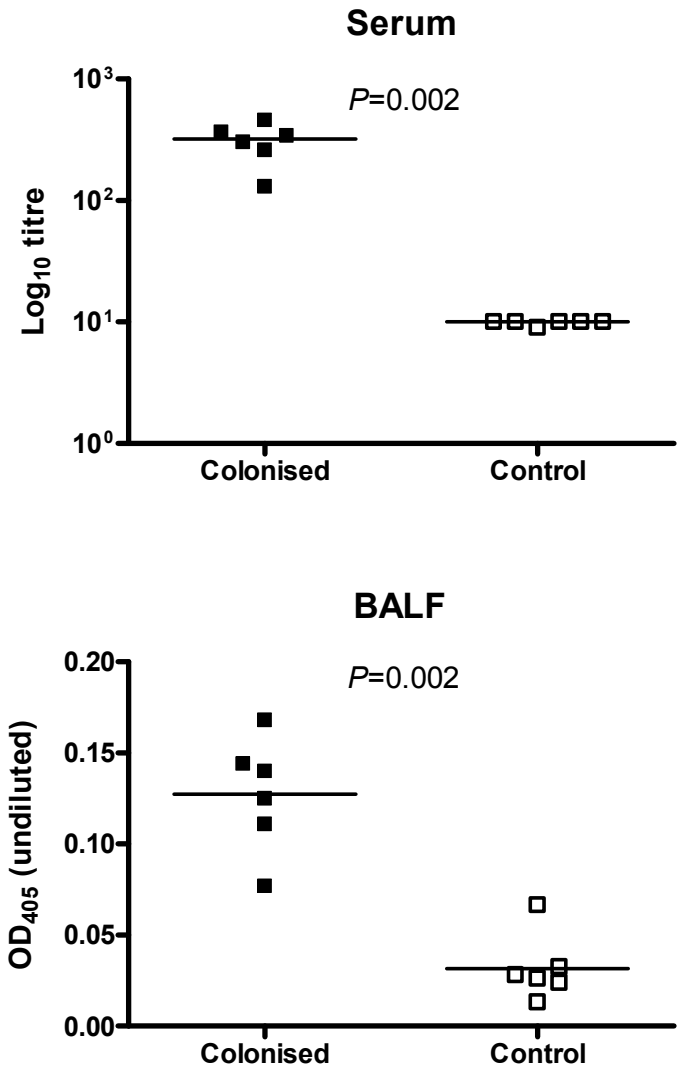

Figure 5.9. Effect of prior colonisation of donor mice on recipient serum and BALF anti-D39 IgG titres.

225 µl of serum pooled from either previously colonised (n=19, pooled titre=1/2260) or control (n=14) CBA/Ca mice was administered to naïve CBA/Ca recipients (n=6 per group) 6h and 30min prior to pneumonia challenge with 8.5×10^6 CFU D39 *S. pneumoniae*. Anti-D39 IgG was measured in recipient serum and BALF collected 18h following challenge. Dots represent data from individual mice, bars represent group medians. Groups compared by Mann-Whitney U-test.

5.3 SUMMARY OF RESULTS

In the absence of CD4+ cells at the time of pneumonia challenge, there was no increase in IL-17 levels in the BALF of previously colonised mice. Furthermore, IL-17 was barely detectable in the serum in either group. Despite this, previously colonised mice were still strongly protected against bacteraemia in the presence of a significant bacterial burden in the lung. These data suggest that memory CD4+ T-cells are a likely source of the enhanced IL-17 levels achieved through prior colonisation, but that such responses are redundant in the protection against bacteraemia. Colonisation did not induce protection in the absence of antibody in μMT mice. Thus colonisation-induced antibody responses are necessary to protect against bacteraemia during D39 pneumonia. Passive i.p. transfer of serum from previously colonised mice to naive recipients was sufficient to protect against bacteraemia. It also led to fewer bacteria within the lungs compared to controls. Anti-D39 IgG was found in both the serum and BALF of recipients. This implies that during infection there is either leak or transfer of systemic antibody into the alveolar spaces. Overall, the data presented in this chapter demonstrate that colonisation-induced serum antibody is both necessary and sufficient to protect against bacteraemia during subsequent pneumonia, and that CD4+ cells are redundant.

6 FUNCTION OF COLONISATION-INDUCED PROTECTIVE SERUM ANTIBODY

6.1 INTRODUCTION

During *S. pneumoniae* pneumonia, if bacterial growth is not controlled within alveolar spaces and lung tissues, bacteria may gain access to the circulation. Antibody present in the alveolar spaces may potentially control early infection by several mechanisms. Neutralisation of specific virulence factors required for invasion (Garcia-Suarez et al., 2004) may contain bacteria within the airways, whilst enhancement of opsonophagocytosis may assist in the airways or within lung tissue. Once in the bloodstream, *S. pneumoniae* replication may lead to lethal progression of disease, unless cleared by phagocytosis within the reticulo-endothelial system. Again, specific antibody may enhance this phagocytosis.

In the previous chapter, colonisation-induced serum antibody was both necessary and sufficient to protect against bacteraemia during subsequent pneumonia (Chapter 5). In this model, antibody was also present in the alveolar spaces during infection. In addition, antibody was able to leak or be actively transferred to the mucosa following systemic administration (Chapter 5). Data obtained following infection with fluorescently labeled bacteria (Chapter 4) suggested that colonisation did not affect early phagocytosis by alveolar macrophages. Furthermore, there were no differences in the numbers of bacteria recovered from BALF and lungs of colonised and control mice during pneumonia (Chapter 4). These observations suggest that serum antibody may be mediating protection primarily by facilitating rapid clearance of bacteria from the bloodstream through opsonophagocytosis. This hypothesis was investigated in this chapter by testing the functional ability of colonisation-induced serum antibody in a series of *in vitro* and *in vivo* assays. Binding of colonisation-induced serum IgG to a range of bacterial strains was assessed. The effect of colonisation on serum

enhancement of bacteria-phagocyte association was explored. Finally, the clearance of bacteria directly inoculated into the bloodstream was studied.

In this chapter, we addressed the following specific questions:

- Does colonisation-induced serum IgG to bind the surface of *S. pneumoniae*?
- Can colonisation-induced serum antibody enhance phagocytosis of *S. pneumoniae*?
- Does colonisation-induced serum antibody enhance bloodstream clearance of *S. pneumoniae*?

6.2 RESULTS

6.2.1 Effect of prior colonisation on serum IgG binding to bacterial surface

Colonisation-induced serum antibody was found to contain IgG that recognised D39 antigens in a whole cell ELISA. To test whether the anti-D39 IgG induced through colonisation specifically recognised surface-located antigens, further immunoassays were performed by flow cytometry using whole viable bacteria in suspension. These assays compared binding of serum IgG pooled from either control CBA/Ca mice ('naïve') or 28 days following D39 colonisation ('immune') to three D39 strains: D39 WT, D39Δ*lgt* (lacking surface lipoproteins) and D39-DΔ (lacking capsule) (see Figure 6.1) and to two TIGR4 strains: TIGR4 WT and TIGR4Δ*cps* (see Figure 6.2).

There was negligible IgG binding with naïve serum to either D39 WT or D39Δ*lgt* (Figure 6.1). However, in the absence of capsule, there was low level IgG binding from naïve serum to D39-DΔ (mean MFI 0.83 ± 0.12) (P=0.002 versus D39WT). There was significantly greater IgG binding from immune than naïve serum for D39WT (P=0.006), D39-DΔ (P<0.001) and D39Δ*lgt* (P<0.001), with stronger binding to D39-DΔ and weaker binding to D39Δ*lgt* than to D39WT with immune serum. This suggests that some IgG targets were non-capsular, and at least some were lipoproteins.

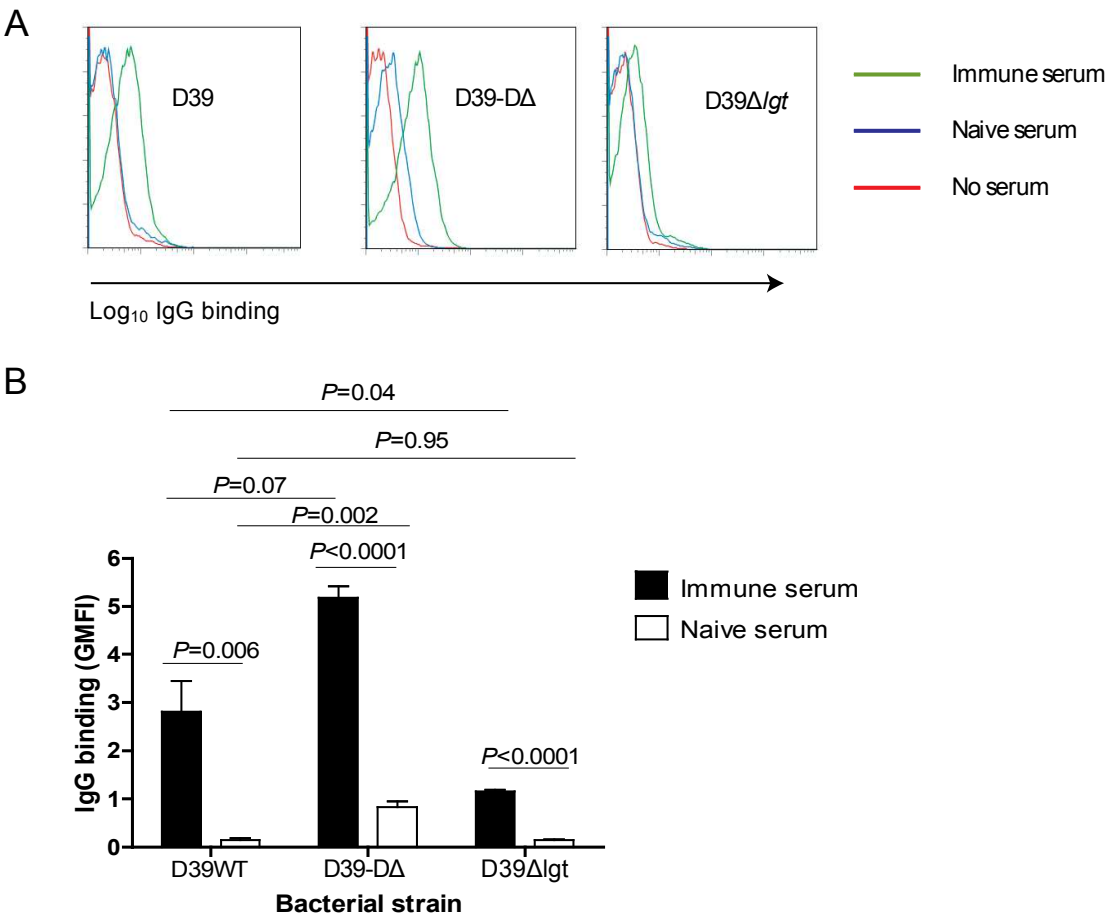

Figure 6.1. Effect of prior D39 colonisation on serum IgG binding to surface of D39 strains

Serum IgG binding from D39-colonised ('immune') or control ('naïve') mice to D39WT, D39-DΔ and D39Δ*lgt* was assessed by flow cytometry. Bacteria were incubated in 50% pooled serum (n=6 per group) then anti-mouse IgG-PE. (A) Typical histograms depicting IgG binding to each bacterial strain using pooled immune or naïve serum or no serum (PBS). There were no differences in background bacterial fluorescence between strains in the absence of serum. (B) Mean and SEM of IgG binding (n=4 replicates per set of conditions) after subtraction of background fluorescence. Groups compared by unpaired Student t-test. Data representative of two experiments performed with separate serum pools with similar results.

There was negligible IgG binding to encapsulated TIGR4 from either naïve or D39-immune serum (Figure 6.2). There was low level IgG binding from naïve serum to unencapsulated TIGR4Δ*cps* ($P<0.0001$ versus TIGR4), and this increased slightly with immune serum, although the level of IgG binding was still low (mean 0.09 ± 0.021 compared to 5.18 ± 0.23 for D39ΔD). This suggests that D39 colonisation induces IgG cross-reacting with TIGR4 non-capsular antigens. However, levels are low and only detectable in the absence of the capsule.

6.2.2 Effect of prior colonisation on in vitro serum opsonophagocytosis

The ability of serum IgG from colonised mice to bind the surface of D39 suggested the possibility that this opsonisation may enhance their phagocytosis. This would explain the marked ability of prior colonisation to protect against bacteraemia secondary to pneumonia. To test whether serum from colonised mice enhanced bacterial association with phagocytes, an *in vitro* neutrophil association assay was kindly performed by Dr Catherine Hyams. This assay utilized freshly isolated human neutrophils which were incubated with FAMSE-labeled D39WT bacteria that had been pre-incubated with serial dilutions of pooled CBA/Ca serum (Figure 6.3). There was a clear dose-response effect of colonised serum enhancing association of D39 bacteria with the neutrophils ($P<0.001$ for all dilutions). When this experiment was repeated using serum from μMT mice which congenitally lack antibody, no enhancement of phagocytosis was observed with serum from colonized mice (Figure 6.4). Thus, colonisation induces serum containing IgG that binds the bacterial surface and enhances *in vitro* association with neutrophils.

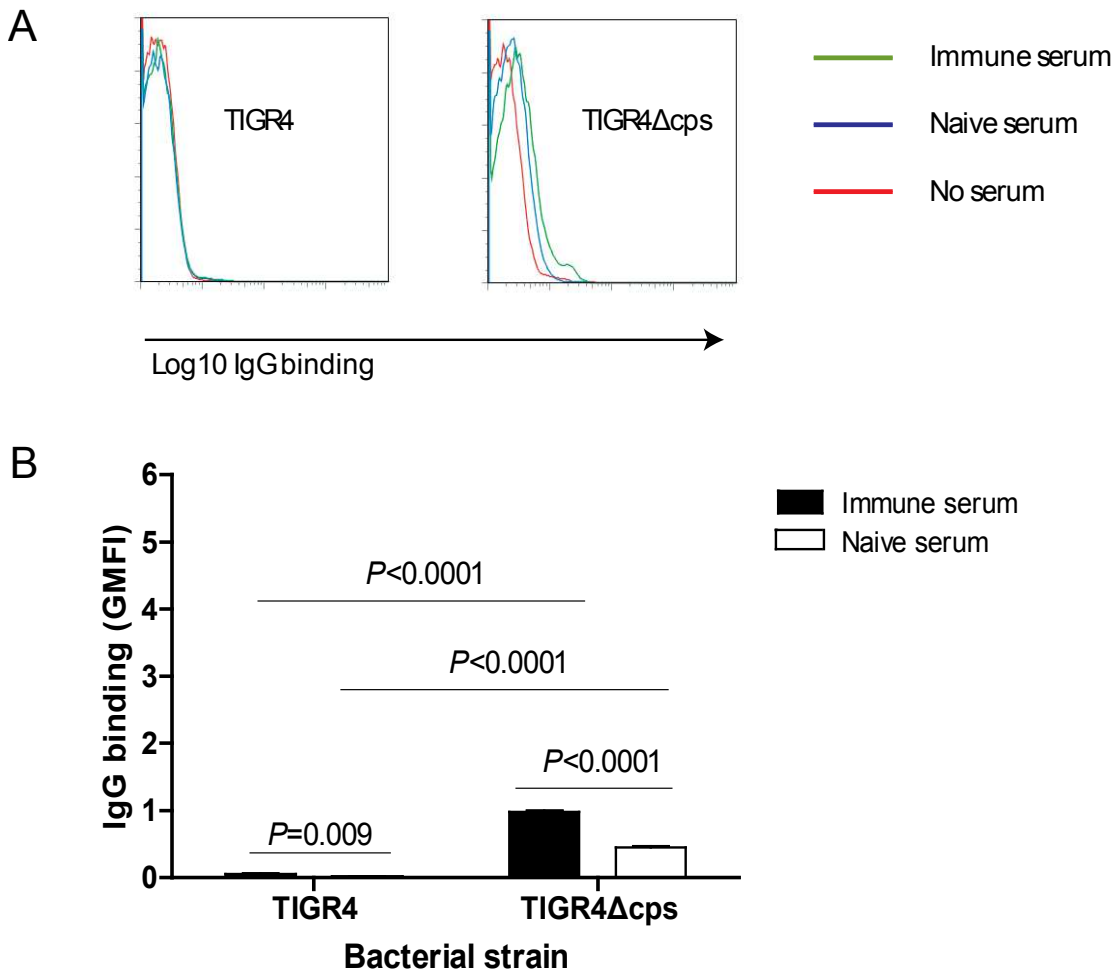

Figure 6.2. Effect of prior D39 colonisation on serum IgG binding to surface of TIGR4 strains

Serum IgG binding from D39-colonised ('immune') or control ('naïve') mice to TIGR4 WT and TIGR4Δ*cps* was assessed by flow cytometry. Washed viable bacteria were incubated in 50% pooled serum (n=6 per group) followed by anti-mouse IgG-PE. (A) Representative histograms depicting IgG binding to each bacterial strain using either pooled immune or naïve serum or no serum (PBS). There were no background differences in bacterial fluorescence between strains in the absence of serum. (B) Mean and SEM of IgG binding (n=4 replicates per set of conditions) after subtraction of background fluorescence. Groups compared by unpaired Student t-test.

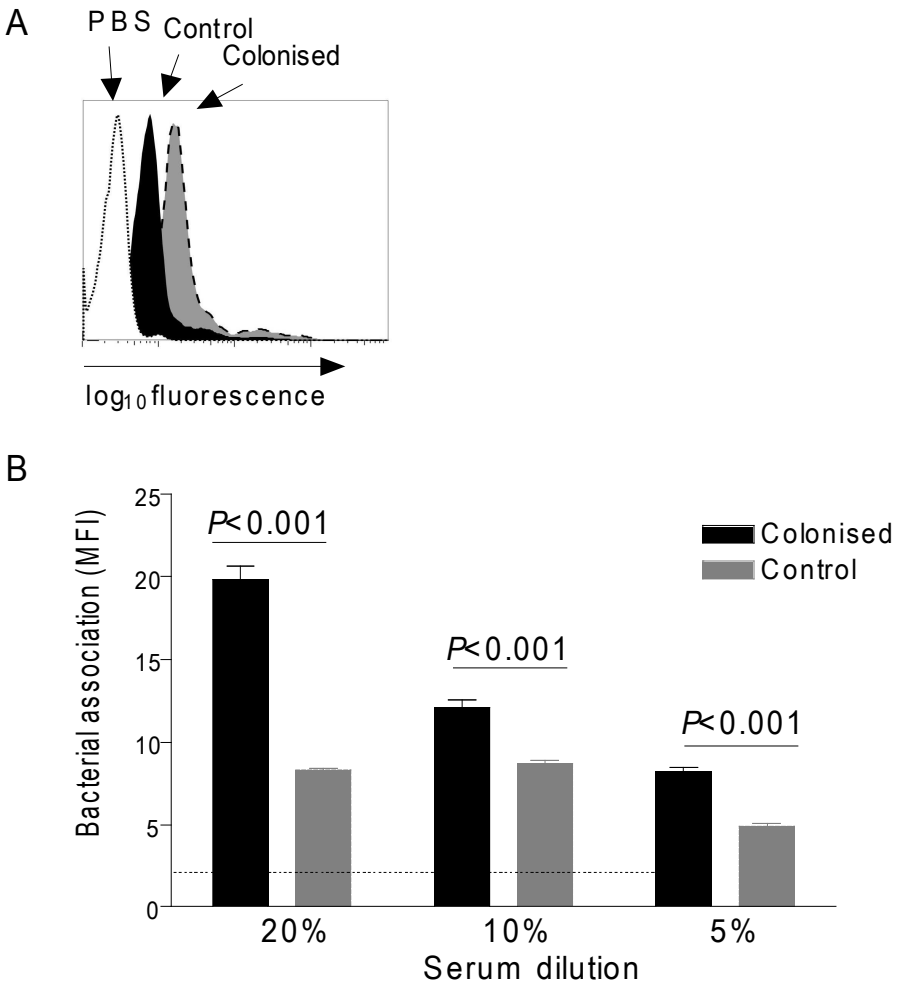

Figure 6.3. Effect of prior D39 colonisation of CBA/Ca mice on serum-mediated enhancement of bacteria-neutrophil association

Flow cytometry assay of human neutrophil association with FAM-SE labelled D39 after incubation in pooled serum from colonised or control CBA/Ca mice (n=6 per group) or PBS. (A) Representative histogram depicting fluorescence of neutrophils after incubation with bacteria pre-incubated with 20% serum from colonised or control mice or with PBS. (B) Mean neutrophil MFI ± SEM (n=4 replicates for each condition). Dotted line represents fluorescence with pre-incubation with PBS alone. Colonised and control serum compared across dilutions by one-way Anova with post-hoc tests.

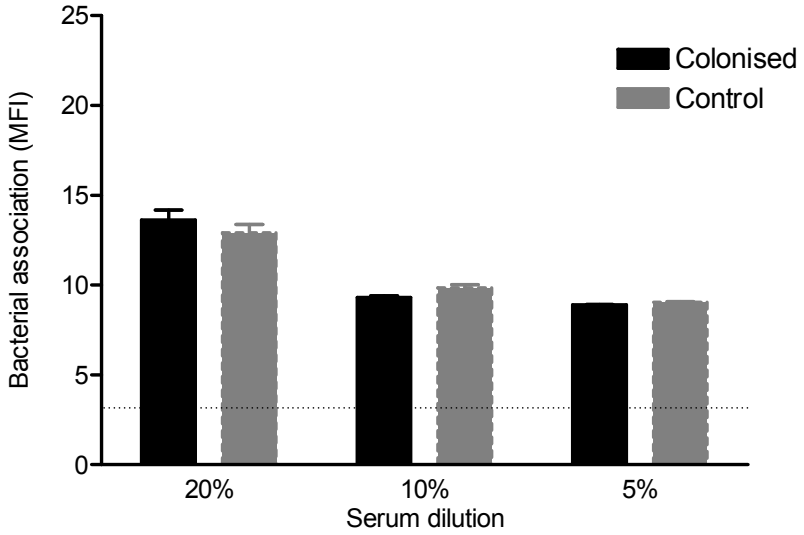

Figure 6.4. Effect of prior D39 colonisation of μMT mice on serum-mediated enhancement of bacteria-neutrophil association

Flow cytometry assay of human neutrophil association with FAM-SE labelled D39 after incubation in pooled serum from colonised or control μMT mice (n=6 per group) or PBS. Mean neutrophil MFI ± SEM are presented (n=4 replicates for each condition). Dotted line represents fluorescence with pre-incubation with PBS alone. No significant differences were observed when colonised and control sera were compared across dilutions by one-way Anova.

6.2.3 Effect of prior colonisation on bloodstream bacterial clearance

6.2.3.1 Effect of prior colonisation on bloodstream bacterial clearance by passive serum pre-opsonisation

To test whether such enhanced phagocyte association can lead to increased clearance of bacteria from the bloodstream, an experiment was performed where bacteria were incubated for 30 min in pooled undiluted serum from either colonised or control CBA/Ca mice, then washed prior to i.v. inoculation. Three naïve CBA/Ca mice received bacteria pre-incubated in serum from colonised mice. Four naïve CBA/Ca mice received bacteria pre-incubated in control serum. The actual inocula measured following pre-incubation were similar for the two groups (4.3×10^5 CFU for colonised and 5.0×10^5 for controls) indicating that pre-incubation had not affected the amount of viable bacteria the two groups received. Mice were tail-bled at two h and terminally bled at five h post-challenge. Viable bacteria were recovered from all mice as shown in Figure 6.5. There was a broad range in the numbers recovered from both groups at both timepoints, with no significant differences between pre-incubation with colonised and control serum.

It was possible in this passive pre-opsonisation model that bacterial cell division within the blood was greater than the enhancement in phagocytosis afforded by colonisation-induced antibody. Thus, although no differences were seen, this may reflect the insufficiency of the amount of antibody remaining on the bacterial surface at the time of inoculation, rather than an ability of this antibody to enhance bloodstream clearance per se.

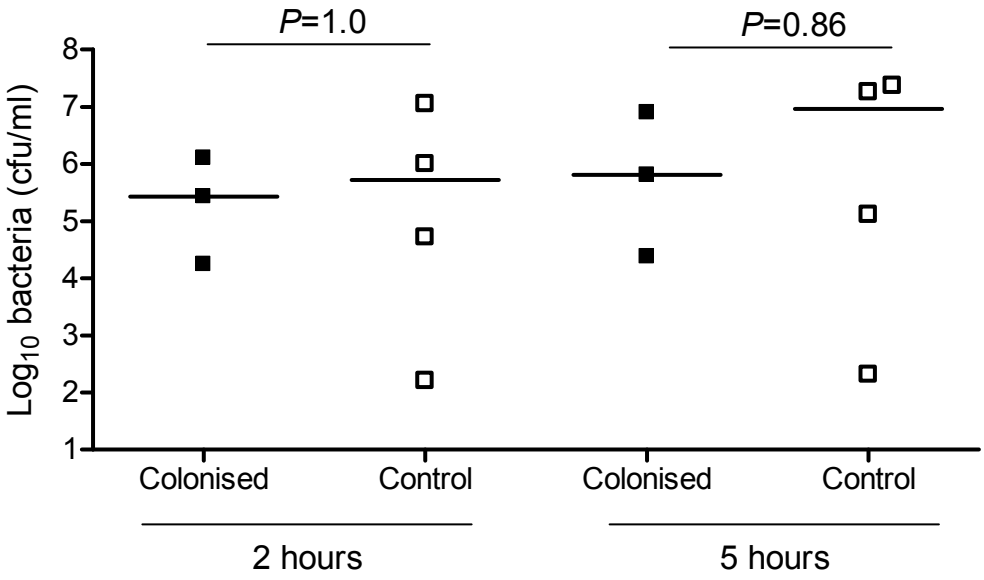

Figure 6.5. Effect on bloodstream clearance of D39 in naïve mice following pre-incubation of bacteria with serum of colonised or control mice

Log_{10} bacterial CFU recovered from the blood of naïve mice challenged by in inoculation with $5x10^5$ CFU D39 following pre-incubation in 50 µl pooled serum of colonised or control mice at 10^7 CFU/ml serum. Dots represent individual mice, bars represent group median. Groups compared at each timepoint by Mann-Whitney U-test.

6.2.3.2 Effect of prior active colonisation on bloodstream bacterial clearance

To test whether there is enhanced clearance of bacteria from the bloodstream of previously colonised mice, actively colonised CBA/Ca mice (colonisation inoculum 1.6 x 10^7 CFU D39) or controls were inoculated with 5 x 10^6 CFU D39 i.v. 28 days post-colonisation. Mice were bled at 2 and 5 h, and recovered CFU are shown in Figure 6.6. There were 10^4-fold fewer bacteria recovered from the bloodstream of previously colonised mice than controls at both timepoints tested (P=0.02 at both times). Thus, prior colonisation with D39 leads to marked enhancement of clearance of D39 bacteria from the bloodstream.

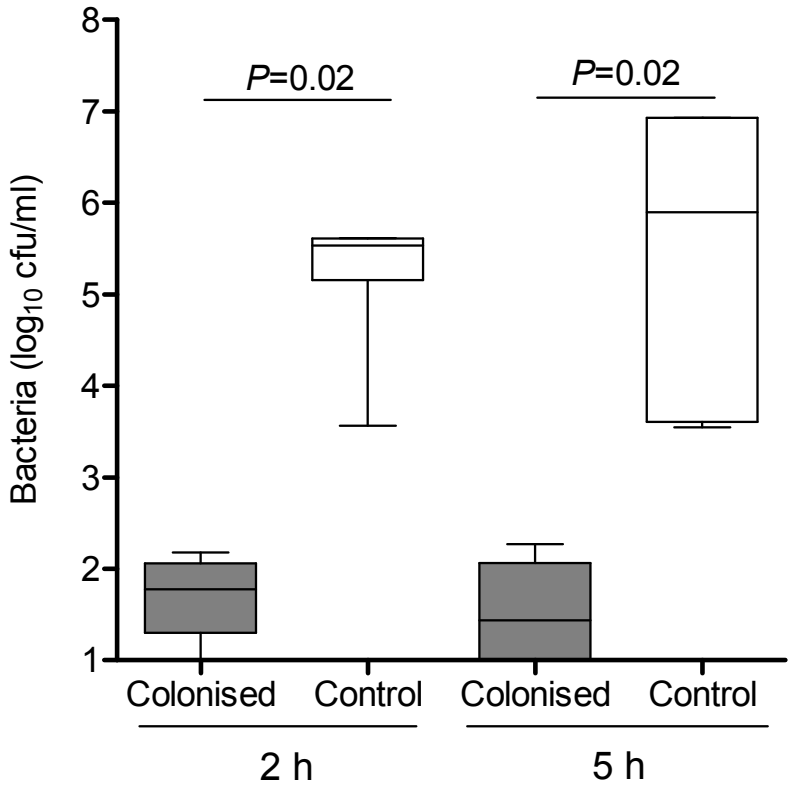

Figure 6.6. Effect of prior colonisation on bloodstream clearance of intravenous inoculum

Box-and-whisker plot showing $\log_{10}$ bacterial CFU recovered at 2 and 5 h from the blood of colonised or control mice inoculated i.v. with $5x10^6$ CFU *S. pneumoniae* D39 on day 28 post-colonisation (n=6 per group). Groups compared at each timepoint by Mann-Whitney U-test.

6.3 SUMMARY OF RESULTS

Colonisation with D39 induces serum IgG that bound the surface of D39. There was greater binding to unencapsulated D39-DΔ than to its parent wild-type strain. There was reduced binding to D39Δ*lgt*. There was negligible binding to the surface of encapsulated WT TIGR4. When unencapsulated, there was more binding to TIGR4Δ*cps*, but still significantly less than to unencapsulated D39. These results suggested that colonisation induced serum IgG that recognised non-capsular antigens located on the surface of D39, and to a lesser extent on TIGR4. At least some of these antigens were likely to be lipoproteins. Serum from colonised CBA/Ca mice enhanced the association of D39 bacteria with human neutrophils, but serum from colonised μMT mice did not. This may indicate enhanced opsonophagocytosis attributable to colonisation-induced antibody. Using serum from colonised mice to pre-incubate D39 bacteria prior to i.v. inoculation did not enhance their clearance from the bloodstream, possibly reflecting the limited amount of antibody present on the bacteria at the time of inoculation. However, previous colonisation led to over 10^4-fold fewer bacteria being recovered from the bloodstream following direct i.v. inoculation, indicating the capacity of colonisation-induced serum to clear bloodstream bacteria.

Overall, colonisation induces serum IgG that binds the bacterial surface, enhances opsonophagocytosis, and leads to rapid clearance of bacteria from the bloodstream.

7 ANTIGENIC TARGETS OF COLONISATION-INDUCED SERUM IgG

7.1 INTRODUCTION

To date, the only proven correlate of protection against invasive human pneumococcal disease is opsonophagocytic anti-capsular antibody (Jokinen et al., 2004). Antibodies induced by immunisation with purified pneumococcal proteins are also protective in animal models of pneumococcal disease (Briles et al., 2000a, Brown et al., 2001b, Giefing et al., 2008). In some cases, anti-protein antibody protects through opsonophagocytosis, indicating that this is not the sole preserve of anti-capsular antibody. Furthermore, natural antibodies to cell-wall polysaccharide can also be protective against pneumococcal sepsis in animal models (Briles et al., 1981, Goldenberg et al., 2004).

The colonised-induced protection against invasive disease demonstrated in Chapter 4 appeared to be mediated by serum antibody (Chapter 5) that is opsonophagocytic and enhances bloodstream clearance (Chapter 6). Colonisation induced serum IgG binding D39 antigens in whole cell ELISA at titres over 1/1000 in nearly all mice (Chapter 4). Similarly, nearly all colonised mice survived lethal pneumonia challenge (Chapter 4). In contrast, serum IgA and IgM that bound D39 antigens was induced in only a small proportion of colonised mice, and therefore cannot explain the degree of protection observed. Thus, colonisation-induced serum IgG is mediating protection against lethal pneumonia in this model.

Binding of serum IgG from colonised mice to the surface of both encapsulated and non-encapsulated D39 strains was demonstrated using flow cytometry (Chapter 6). This would suggest that at least some antigenic targets of colonisation-induced IgG are non-capsular antigens. Reduced binding to the D39Δ*lgt* mutant implies that a proportion of these antigens may be surface lipoproteins. There was also weak

binding to the surface of unencapsulated TIGR4Δ*cps*, suggesting that serum IgG may cross-react with surface-expressed proteins from other strains of *S. pneumoniae*.

This chapter attempted to identify antigenic targets of colonisation-induced serum IgG which may be mediating its protective effects. Individual and pooled sera from colonised and control mice were tested for their ability to bind a range of pneumococcal polysaccharide and protein antigens. Anti-capsular and anti-CWPS responses were measured by ELISA. Serum IgG binding to pneumococcal proteins was explored using immunoblotting against lysates of different pneumococcal strains. Finally, binding to specific recombinant pneumococcal proteins was tested by immunoblotting and Luminex bead immunoassay techniques.

In this chapter, we addressed the following specific questions:

- Does colonisation induce serum anti-capsular antibody?
- Does colonisation induce serum anti-CWPS antibody?
- Does colonisation-induced serum IgG bind to pneumococcal proteins, and if so, which proteins?

7.2 RESULTS

7.2.1 Anti-polysaccharide antibody responses to colonisation

Serum antibody to both capsular and cell wall polysaccharide can be protective in animal models of invasive pneumococcal disease. Anti-capsular polysaccharide antibody can be induced through colonisation, although this appears to be very variable in both humans and mouse models, and may depend on serotype. Serum anti-type 2 capsular polysaccharide and anti-cell wall polysaccharide were therefore measured by ELISA in the serum of D39 colonised (n=11-13) or PBS controls (n=5-8). Colonised mice received 0.6 x 10^7 CFU D39. All mice were bled after 28 days.

7.2.1.1 Anti-capsular polysaccharide antibody responses

No anti-type 2 capsular IgG or IgA was detected in the serum of either colonised or control mice (Figure 7.1). Whilst serum IgM against capsule was detected, the levels were very low, with a median OD_{405} at 1:20 serum dilution of 0.29 (IQR 0.21-0.52) in colonised mice and 0.16 (0.05-0.25) in controls (P=0.057). These median OD levels were equivalent to titres of approximately 1/64 for colonised mice and 1/38 for controls. Thus, no IgG or IgA against capsular polysaccharide was induced through colonisation. Whilst there was a trend towards marginally higher levels of anti-capsule IgM in colonised mice, this difference was no greater than the background levels observed in the controls themselves.

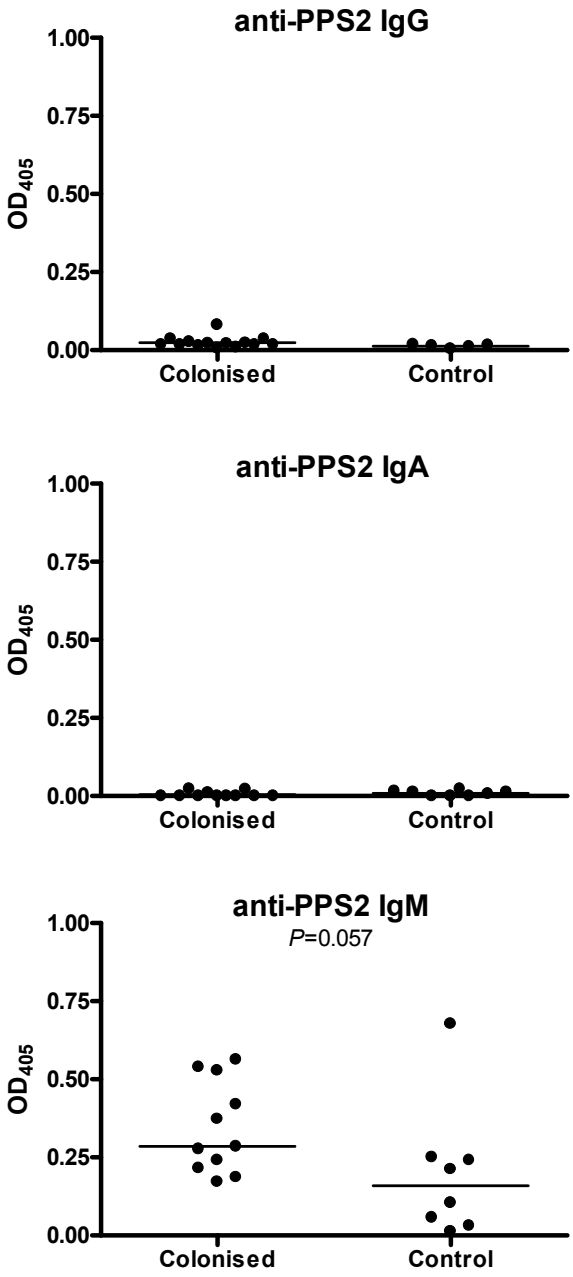

Figure 7.1. Effect of D39 colonisation on serum anti-type 2 capsular polysaccharide antibody.

Serum anti-type 2 capsular polysaccharide (PPS2) antibody responses 28 days following colonisation with *S. pneumoniae* strain D39 measured by ELISA at 1:20 serum dilution. (n=14 for colonised mice, n=8 for controls). Dots represent individual mice, bars represent group medians. Anti-PPS2 IgM levels in colonised and control mice compared by Mann-Whitney U-test.

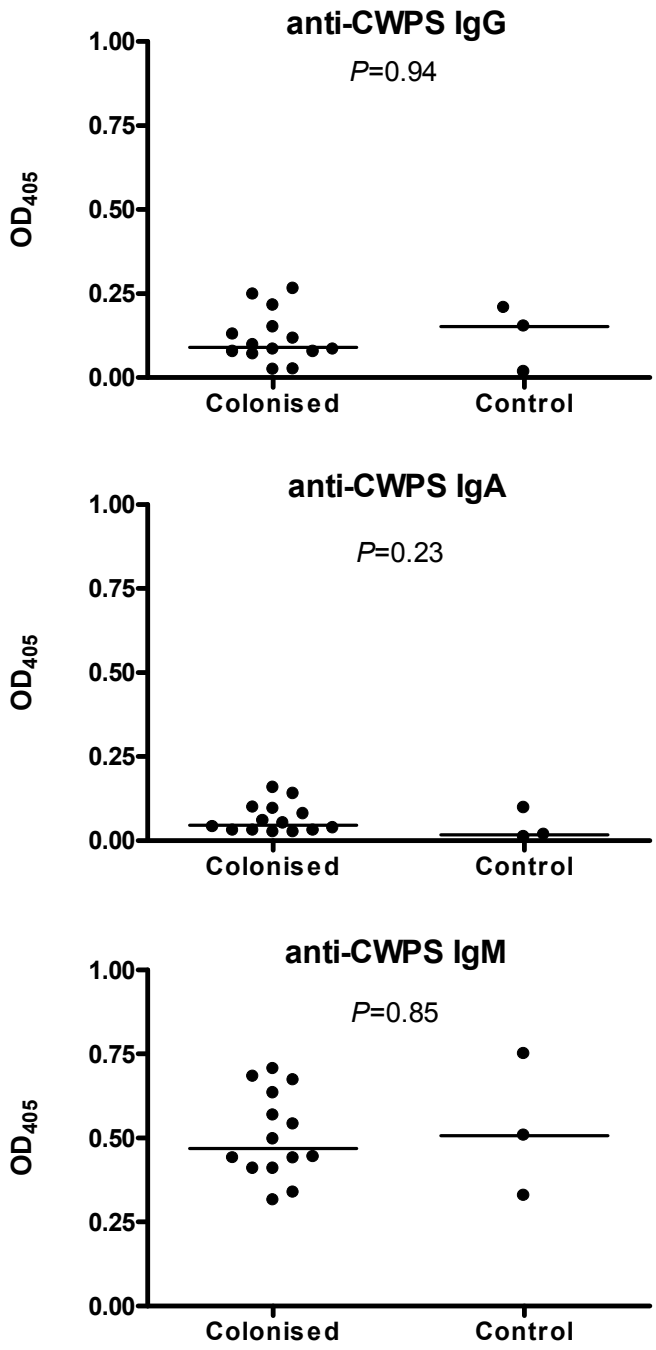

Figure 7.2. Effect of D39 colonisation on serum anti-cell wall polysaccharide antibody. Serum anti-cell wall polysaccharide (CWPS) antibody responses 28 days following colonisation with *S. pneumoniae* strain D39 measured by ELISA at 1:20 serum dilution. (n=14 for colonised mice, n=3 for controls). Dots represent individual mice, bars represent group medians, comparison by Mann-Whitney U-test.

7.2.1.2 Anti-cell wall polysaccharide antibody responses

Limited serum samples from control mice were available for this analysis. Nevertheless, there was no evidence of either serum anti-cell wall polysaccharide IgG, IgA or IgM responses induced by colonisation (Figure 7.2). Thus, it appeared that the marked efficacy of colonisation-induced systemic antibody in protecting against bacteraemia in this model was not being mediated by anti-polysaccharide responses.

7.2.2 Serum anti-protein IgG responses detected by immunoblot of bacterial lysates

To screen for the presence of IgG against unknown bacterial proteins, lysates of *S. pneumoniae* were prepared, the proteins separated by SDS-PAGE, and the blotted membranes probed with serum pooled from either colonised or control mice (n=6 mice per pool) (Figure 7.3). These serum pools are referred to as 'immune' or 'control' serum hereafter. Coomassie blue stained gel and immunoblots of proteins from lysates of D39 WT and D39Δ*lgt* are shown in Figure 7.3. Staining the gel revealed a large number of proteins present. When the D39 WT immunoblot was probed with immune serum, however, only four strong bands and a limited further number of weaker bands were visible. No bands were visible when membranes were probed with control serum. When the immune serum was used to probe proteins from the D39Δ*lgt* lysates, several bands of approximately 35-45kDa were absent. A number of pneumococcal lipoproteins are known to be in this size region. Thus, colonisation with D39 induced serum IgG against a limited number of protein antigens, including some lipoproteins. As the sequence of many pneumococcal proteins is conserved between serotypes, the ability of D39 colonisation-induced serum IgG to bind proteins derived from other *S. pneumoniae* strains was tested.

Lysates were prepared of strains TIGR4 and 0100993 and immunoblotted to detect IgG binding from colonised serum (Figure 7.3). Although there were some differences, especially in the larger proteins, similar patterns of bands to D39 were detected. This suggested that serum IgG induced by D39 colonisation cross-reacted with similar proteins in different pneumococcal strains. To identify whether there is variation in which proteins dominated the serum IgG response to D39 colonisation between mice, lysates of D39 were also probed by individual sera from seven colonised mice (Figure 7.4). Similar binding patterns were seen between sera, differing only in overall binding strength. This suggested that specific proteins dominate the immune response to colonisation in this inbred mouse strain.

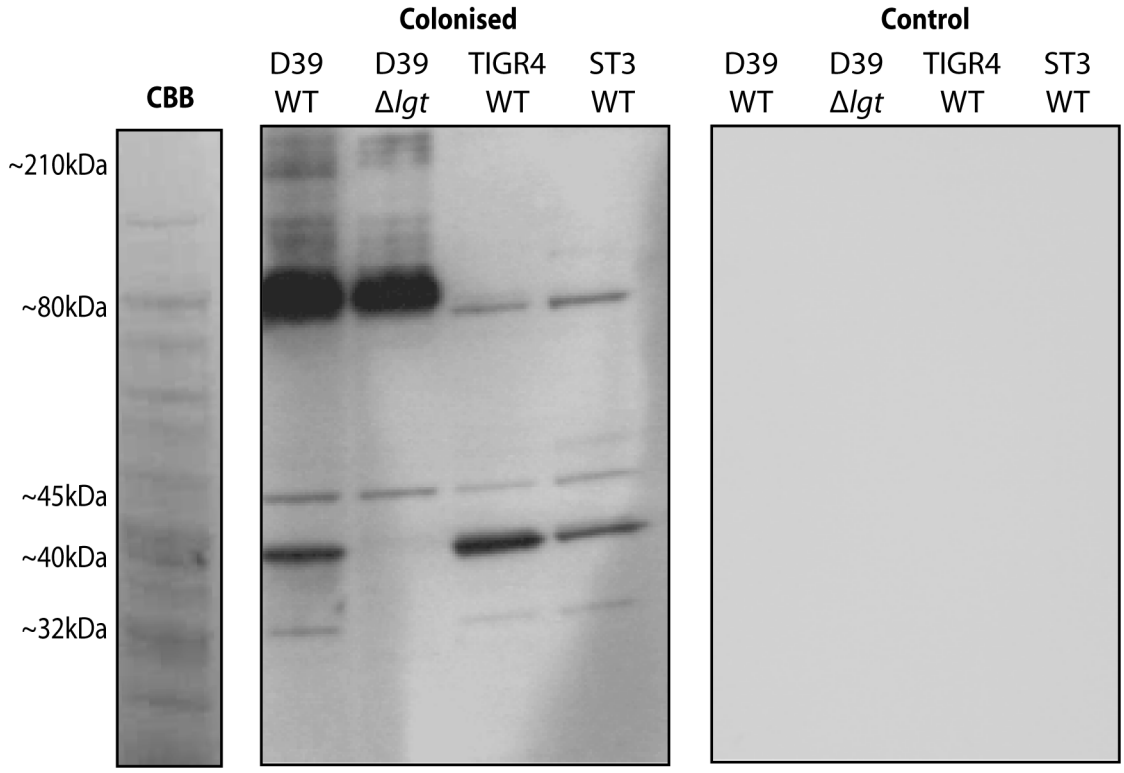

Figure 7.3. Serum IgG binding to pneumococcal lysate proteins in pooled sera of colonised and control mice.

Immunoblots of IgG binding in pooled sera from colonised and control mice to whole cell lysates of D39, D39Δlgt, TIGR4 and ST3 *S. pneumoniae* strains separated by SDS-PAGE. A Coomassie brilliant blue stained SDS-PAGE of the D39 lysate is shown (CBB). Data representative of two experiments with different serum pools with similar results.

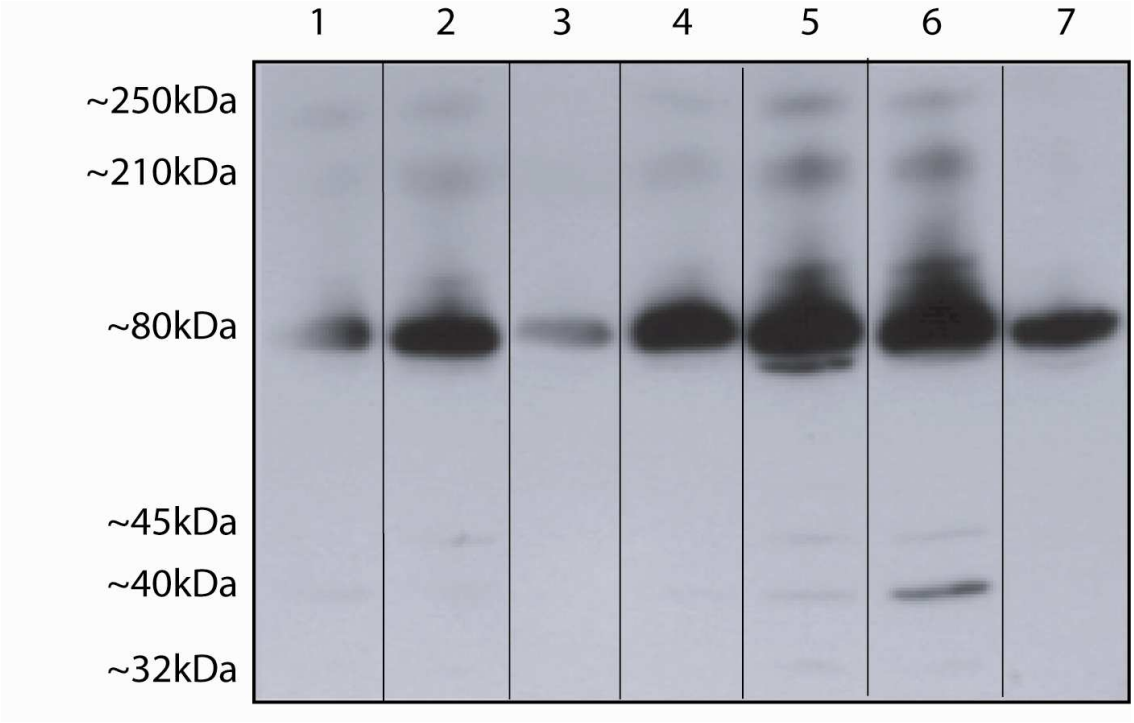

Figure 7.4. Serum IgG binding to pneumococcal lysate proteins in individual sera of colonised mice.

Immunoblots of IgG binding to whole cell lysates of D39 using individual sera from 7 colonised mice.

7.2.3 Identification of specific protein targets of colonisation-induced serum IgG

To identify specific protein targets of colonisation-induced serum anti-D39 IgG, two approaches were taken. Firstly, immunoblotting was performed using two sets of recombinant pneumococcal proteins. One set consisted of fifteen proteins derived from the genome of the TIGR4 strain of *S. pneumoniae*. The second set consisted of proteins derived from the genome of the 0100993 strain (Table 7.1). These TIGR4 proteins were selected as they are known to induce serum antibody responses in humans following either colonisation or pneumonia (Giefing et al., 2008). The second approach was bead immunoassay using Luminex beads conjugated to sixteen pneumococcal proteins from several strain sources (Table 7.1). The broadly similar immunoblot binding patterns seen with bacterial lysates of D39, 0100993 and TIGR4 suggested that using proteins from heterologous strains was likely to positively identify some target antigens. As negative controls, both assays incorporated TIGR4 pilus proteins absent in D39 (RrgB in immunoblots and RrgA in bead immunoassay (Gianfaldoni et al., 2007)). To aid the interpretation of negative results which could be due to antigenic variation rather than lack of antibody against homologous antigen, the predicted amino acid sequences of the proteins based on the D39 and TIGR4 genomes were compared. These were obtained by BLAST analysis of the amino acid sequences. All protein antigens and the BLAST results are listed in Table 7.1, along with summarized results of the immunoassays described below.

Table 7.1. ***S. pneumoniae*** **protein antigens for which antibody responses in colonised sera were investigated by immunoblots and Luminex bead assays.**

Assay antigen[6]	D39 gene identifier	%Identity[7]	Results for colonised sera[8]		Antigen protects against sepsis[9]	Antigen protects against pneumonia
			Bead assay	Immunoblot		
Choline-binding proteins						
PspA	SPD0126	57	+	+	(Wu et al., 1997a)	(Briles et al., 2003)
PspC	SPD2017	73	-	-	(Ogunniyi et al., 2001)	
CbpD*	SPD2028	97	-	nt		
Lipoproteins						
PsaA	SPD1463	99	+	+	(Talkington et al., 1996)	
SlrA*	SPD0672	99	-	nt	(Audouy et al., 2007)	(Audouy et al., 2007)
PpmA*	SPD0868	99	+	nt		
AmiA	SPD1671	99	nt	+		
AliB	SPD1357	100	nt	-		
PiaA+	SPD0916	99	nt	-	(Brown et al., 2001b)	(Jomaa et al., 2006)
PiuA+	SPD1652	99	nt	-	(Brown et al., 2001b)	(Jomaa et al., 2006)

[6]Proteins expressed using TIGR4 gene unless otherwise specified as *D39, +ST3 or §serotype 23 strain
[7]Percentage amino acid identity between TIGR4 and D39 homologues
[8]For sera from colonised mice, + = IgG detected and - = IgG not detected, nt = not tested
[9]Recombinant protein antigen protective when used as a vaccine in animal models of disease

SP0149	SPD0151	99	nt	-		
SP0749	SPD0652	100	nt	-		
MalX	SPD1934	99	nt	+		
Other						
PsaD	SPD1464	100	-	nt		
PhtD	SPD0889	94	+	nt	(Adamou et al., 2001)	
PhtE	SPD0890	99	-	nt	(Hamel et al., 2004)	(Hamel et al., 2004)
SP0376	SPD0344	99	-	nt		
Eno*	SPD1012	99	-	nt		
Ply*	SPD1726	99	-	-	(Alexander et al., 1994)	(Alexander et al., 1994)
NanA	SPD1504	99	-	nt	(Long et al., 2004)	
Hyaluronidase§	SPD0287	98	-	nt		
IgA1 protease	SPD1018	87	-	nt	(Audouy et al., 2007)	(Audouy et al., 2007)
PavB	SPD0080	99	nt	-		
LysM	SPD0104	85	nt	-		
SP1522	SPD1350	99	nt	-		
StkP[10]	SPD1542	99	nt	+	(Giefing et al., 2008)	
SP1954	SPD1753	99	nt	-		
Cbf1	SPD1777	99	nt	-		
SP2141	SPD1969	99	nt	+		

[10]C-terminal truncated protein as described (Giefing et al., 2008)

PcsB[11]	SPD2043	100	nt	+	(Giefing et al., 2008)
Negative control proteins[12]					
RrgA	-	-	-	nt	(Gianfaldoni et al., 2007)
RrgB	-	-	nt	-	(Gianfaldoni et al., 2007)

[11]N-terminal truncated protein as described (Giefing et al., 2008)
[12]Negative control TIGR4 pilus proteins from TIGR4 absent in D39

7.2.3.1 Detection of serum IgG binding to *S. pneumoniae* proteins by immunoblot

To identify binding of IgG in immune and control serum to TIGR4 proteins, the proteins were separated by SDS-PAGE and IgG binding with immune and naive serum to specific proteins identified by Western blotting (Figure 7.5). When probed with immune serum there were dense bands indicating IgG binding to SktP (lane 1), PsaA (lane4) and PcsB (lane 6). Binding was also evident to MalX and AmiA (lane 2), PspA (lane 5) and SP2141 (lane 6). The densest band when probed with immune serum was SktP (lane 1). Although there was a faint band when this protein was probed with control serum, the marked difference was indicative of colonisation augmenting the level of IgG to this antigen. Similar faint binding was also seen for Ply (lane 4) with both immune and control serum. There was no IgG binding to LysM, SP1954, AliB, PspC, Cbf1, PavB or the negative control protein RrgB with either immune or naïve serum. Thus, colonisation with *S. pneumoniae* strain D39 appeared to induce serum IgG capable of binding to the TIGR4 recombinant proteins PsaA, PcsB, MalX, AmiA, PspA, SP2141 and SktP.

Further immunoblots were performed as above against four pneumococcal lipoproteins synthesised from strain ST3 genes, as shown in Figure 7.6. There was faint binding of both immune and naïve serum IgG to PiuA and SP0149. No binding was evident for PiaA or SP0749. Thus, colonisation did not appear to induce IgG to any of these four proteins.

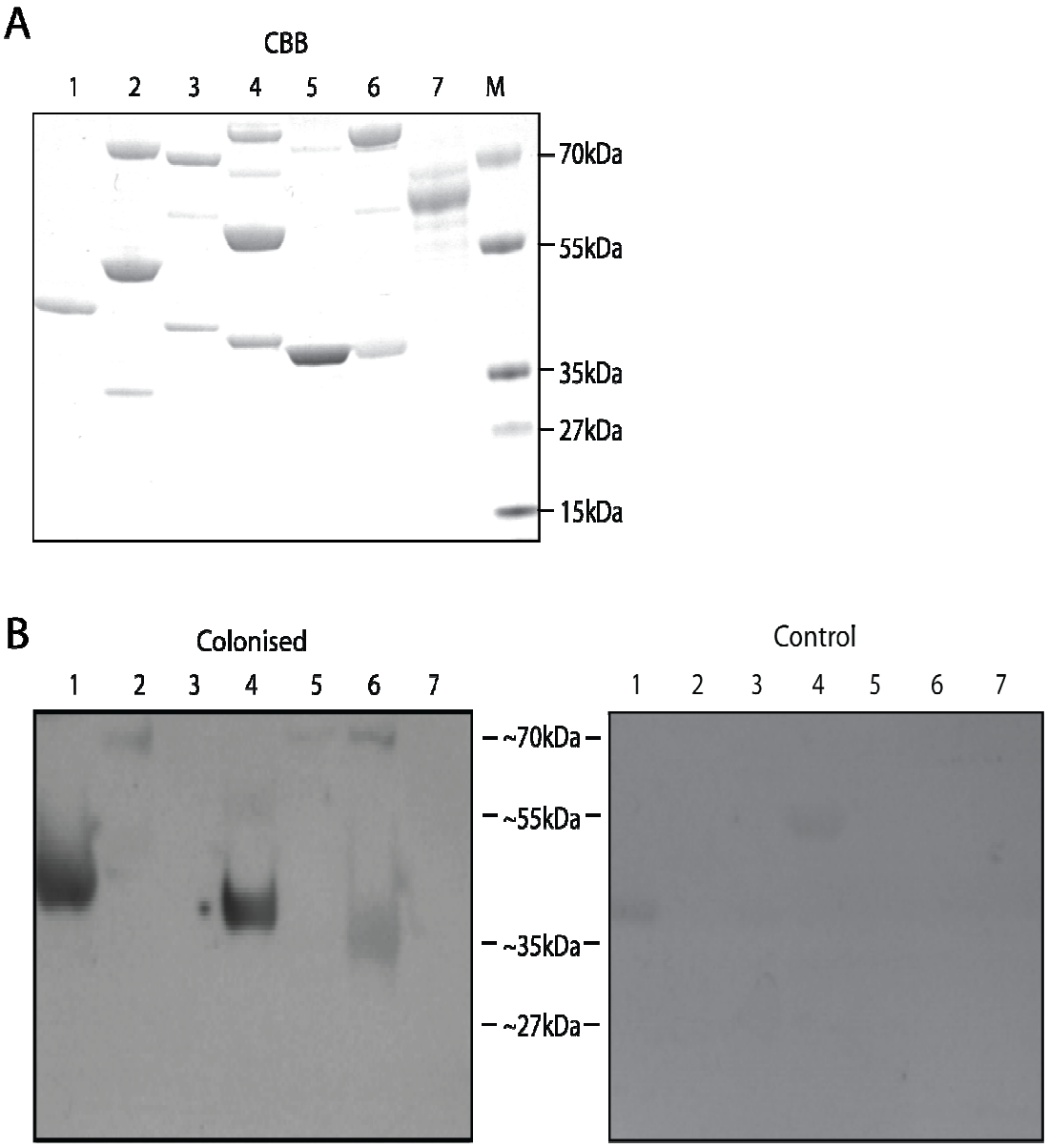

Figure 7.5. Serum IgG binding to individual *S. pneumoniae* TIGR4 proteins.

(A) Coomassie brilliant blue stained gel of recombinant pneumococcal proteins separated by SDS-PAGE. The recombinant protein antigens (predicted size in kDa) in each lane were: 1, SktP-C (35.1); 2, LysM (19.2), MalX (43.9), and AmiA (71.8); 3, SP1954 (50.8) and AliB (70.7); 4, PsaA (33.7), Ply (52.9) and PspC (55.9); 5, Cbf1 (36.5) and PspA (62); 6, PcsB-N (28.3) and SP2141 (72.7); 7, RrgB (negative control, 66) and PavB (83); M, size markers. (B) Immunoblots of IgG binding in pooled sera from colonised and control mice to recombinant Pnc protein antigens. Data representative of two experiments with different serum pools with similar results.

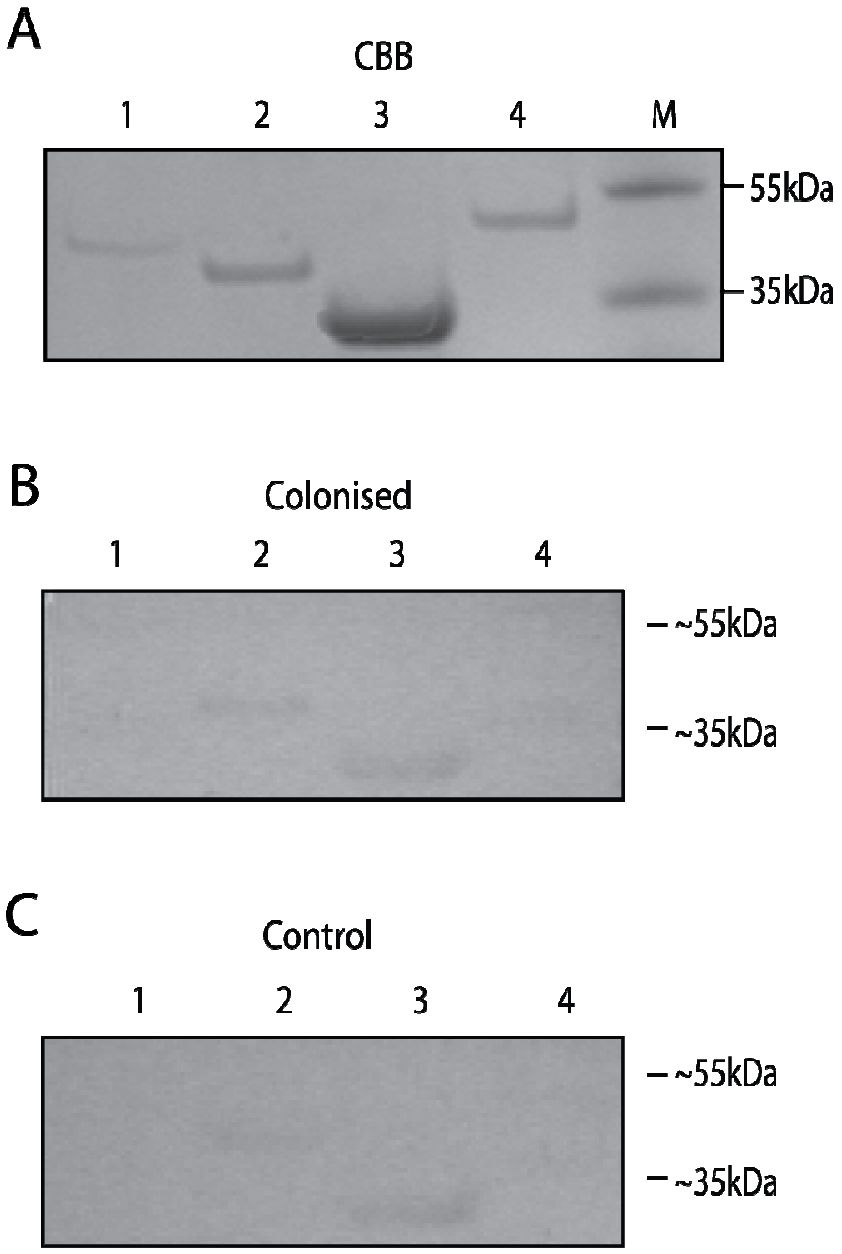

Figure 7.6. Serum IgG binding to individual pneumococcal ST3 prolipoproteins.

(A) Coomassie brilliant blue stained gel of recombinant pneumococcal prolipoproteins separated by SDS-PAGE. The protein antigens in each lane were: 1, PiaA; 2, PiuA; 3, SP0149; 4, SP0749; M, size markers. (B-C) Immunoblots of IgG binding in pooled sera from colonised (B) and control (C) mice to recombinant Pnc prolipoprotein antigens.

7.2.3.2 Detection of serum IgG binding to *S. pneumoniae* proteins by Luminex bead immunoassay

A Luminex bead immunoassay for 16 Pnc proteins was used to partially confirm the immunoblot results and identify additional antigens recognised by sera from colonised mice, as shown in Figure 7.7. Conjugated beads were kindly provided for this purpose by Prof Alex van Belkum (Erasmus MC, Rotterdam, The Netherlands). IgG binding to *S. pneumoniae* proteins was assessed at 10% and 1% dilution of serum pooled from colonised and control mice. The Luminex assay demonstrated IgG binding to PspA, PsaA, PhtD and PpmA in immune serum (Figure 7.7A) but not control serum (Figure 7.7B). Comparison of binding levels of binding to these antigens between the two serum pools demonstrated greater than 10-fold difference in MFI for these four antigens at both dilutions (Figure 7.7C-D). There was no binding to the other protein antigens tested by Luminex with either serum (PspC, CbpD, SlrA, PsaD, PhtE, SP0376, Eno, Ply, NanA, Hyal, IgAse, negative control RrgA). For those antigens tested in both immunoblot and bead immunoassay, concordant results were obtained for the two methods (positive for PspA and PsaA; negative for PspC and Ply).

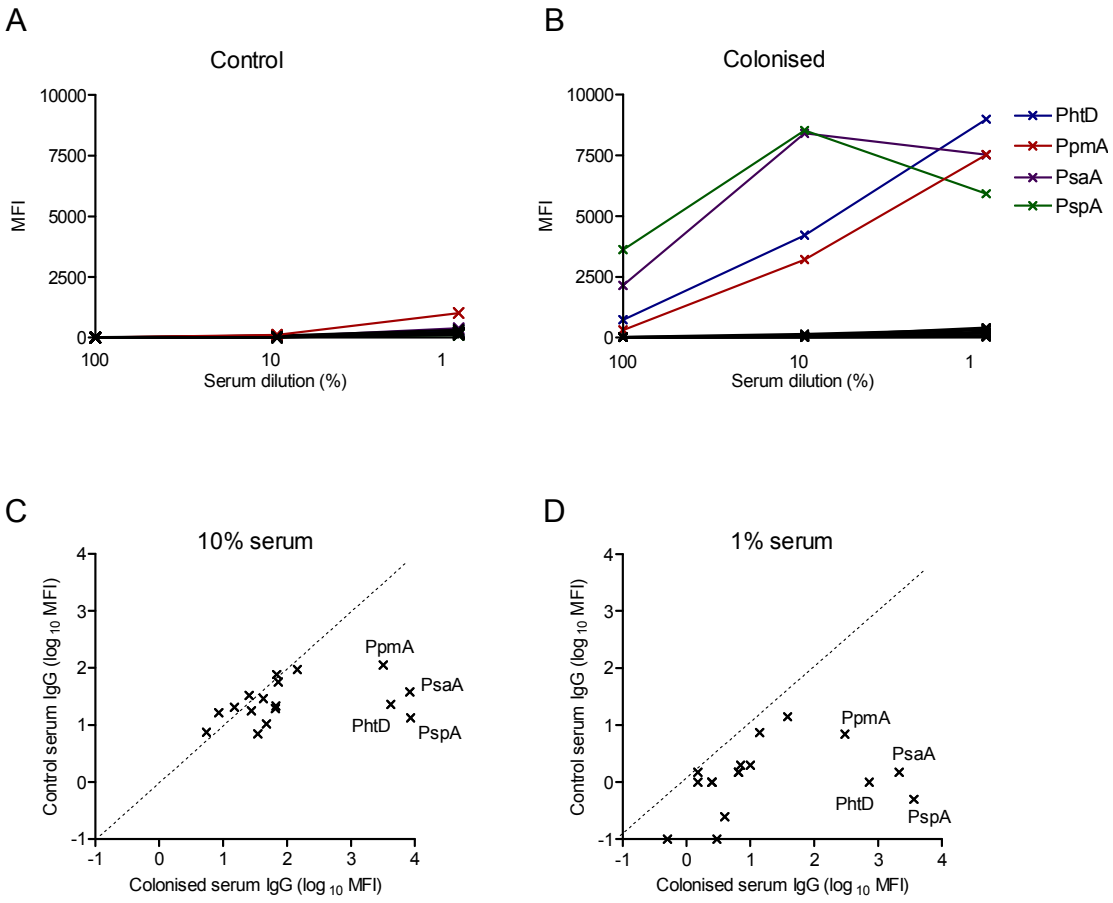

Figure 7.7. Serum IgG binding to individual pneumococcal proteins by Luminex bead assay.

IgG binding to Pnc protein-conjugated beads (Luminex) in pooled sera from colonised or control serum, detected with anti-mouse IgG-PE. Data points represent means of two replicate wells. Data are shown as (A) MFI for each protein against serum dilutions from colonised (A) and control (B) mice. For each protein antigen, IgG binding as $\log_{10}$ MFI in serum from control mice is plotted against that from colonised mice at 10% (C) and 1% (D) serum dilution. Only for PhtD, PsaA, PspA and PpmA (labelled) were there greater than tenfold differences in MFI between colonised and control sera. Data representative of two experiments with different serum pools with similar results.

7.3 SUMMARY OF RESULTS

Colonisation with D39 did not induce significant antibody responses against either type 2 capsular polysaccharide or against CWPS. Immunoblotting against lysates of D39 using pooled colonised serum demonstrated IgG binding to several proteins, with similar patterns in individual sera of colonised mice. Several bands were absent using D39Δ*lgt*, confirming earlier flow cytometry data that suggested some lipoprotein targets. Furthermore, similar cross-reactive binding patterns were seen with *S. pneumoniae* strains TIGR4 and ST3. Thus, colonisation with D39 induced serum IgG against a limited number of conserved protein antigens, including some lipoproteins.

Immunoblotting demonstrated that D39 colonisation induced serum IgG that bound seven proteins expressed from a TIGR4 genetic background: PsaA, PcsB, MalX, AmiA, PspA, SP2141 and SktP. Luminex bead immunoassay confirmed the results for PsaA and PspA, and identified PhtD and PpmA in addition. In total, nine antigenic targets of protective serum IgG were identified (Table 7.1), the majority of which are known to be protective in animal models of pneumococcal disease.

8 DISCUSSION

8.1 GENERAL DISCUSSION

Observational human data (Lipsitch et al., 2005) and investigations using animal models of disease (Roche et al., 2007, Richards et al., 2010) suggest that exposure of the immune system to *S. pneumoniae* during carriage events may induce adaptive responses that protect against subsequent disease. In this thesis, colonisation-induced protection against lethal *S. pneumoniae* strain D39 pneumonia was shown to be mediated by induction of serum anti-protein IgG capable of clearing bloodstream bacteria via opsonophagocytosis. Protection was demonstrated in both inbred (CBA/Ca) and outbred (CD1) strains of mice. Furthermore, the ability to replicate has been shown to be required to induce protection in the CD1 model.

8.1.1 Colonisation model

Nasopharyngeal bacterial load was assessed both to confirm that the WT strains were able to establish colonization, and to compare the bacterial load with that of mutant strains. This was done by culture of nasopharyngeal wash fluid, an established method (Wu et al., 1997b, Matthias et al., 2008, Magee and Yother, 2001). This technique is not expected to isolate every bacterial cell from within the nasopharynx. However, numbers of bacteria recovered by wash are similar to those recovered by homogenisation of nasopharyngeal tissue (Briles et al., 2005). Thus, this method is likely to be a robust means of detecting meaningful changes in colonising load and eventual clearance.

Using inocula of approximately 10^7 CFU, colonisation of the nasopharynx was achieved with both WT *S. pneumoniae* strains D39 and TIGR4. This is similar to the inoculum size used by both Wu (Wu et al., 1997b) and Weiser (McCool and Weiser,

2004) in their WT colonisation models. It is interesting to note that having tested a range of inoculum sizes, Wu found that this inoculum generally led to the most consistent colonisation without causing disease. Richards (Richards et al., 2010) established D39 colonisation in outbred MF1 mice using a much smaller inoculum of 10^5 CFU, and found this offered lasting colonisation at high density over 28 days. Although they measured bacterial load in tissue homogenate rather than wash fluid, it is unlikely that this explains the difference between their model and the one described in this study. It may be a reflection of the mouse strain. Indeed, Joyce colonised BALB/c mice with 10^8CFU D39 leading to a time course more similar to that seen in this study (Joyce et al., 2009).

The inoculum required to establish colonisation naturally in humans in unknown. It would be methodologically challenging to measure the number of bacterial CFU actually entering the nares during aerosol or droplet transmission. In the only reported study of experimental human colonisation, doses of 5-17 x 10^3 CFU of serotype 23F strain suspended in 100 µl were sufficient to establish colonisation in 6 of 14 volunteers. Compared to total body size this volume is proportionally much smaller than the 10 µl used in all reports of experimental mouse colonisation. Much of the inoculum given to a mouse may be lost due to physical factors (swallowing or sneezing), whereas most inoculum given to humans is likely to initially remain within the upper airway, and this may explain the need for the larger inocula required in mice. However, *S. pneumoniae* is not a natural pathogen of mice and may therefore find it more difficult to establish nasopharyngeal colonisation in a mouse compared to a human.

8.1.2 Challenge model

For the challenge experiments a large inoculum leading to rapidly progressive disease in the majority of mice was chosen in order to test whether colonisation induced a strong enough immune responses to protect against severe disease. Colonisation of both outbred CD1 and inbred CBA/Ca mice with strain D39 protected against subsequent lethal challenge with D39. Protection was stronger in the CBA/Ca model than the CD1 model, probably reflecting several factors. In repeat experiments, immunogenicity of colonization was more uniform in the CBA/Ca mouse strain. This may reflect greater consistency of colonization following nasal inoculation. In addition, genetic variation between outbred CD1 mice may result in varying immunogenicity of specific antigens reflecting different MHC binding potential. Colonization of CD1 mice with the TIGR4 strain did not protect against TIGR4 challenge. The rapid progression of disease in control mice in this model suggests the challenge dose may be too large to identify a modest protective effect. Thus, only limited conclusions can be drawn in this regard.

8.1.3 Innate cytokines

This study utilised a challenge model whereby unprotected mice developed lethal bacteraemia secondary to pneumonia. In such a setting, it is expected that a large challenge inoculum introduced into the lungs would lead to robust inflammatory responses. Thus, the adaptive responses induced through prior colonisation would only be evident over and above those already evoked by this strong innate response. The effects of previous colonisation were tested against this background.

The concentration of inflammatory mediators were measured in BALF at 4 and 18 h following infection. Significant levels of pro-inflammatory cytokines were found in BAL within 4 h of infection. The levels fell from 4 h to 18 h for KC, IL-6, TNF-α,

GM-CSF and IL-1β, consistent with previous reports of models of pneumococcal pneumonia with differing mouse and bacterial strains (Wang&Bergeron I&I 2001; Beg&Berg I&I 1998). There was a trend towards slightly higher levels of these cytokines in previously colonised mice at 4 h, significantly so for IL-6 and TNF-α. The biological significance of these small differences is not clear. They may have contributed to the greater neutrophil recruitment that had occurred by 4 h, itself a modest increase relative to that observed in naïve mice. There are several potential sources for these cytokines that may have been affected by previous colonisation. A major contributor is likely to be alveolar macrophages. Sub-lethal lung infection with *S. pneumoniae* is reported to have prolonged effects on AM recruitment and phenotype lasting at least until day 14 post-infection (Kirby et al., 2006). It is expected that nasopharyngeal colonisation will lead to aspiration of small numbers of bacterial cells into the lower respiratory tract. These are rapidly cleared, and hence negligible numbers were recovered in BALF and none in lung homogenate in this study. Whether colonisation with asymptomatic micro-aspiration is sufficient to induce similar changes within the lung is not known, but this could explain why previously colonised mice had higher levels of these cytokines even by 4 h post-infection. Other rarer pulmonary-resident cells types may be similarly affected by small sub-clinical inocula aspirated during carriage including γδ-T-cells. These expand and develop an activated phenotype in sub-lethal infection models (Kirby et al., 2007). Furthermore, through direct contact, γδ-T-cells could modulate responses from AMs themselves (Wands et al., 2005).

8.1.4 CD4 cells and IL-17

Pulmonary infection of naïve mice with *S. pneumoniae* also leads to local innate production of IL-17. This is IL-23 and γδ-T-cell dependant (Ma et al., 2009). In

addition, nasopharyngeal colonisation with *S. pneumoniae* is sufficient to induce both mucosal and systemic Th17-cell memory responses (Zhang et al., 2009). The ability of Th17 cells, primed during previous colonisation, to respond during pneumococcal pneumonia, and add to innate IL-17 during infection, has not been demonstrated to date. In this study, following infection, previously colonised mice had higher levels of IL-17 in both BALF and serum. Depletion of CD4+ cells, prior to challenge, led to abolition of the enhanced BALF IL-17 levels and to a substantially lower level of IL-17 in the serum. Thus, the difference between colonised and control mice was CD4+ cell dependant. Such responses were however redundant in preventing the development of bacteraemia in previously colonised mice. Depletion of CD4+ cells also had little effect on numbers of bacteria within the lungs. Thus, if these CD4+ cell dependant differences in IL-17 do represent a Th17 cell response, it would appear redundant in effecting protection in this challenge model.

This rapidly lethal model utilises a large challenge inoculum, and there is a significant rise in IL-17 levels in BALF even in naïve mice from 4 h to 18 h. This is associated with a brisk neutrophil influx. It may be that any potential benefits of a Th17-cell response are overwhelmed at the mucosal level in this model. It is possible that challenge with a smaller inoculum would lead to a more modest innate response, and that the Th17-cell response to previous colonisation would become more important in enhancing local clearance of bacteria. This hypothesis requires further investigation.

The mucosal microenvironment itself also appears to preferentially polarise naïve T-cells towards a Th17 rather than a Th1 phenotype (Zygmunt et al., 2009, Pepper et al., 2009), perhaps due to higher levels of factors such as TGF-β and IL-6. The ability of nasal immunisation with heat-killed whole cell *S. pneumoniae* to induce Th17-cell responses is dependant on the presence of CT (Malley et al., 2001). CT enhances production of IL-6 and thus may contribute to Th17 cell polarisation. Where viable

bacteria are used to colonise the nasopharynx, induction of Th17 memory is possible without CT. This process is TLR2 dependant (Zhang et al., 2009). The importance for TLR2 in induction of Th17 responses in this model is not known, but could involve TLR2-dependant production of cytokines such as IL-6, IL-1β or TGF-β from APCs. Alternatively, the recognised ability of TLR2 signalling to polarise dendritic cells towards production of IL-23 (Roses et al., 2008), required for the maintenance of Th17 cells, may be important.

8.1.5 Antibody responses

D39 colonisation of WT CBA/Ca mice led to protection against subsequent lethal challenge. μMT mice however were not protected. Duration of nasopharyngeal colonisation in μMT mice is similar to that in WT mice (van Rossum et al., 2005). Thus they represent an appropriate model to test the contribution of colonisation-induced antibody at the time of challenge, without significant effect on T-cell responses. Passive transfer of serum pooled from colonised mice was sufficient to protect naïve recipients from bacteraemia during subsequent challenge. Thus, colonisation-induced serum antibody is both necessary and sufficient to protect in this model.

Colonisation of CBA/Ca mice with *S. pneumoniae* strain D39 induced IgG in serum that bound D39 antigens in whole cell ELISA in nearly every mouse. Levels of serum IgM against D39 were marginally higher in colonised mice than controls, but this mainly reflected responses in a small number of individual mice, as for serum IgA. Passive transfer of this serum was sufficient to protect against bacteraemia and limit bacterial CFU in the lungs. Thus, colonisation-induced D39-specific IgG is the most likely mediator of the protective effect of serum.

In CD1 mice levels of D39-specific serum IgG correlated with levels of protection against lethal pneumonia. Thus, groups of mice receiving a booster dose of WT D39 were more protected than those receiving a single dose, and those receiving mutant strains were less protected. The precise mechanism of protection has yet to be determined in the CD1 model, as the greater consistency of responses in the CBA/Ca model meant this model was chosen for the detailed evaluation of immune effectors required for protection. Hence, it is theoretically possible that factors other than serum IgG were responsible for protection in the CD1 model, and that serum IgG was merely a correlate of some other effecter mechanism. Indeed, following nasal vaccination with heat-killed whole cell vaccine protection against re-colonisation correlates with serum antibody levels against various Pnc proteins, but antibody is entirely redundant in effecting protection (Trzcinski et al., 2005). Antibody induction through colonisation merely correlates with the induction of the Th17–cell response that is actually responsible for protection. Whether this is the case in the CD1 model would require further investigation.

The specificity of D39-colonisation induced antibody responses was assessed by flow cytometry, whole cell and polysaccharide ELISA, immunoblot and multiplex bead immunoassay. D39-DΔ bound more IgG from serum pooled from colonised CBA/Ca mice than its encapsulated parent strain in flow cytometry experiments. This supports the argument that there is binding to non-capsular antigens, but itself does not prove absence of IgG binding to capsule. The strongest evidence that no IgG against capsular antigen is induced through colonisation is derived from the ELISA data. Neither D39 nor TIGR4 colonisation of CD1 mice, nor D39 colonisation of CBA/Ca mice induced serum IgG against the homologous capsular polysaccharide. Thus the IgG that bound D39 antigens in whole cell ELISA at high titre was binding non-

capsular antigen. This was confirmed by competitive inhibition assay in which excess soluble CPS did not abrogate IgG binding in whole cell ELISA.

Type 4 CPS is known to be immunogenic in mice (Jones et al., 2009) and humans as either plain (Cadoz et al., 1985) or conjugate polysaccharide (Rennels et al., 1998). Absence of serum anti-type 4 CPS IgG following TIGR4 colonisation of CD1 mice must reflect how the polysaccharide antigen is recognised by the immune system in this context. Little is known about induction of anti-type 2 CPS responses. There are no published data on its immunogenicity in humans, and very limited data from murine models. Colonisation of MF1 mice appeared to induce low level serum IgM against type 2 capsule, but no IgG response was reported (Richards et al., 2010). Immunisation of BALB/c mice with a TIGR4 strain induced low level anti-type 4 CPS serum antibody responses which were boosted following PCV immunisation (Rabquer et al., 2007). Colonisation has a variable effect on induction of serum anti-CPS IgG responses in humans, which may depend on bacterial strain, CPS type and previous host exposure. In a longitudinal family study, serotypes 9V, 14, 18C, 19F and 23F induced anti-CPS responses, but serotype 6B did not (Goldblatt et al., 2005). Following carriage in a childhood study, responses were detected to of serotypes 11A and 14, but not to serotypes 6B, 19F and 23F (Soininen et al., 2001). The data generated in this thesis support these findings that, in mice, a single nasopharyngeal is not sufficient to induce a serum anti-CPS IgG response. This may depend on interactions between particular CPS serotypes with host genetic background and this may also explain data obtained from human observations.

Flow cytometry studies of binding to the bacterial surface suggested that colonisation induced serum IgG to subcapsular antigens, a proportion of which were likely to be lipoproteins as there was reduced binding to the Δ*lgt* mutant. This was confirmed by IgG binding to bacterial proteins and lipoprotein antigens in immunoblots of bacterial

lysates. Interestingly, immunoblots with sera from individual mice gave very similar patterns to one another, suggesting that there were several immunodominant proteins causing similar IgG responses to colonisation amongst these inbred mice. It would be interesting to investigate whether this is true for other inbred mouse strains, and whether responses in outbred CD1 mice are more diverse, perhaps contributing to the weaker protection seen in this model.

This study did not attempt to identify the site of induction of antibody responses following colonisation. It is expected that both NALT and cervical lymph nodes would house germinal centres where B-cells would encounter bacterial antigens. Some antigen is likely to reach the lungs during colonisation, and could be trafficked to mediastinal lymph nodes by dendritic cells or AMs (Kirby et al., 2009). However, the significantly higher bacterial load in the upper airway would predict that its associated lymphoid organs would be most important. This could be tested using antigen specific B-cell ELISPOT assays to identify antigen specific memory B-cell populations from various lymphoid organs, as has been attempted by others (Richards et al., 2010).

8.1.6 Δpab mutants

Bacterial viability during colonisation is likely to contribute to both colonisation duration and immunogenicity. Several factors serve to reduce the number of viable bacteria at any given timepoint following inoculation. These exclude extracellular bacterial death, physical removal in mucus (Nelson et al., 2007), and in situ phagocytosis (Matthias et al., 2008). Using mutant bacteria (D39Δ*pab* and TIGR4Δ*pab*) the role of active replication in the nasopharynx was explored. Both the D39Δ*pab* and the TIGR4Δ*pab* strains were rapidly cleared within 2 days of inoculation. With PABA supplementation, the ability of D39Δ*pab* to colonise was

restored, paralleling the restoration of virulence of the Δ*pab* mutant in the i.p. sepsis model (Dr Khandavilli, unpublished data described in Introduction). Using this technique to control duration of colonisation may avoid pro-inflammatory effects associated with bacterial lysis after exposure to antibiotics to terminate colonization which may have secondary effects on adaptive immune response (Moore et al., 2005). Compared to its WT parent strain, D39Δ*pab* was poorly immunogenic following colonisation. Supplementation with PABA for five days restored the ability of D39Δ*pab* to colonise, and enhanced the speed of anti-D39 IgG seroconversion. It may be that supplementation for longer would have greater impact on immunogenicity and lead to significantly enhanced protection. The data support the hypothesis that for a given strain of *S. pneumoniae*, the duration of colonisation is important in generating protective immunity. Whether the 'area under the curve' (reflecting total antigen present over time ie extent as well as duration of colonisation) is more important than duration alone is not clear, but could be explored using the conditional colonization of the D39Δ*pab* strain. Minimum duration is likely to vary with both mouse and bacterial strain. Mutants have been reported which are attenuated in their colonisation density and duration yet colonisation is still sufficient to induce protective immune responses (Roche et al., 2007). Extrapolating from the data in this thesis, the wild-type parent strains of those mutants may be even more protective.

Although a strain that is not capable of replication at all is unlikely to induce protection via this route, this can be overcome by use of an adjuvant. Indeed, the unencapsulated heat-killed vaccine studied by Malley (which is by definition incapable of replication) (Malley et al., 2001) is only immunogenic when used with CT. Repeat colonisation can boost immunogenicity and protection as reported here

and by others (Roche et al., 2007). Thus, repeat administration of a replication-incompetent strain, such as D39Δ*pab*, may increase immunogenicity.

8.1.7 Unencapsulated mutants

There are no previous reports that directly compare the immunogenicity of otherwise identical encapsulated and unencapsulated strains. Compared to its parent WT strain, colonisation with D39-DΔ and TIGR4Δ*cps* led to lower levels of CFU and more rapid clearance in CD1 mice. In addition, D39-DΔ was les immunogenic and not protective in CD1 mice compared to its WT parent strain, suggesting that several doses of D39-DΔ may be required to induce sufficient protection against D39WT challenge.

The primary clearance of encapsulated Pnc from the nasopharynx appears to be mediated by an adaptive Th17-cell response (Zhang et al., 2009). The early neutrophil influx in the first 2-3 days is insufficient to clear encapsulated Pnc (Matthias et al., 2008). Clearance depends on the evolution of a Th-17 cell orchestrated influx of mononuclear phagocytes later in colonisation. Whilst unencapsulated *S. pneumoniae* are more readily phagocytosed by neutrophils *in vitro* than their encapsulated parent strains (Hyams et al., 2010a), neutrophils appear redundant in clearing unencapsulated *S. pneumoniae* from the nasopharynx (Nelson et al., 2007). They are cleared more rapidly due to increased mucus trapping (Nelson et al., 2007). This may provide an additional reason for reduced immunogenicity of unencapsulated strains. Removal of unencapsulated bacteria from the nasopharynx by purely physical factors would leave fewer bacteria available for neutrophil phagocytosis, a component of antigen pre-processing necessary for the induction of adaptive immune responses to colonisation (Matthias et al., 2008). In this regard, it would be interesting to directly compare the immunogenicity and protective efficacy of colonisation with those unencapsulated strains that are known to protect (Roche et

al., 2007) with those of their WT parent strains. It is possible that WT strains in general would emerge as more immunogenic than unencapsulated isogenic mutants.

8.1.8 Δlgt mutants

D39Δ*lgt* also colonised with fewer CFU and was cleared more rapidly in CD1 mice. Unfortunately, a strain with this mutation on the TIGR4 background was not available during this thesis to confirm that this observation was specific to this strain. Many lipoproteins provide important nutrient transporter functions (Basavanna et al., 2009), and their absence impairs bacterial growth (Khandavilli, unpublished data [see Introduction]). Thus, the effects on growth of lack of lipoproteins may contribute to the reduced colonisation ability of the D39Δ*lgt* strain. Furthermore, several lipoproteins are important in mediating adherence to nasopharyngeal epithelium. Mutants lacking PpmA (Cron et al., 2009), SlrA (Hermans et al., 2006) and the Ami-AliA/AliB complex (Kerr et al., 2004) are cleared more rapidly from the nasopharynx than their parent WT strains, whilst antibodies against PsaA can prevent nasopharyngeal colonisation (Johnson et al., 2002). Since D39Δ*lgt* lacks all surface lipoproteins, it is likely to have significantly impaired adhesion to the epithelium, contributing to its more rapid clearance. This could be explored further using *in vitro* models of epithelial adherence (Rose et al., 2008).

TLR2 signalling is important in the induction of Th17-cell responses through Pnc colonisation. Thus, mice lacking TLR2 have delayed clearance of *S. pneumoniae* (van Rossum et al., 2005, Zhang et al., 2009). Reduced TLR2 signalling from D39Δ*lgt* may therefore impair the induction of the Th17 response which ultimately facilitates clearance of colonisation. Theoretically, this may facilitate prolonged colonisation compared to WT. However, the reduced growth and adherence of

D39Δ*lgt* appears to be dominant and thus the mutant is cleared more rapidly than its parent wild-type strain.

D39Δ*lgt* was less immunogenic and not protective compared to its parent WT strain in CD1 mice. This could reflect several phenotypic differences between the strains, arising from the Δ*lgt* mutation. Firstly, impaired immunogenicity may simply reflect shortened colonisation duration, as for the other mutant strains and this could possibly be overcome by using administration of multiple doses. Secondly, it may be that serum antibody against lipoprotein makes up a significant part of the response to colonisation with the WT strain in CD1 mice (as was shown by flow cytometry with serum from CBA/Ca mice). Thus, absence of these antigens may lead to reduced IgG detected in whole cell ELISA. Finally, the absence of bacterial lipoproteins (important TLR2 agonists) in D39Δ*lgt* may lead to reduced TLR2 signaling. TLR2 signaling is important in the induction of Th17-cell responses through Pnc colonisation (van Rossum et al., 2005, Zhang et al., 2009). Whether TLR2 signaling is important in the induction of antibody responses to colonisation is not known and requires further investigation. However, TLR2 signaling has been shown to be important in the induction of anti-protein and anti-carbohydrate antibody responses (Sen et al., 2005). Distinguishing between these factors to identify why D39Δ*lgt* colonisation has reduced protective efficacy would be difficult, and it is likely to reflect the complex interaction of all these processes which makes their *in vivo* dissection challenging. Studies using strains containing multiple mutations, possibly administered with multiple doses to WT and TLR2-deficient mice may help to determine the key factors in colonisation-induced protective immunity.

8.1.9 Opsonophagocytosis

Serum from colonised mice contained IgG that bound the bacterial surface. Serum from colonised CBA/Ca mice also enhanced the ability to *S. pneumoniae* D39 to associate with human neutrophils. These were used as large numbers of phagocytic cells were readily available, unlike the smaller numbers that could be obtained from mice. Serum from colonised μMT mice which lack antibody did not enhance this interaction, demonstrating that it is likely to be the antibody component of serum from colonised mice which is responsible. If sufficient serum was available, antibody depletion of serum would further support this. In this flow cytometry assay, results reflect both internalisation of the bacteria through phagocytosis and binding to the phagocyte surface without internalisation (Hyams et al., 2010a, Yuste et al., 2008). Previous studies of the relative contribution of internalisation and phagocytosis to total bacterial association using quenching of surface fluoresence with Trypan blue and blocking phagocytosis with cytochalasin D suggest most of the neutrophil association is due to phagocytosis (Hyams et al., 2010a). Limited quantities of serum precluded these approaches in this study, and it was considered more important to demonstrate the functional consequences of the interaction with phagocytes *in vivo*.

Previous colonisation did not significantly affect the numbers of bacteria recovered from BALF at any timepoint, and *in vivo* alveolar macrophage phagocytosis measured using fluorescently-labelled bacteria at four h post-infection was not enhanced by prior colonisation despite the presence of mucosal IgG and IgA against D39 antigens in BALF. Furthermore, although there was a trend towards reduced lung bacteria by 18 h in previously colonised mice, this difference was also present following passive transfer, demonstrating that serum antibody is responsible for this difference and that mucosally-produced antibody is not required. Overall, the data suggest that mucosal

antibody responses were not important for the protection seen after colonisation. This may be because the levels were too low relative to the large challenge inoculum. Future experiments will address whether mucosal antibody can help clearance of a lower challenge inoculum, which might more accurately reflect the normal situation found during microaspiration of *S. pneumoniae* from the nasopharnyx in colonised humans.

In addition to opsonisation of bacteria, colonisation-induced serum antibody may also protect at the mucosal level by neutralising the bacterial factors required for invasion. Rapid clearance of bloodstream bacteria following i.v. challenge in previously colonised mice indicates that enhanced opsonophagocytosis alone would be sufficient to explain the absence of bacteraemia in previously colonised mice following pneumonia challenge. It is difficult to demonstrate in the presence of this opsonophagocytosis whether colonisation-induced antibody is capable of neutralising bacterial invasion, such that bacteria enter the bloodstream at a slower rate in previously colonised mice. The ability of colonisation-induced serum antibody to block bacterial adhesion to epithelial cells could be explored *in vitro*. It may be possible to investigate this *in vivo* on a background of selective phagocyte depletion (Zhang et al., 2009) prior to pneumonia challenge.

8.1.10 Cross-reactive antibody

Colonisation of CD1 mice with D39WT induced serum IgG that cross-reacted in a whole cell ELISA with TIGR4 and ST3 antigens. A quantitative comparison was not possible as these assays have not been standardized to quantify absolute antibody concentrations. The presence of cross-reactive serum IgG following D39 colonisation of CBA/Ca mice was also demonstrated by immunoblotting against bacterial lysates of ST3 and TIGR4 strains. Other than bands representing proteins larger than 100

kDa (which might reflect dimers or trimers of common antigens rather than separate D39-specific antigens), this revealed a broadly similar pattern of bands to the homologous D39 strain, as has been recently reported following colonisation with acapsular strains (Roche and Weiser, 2010). Flow cytometry studies demonstrated cross-reactive IgG binding to the TIGR4 strain, but less than to D39, and only in the absence of the capsule. Presence of IgG against a range of recombinant TIGR4 protein antigens was demonstrated by immunoblot. It is possible that the serotype 4 capsule may impede the binding of cross-reacting antibody to subcapsular protein antigens more effectively than the serotype 2 capsule. This hypothesis could be explored using capsular switch strains which are genetically identical with regard to protein antigens and differ solely in their capsular types (Hyams et al., 2010b).

Cross-protection induced through colonisation with unencapsulated mutants has been previously reported (Roche et al., 2007). Despite inducing serum IgG against D39 antigens, colonisation of CD1 mice with D39 did not protect against subsequent TIGR4 challenge. However, even colonisation with the TIGR4 strain failed to prevent fatal challenge with the TIGR4 strain, suggesting for this strain nasopharyngeal colonisation of CD1 mice may not induce effective protective immune responses. Interpretation is limited by the high mortality in the control group in this experiment. There was a strong trend towards some cross-protection against ST3 (P=0.057). There was only 10% survival in the ST3-challenged controls, and it is possible that challenge of D39-colonised mice with a smaller ST3 inoculum would demonstrate statistically significant cross-protection. Given the stronger homologous protection induced by D39 colonisation of CBA/Ca mice, it is possible that these mice would be cross-protected against heterologous challenge.

Data from the CD1 model suggest that cross-reaction in WC ELISA does not correlate with cross-protection. Future studies may be able to clarify whether IgG

binding to the bacterial surface measured by flow cytometry correlates better with cross-protection, and whether allelic protein antigen variation or hindrance of anti-protein binding by capsule type is more important.

8.1.11Specific protein antigens

The combination of immunoblotting and Luminex assay identified nine specific proteins, including four lipoproteins, that are immunogenic following D39 colonisation of CBA/Ca mice: PspA, PsaA, PpmA, AmiA, MalX, PhtD, StkP, SP2141 and PcsB. Except for PpmA (see Table 7.1), the recombinant proteins used for these assays were obtained from heterologous strains, demonstrating that these are likely to be target antigens for cross-reactive antibody. A negative result obtained with these assays must be taken with caution, as it may not necessarily indicate that the antigen is not immunogenic during colonisation. A number of proteins were expressed in truncated form, and antibody binding antigens in non-expressed domains or conformational antigens affected by truncation would not be identified by this approach. Although nearly all antigens tested were highly conserved (see Table 7.1), a smaller number such as PspC are not, and failure to detect IgG binding may reflect absence of cross-reaction rather than absence of antibody against these antigens. If recombinant D39 antigen had been used in these assays, binding may have been identified.

Despite only 57% identity in amino acid sequence, colonisation with the D39 strain induced serum IgG that bound TIGR4 PspA in both immunoblot and Luminex assay. This would imply that this variation did not affect epitopes recognised by colonisation-induced antibody. D39 colonisation of MF1 mice has also been shown to induce anti-PspA serum IgG (Richards et al., 2010). As for all the specific antigens identified, it is not known whether the IgG is binding to PspA epitopes which are

important for protection or whether anti-PspA is functionally important in the protection demonstrated here.

Recombinant PspA (Wu et al., 1997a), PhtD (Adamou et al., 2001), StkP and PcsB (Giefing et al., 2008) are individually protective in animal models of pneumococcal disease. The protective serum antibody induced by murine immunisation with SktP or PcsB demonstrates opsonophagocytic killing *in vitro*. Recombinant PpmA is also protective when administered in conjunction with other protein antigens (Audouy et al., 2007). Immunisation with PsaA alone protects against colonisation, but not against disease. To date, there is no evidence that immunisation with AmiA, MalX or SP2141 is protective against disease. Conversely, the antigens PiuA, PiaA and PspC are strongly immunogenic and protect against disease when given as recombinant proteins in mouse models. However, colonization did not induce a detectable antibody response to these antigens. For PspC this could be due to allelic variation, as discussed above. However, PiuA and PiaA are very highly conserved between *S. pneumoniae* strains and known to be expressed during infection (LeMessurier et al., 2006) Brown unpublished data), so it is unclear why they do not induce an antibody response after nasopharyngeal colonisation with live bacteria. Which of the antigens for which antibody responses were found were responsible for the marked protection against bacteraemia in CBA/Ca mice is not known. The degree of protection perhaps suggests antibodies to multiple antigens were responsible, as protein antigens administered in combination offer stronger protection (Ogunniyi et al., 2007). It may be possible to deplete serum of specific antibodies to reveal the non-redundancy of specific responses in effecting protection. Such studies are likely to require larger quantities of serum, which may necessitate establishing colonisation models in larger animals. The weak binding in immunoblots of serum IgG from control mice to Ply, PiuA and SP0149 was similar to that in serum of colonised mice. This may reflect

cross-reactive natural antibody following previous exposure to other non-pneumococcal *Streptococcus* species which posses structurally related cytolysins (Jefferies et al., 2007) or lipoproteins (Whalan et al., 2006).

Overall, it appears that experimental colonisation of mice biases the adaptive immune response towards anti-protein antibodies. Perhaps their inherent T-dependant nature leads to their immunodominance during colonisation.

The importance of TLR signalling in the induction of antibody responses has recently become apparent (Ganley-Leal et al., 2006). Specifically, TLR2-deficient mice have poor anti-phosphorylcholine IgG responses to *S. pneumoniae* immunisation (Vasilevsky et al., 2008), and poor anti-surface protein IgG responses to *Salmonella enterica* (Cervantes-Barragan et al., 2009). Even commercially available PPS and PCV formulations have been shown to be routinely contaminated with TLR2 ligands that are indispensible for induction of anti-CPS IgG responses (Sen et al., 2005). It is possible that the potent TLR2 signalling capacity of antigens such as lipoproteins contributes to their immunodominance during colonisation. That may explain the dominance of lipoproteins as target for colonisation-induced serum IgG in this study, and the dominance of PsaA over PspA, PspC and Ply in other reports (Palaniappan et al., 2005).

Although a number of Pnc proteins have been identified in this study, it is likely that there are further antigens recognised by sera from colonised mice. Other approaches such as 2D gel electrophoresis or ANTIGENome screening (Giefing et al., 2008) may be required to obtain greater resolution enabling more comprehensive identification of immunodominant responses.

It is likely that the immunodominant proteins will vary between different colonising strains. This has been reported following colonisation with unencapsulated TIGR4 and serotype 6A and a wild-type serotype 23 strain. Whilst antibody against several

proteins was induced, TIGR4Δ*cps* induced antibody predominantly against PspA and PpmA, 6AΔ*cps* induced antibody against PsaA, and the serotype 23F strain induced antibody against just PpmA. Interestingly, none of these responses were necessary as colonisation with a mutant deficient in all three proteins still induced protection. This is a useful strategy for determining redundancy but cannot prove that a specific antibody target is necessary. Were protection not seen following colonisation with a mutant strain, it may reflect the impact of the mutation on colonisation itself (e.g. duration) as found with the D39 and TIGR4 mutant strain studied in this thesis, rather than lack of antibody against a specific protein target. How and why the bacterial or mouse strain background affects the pattern of antibody responses to colonisation warrants further detailed investigation, These studies will be difficult, however, due to the effects of specific mutations on the dynamics of infection and interaction with host immune effectors independent of their effect on immune responses.

8.1.12 Relevance of findings for human populations

Are such adaptive anti-protein responses important in protecting humans? It is difficult to separate the effects of the developing innate immune responses from those of the adaptive immune response following such ubiquitous natural exposure. Human *S. pneumoniae* nasopharyngeal colonisation induces antibodies to a range of *S. pneumoniae* protein antigens (Giefing et al., 2008, Laine et al., 2004, Goldblatt et al., 2005, Simell et al., 2009, Melin et al., 2008). The epidemiology of *S. pneumoniae* disease is also consistent with colonisation inducing protective humoral immunity against proteins antigens, with anti-protein responses increasing and the incidence of invasive *S. pneumoniae* infection, especially septicaemia, falling in older children (Laine et al., 2004, Lipsitch et al., 2005). If anti-protein responses are cross-protective this would explain why the incidence of invasive *S. pneumoniae* disease

caused by all serotypes falls after infancy at similar rates, irrespective of prevalence of carriage (Lipsitch et al., 2005). Furthermore, immune senescence affects antibodies to *S. pneumoniae* proteins to a greater degree than antibodies to the capsule (Simell et al., 2008) which could contribute to the increased incidence of invasive *S. pneumoniae* infection in the elderly.

Affinity purified natural IgG against StkP and PcsB has recently been shown to promote opsonophagocytic killing *in vitro*. Thus, it is possible that naturally acquired anti-protein IgG in human serum may facilitate killing of *S. pneumoniae in vivo*. *S. pneumoniae* pneumonia in humans is often associated with negative blood cultures (Werno and Murdoch, 2008). Furthermore, blood culture is also often negative in focal pneumococcal infection such as meningitis, despite infection arising through haematogenous spread (Kirkpatrick et al., 1994). This could be explained by the presence in serum of natural antibodies against *S. pneumoniae* antigens sufficient to enhance bloodstream clearance but not to protect against occasional colonies establishing focal infection in protected 'sanctuary' sites. It has been observed that patients presenting with non-bacteraemic pneumococcal pneumonia have higher serum anti-Ply IgG than those with bacteraemic pneumonia (Musher et al., 2001). Such observations do not prove that pre-existing serum anti-protein antibody prevents progression to bacteraemia in humans, but they are consistent with this hypothesis.

At present, vaccine-induced opsonophagocytic antibodies to capsular polysaccharide are the only known correlate of human protection against invasive *S. pneumoniae* disease (Bogaert et al., 2004b), and serological surveys in children have only investigated antibody responses to a limited number of *S. pneumoniae* protein antigens. Murine data identifying important immunodominant antigens to *S. pneumoniae* colonisation could inform which protein antigens should be included in serological studies of human responses to *S. pneumoniae* colonisation. There is

increasing interest in the identification of protein antigens which are immunogenic following natural exposure. Studies are in progress in several geographical locations investigating these responses in the context of *S. pneumoniae* carriage particularly in areas of high prevalence (Prof David Goldblatt, personal communication). Future studies may investigate whether risk of subsequent recolonisation or disease correlates with naturally-induced anti-protein antibody responses. This may guide future multi-valent protein vaccine design. It is of note that several of the protein antigens currently in early clinical phase vaccine studies were discovered through empiric analysis of *S. pneumoniae* proteome using sera from exposed humans. Since over 12% of TIGR4 genes are conserved hypothetical proteins of unknown function (The Institute for Genomic Research, 2010), there are likely to be other protective antigens to be discovered.

8.2 SUMMARY OF FINDINGS

Using mouse models, this thesis has demonstrated that nasopharyngeal colonisation is immunogenic, inducing both mucosal and systemic antibody responses to bacterial antigens. Furthermore, previous colonisation is protective against subsequent lethal pneumonia in both inbred and outbred mice.

Colonisation with WT strains led to higher levels and longer duration of colonisation than with unencapsulated, Δ*pab* or Δ*lgt* strains. Unlike the D39 WT strain, D39 mutants were less immunogenic and were not protective against lethal pneumonia, perhaps reflecting the reduced numbers of bacteria present during shorter periods of colonisation. Extending the duration of colonisation of the D39Δ*pab* strain with PABA supplementation led to enhanced immunogenicity, supporting the hypothesis that a persistent bacterial load in the nasopharynx has a direct influence on the speed and magnitude of the immune response. The contribution of bacterial lipoproteins as both antigens and TLR2 agonists may also be important in inducing protective responses to colonisation.

Protection against lethal *S. pneumoniae* pneumonia in previously colonised mice strongly correlated with the absence of bacteraemia. During pneumonia, previously colonised mice had higher mucosal levels of IL-6 and TNF-α and more rapid recruitment of neutrophils to alveolar spaces. Previously colonised mice also had significantly enhanced levels of mucosal and systemic IL-17. This enhancement was dependent on the presence of CD4+ cells at the time of challenge. Despite this, previous colonisation did not lead to a reduced mucosal bacterial load in this infection model. CD4+ cells and the enhanced IL-17 response were redundant in protecting against progression to bacteraemia. However, serum antibody induced through

colonisation was both necessary and sufficient to protect against the bacteraemia associated with lethal progression of disease.

Serum IgG induced through colonisation bound non-capsular surface antigens including lipoproteins, but not capsular polysaccharide. This serum enhanced phagocytosis of *S. pneumoniae in vitro* and the clearance of bacteria from the bloodstream *in vivo*. Nine pneumococcal protein targets of protective colonisation-induced serum IgG were identified, including four lipoproteins. Most of these antigens are highly conserved between *S. pneumoniae* strains, explaining the induction of cross-reactive serum IgG through colonisation with the D39 strain.

These data support the hypothesis that induction of serum anti-protein IgG through colonisation protects against invasive pneumococcal disease. This finding provides important insight into the dynamics of pneumococcal disease in repeatedly exposed human populations, and may direct future work to the identification of novel antigens for inclusion in future pneumococcal vaccines.

REFERENCES

Abdullahi O, Nyiro J, Lewa P, Slack M, Scott J A. The Descriptive Epidemiology of *Streptococcus pneumoniae* and Haemophilus influenzae Nasopharyngeal Carriage in Children and Adults in Kilifi District, Kenya. The Pediatric Infectious Disease Journal 2008; (27) 59-64.

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. Identification and Characterization of a Novel Family of Pneumococcal Proteins That Are Protective against Sepsis. Infect Immun 2001; (69): 949-958.

Adrian P V, Bogaert D, Oprins M, Rapola S, Lahdenkari M, Kilpi T, de Groot R, Kayhty H, Hermans P W M. Development of antibodies against pneumococcal proteins [alpha]-enolase, immunoglobulin A1 protease, streptococcal lipoprotein rotamase A, and putative proteinase maturation protein A in relation to pneumococcal carriage and Otitis Media. Vaccine 2004; (22): 2737-2742.

Ahman H, Kayhty H, Lehtonen H, Leroy O, Froeschle J, Eskola J. *Streptococcus pneumoniae* capsular polysaccharide-diphtheria toxoid conjugate vaccine is immunogenic in early infancy and able to induce immunologic memory. The Pediatric Infectious Disease Journal 1998; (17): 211-216.

Albiger B, Dahlberg S, Sandgren A, Wartha F, Beiter K, Katsuragi H, Akira S, Normark S, Henriques-Normark B. Toll-like receptor 9 acts at an early stage in host defence against pneumococcal infection. Cell Microbiol 2007; (9): 633-644.

Albiger B, Sandgren A, Katsuragi H, Meyer-Hoffert U, Beiter K, Wartha F, Hornef M, Normark S, Normark B H. Myeloid differentiation factor 88-dependent signalling controls bacterial growth during colonization and systemic pneumococcal disease in mice. Cell Microbiol 2005; (7): 1603-1615.

Alexander J E, Lock R A, Peeters C C, Poolman J T, Andrew P W, Mitchell T J, Hansman D, Paton J C. Immunization of mice with pneumolysin toxoid confers a significant degree of protection against at least nine serotypes of *Streptococcus pneumoniae*. Infect Immun 1994; (62): 5683-5688.

Ali H, Richardson R M, Haribabu B, Snyderman R. Chemoattractant Receptor Cross-desensitization. J Biol Chem 1999; (274): 6027-6030.

Aliprantis A O, Yang R B, Mark M R, Suggett S, Devaux B, Radolf J D, Klimpel G R, Godowski P, Zychlinsky A. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science 1999; (285): 736-739.

Anderson P, Pichichero M E, Insel R A. Immunogens consisting of oligosaccharides from the capsule of Haemophilus influenzae type b coupled to diphtheria toxoid or the toxin protein CRM197. J Clin Invest 1985; (76): 52-59.

Aniansson G, Alm B, Andersson B, Larsson P, Nylen O, Peterson H, Rigner P, Svanborg M, Svanborg C. Nasopharyngeal colonization during the first year of life. J Infect Dis 1992; (165 Suppl 1): S38-S42.

Arita M, Ohira T, Sun Y P, Elangovan S, Chiang N, Serhan C N. Resolvin E1 Selectively Interacts with Leukotriene B4 Receptor BLT1 and ChemR23 to Regulate Inflammation. J Immunol 2007; (178): 3912-3917.

Armstrong L, Medford A R L, Uppington K M, Robertson J, Witherden I R, Tetley T D, Millar A B. Expression of Functional Toll-Like Receptor-2 and -4 on Alveolar Epithelial Cells. Am J Respir Cell Mol Biol 2004; (31): 241-245.

Arredouani M S, Yang Z, Imrich A, Ning Y, Qin G, Kobzik L. The macrophage scavenger receptor SR-AI/II and lung defense against pneumococci and particles. Am J Respir Cell Mol Biol 2006; (35): 474-478.

Audouy S A L, van Selm S, van Roosmalen M L, Post E, Kanninga R, Neef J, Estevo S, Nieuwenhuis E E S, Adrian P V, Leenhouts K, Hermans P W M. Development of lactococcal GEM-based pneumococcal vaccines. Vaccine 2007; (25): 2497-2506.

Aujla S J, Chan Y R, Zheng M, Fei M, Askew D J, Pociask D A, Reinhart T A, McAllister F, Edeal J, Gaus K, Husain S, Kreindler J L, Dubin P J, Pilewski J M, Myerburg M M, Mason C A, Iwakura Y, Kolls J K. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. Nat Med 2008; (14): 275-281.

Aujla S J, Dubin P J, Kolls J K. Interleukin-17 in pulmonary host defense. Experimental Lung Research 2007; (33): 507-518.

Austrian R, Douglas RM, Schiffman G, Coetzee A, Koornhof H, Hayden-Smith S, Reid R D. Prevention of pneumococcal pneumonia by vaccination. Trans Assoc Am Physicians 1976; (89): 184-194.

Austrian R. *S. pneumoniae* infections. In: Bacterial vaccines. (Ed.Germanier R). Orlando, Florida: 1984; 257-288.

Avery O T, Macleod C M, McCarty M. Studies on the chemical nature of the substance inducing transformation of pneumococcal types: induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. J Exp Med 1944; (79): 137-158.

Balmer P, Borrow R, Findlow J, Warrington R, Frankland S, Waight P, George R, Andrews N, Miller E. Age-stratified prevalences of pneumococcal-serotype-specific immunoglobulin G in England and their relationship to the serotype-specific incidence of invasive pneumococcal disease prior to the introduction of the pneumococcal 7-valent conjugate vaccine. Clin Vaccine Immunol 2007; (14): 1442-1450.

Basavanna S, Khandavilli S, Yuste J, Cohen J M, Hosie A H F, Webb A J, Thomas G H, Brown J S. Screening of *Streptococcus pneumoniae* ABC transporter mutants demonstrates that LivJHMGF, a branched-chain amino acid ABC transporter, is necessary for disease pathogenesis. Infect Immun 2009; (77): 3412-3423.

Basset A, Thompson C M, Hollingshead S K, Briles D E, Ades E W, Lipsitch M, Malley R. Antibody-Independent, CD4+ T-Cell-Dependent Protection against Pneumococcal Colonization Elicited by Intranasal Immunization with Purified Pneumococcal Proteins. Infect Immun 2007; (75): 5460-5464.

Baxendale H E, Davis Z, White H N, Spellerberg M B, Stevenson F K, Goldblatt D. Immunogenetic analysis of the immune response to pneumococcal polysaccharide. Eur J Immunol 2000; (30): 1214-1223.

Baxendale H E, Johnson M, Stephens R C M, Yuste J, Klein N, Brown J S, Goldblatt D. Natural human antibodies to pneumococcus have distinctive molecular characteristics and protect against pneumococcal disease. Clinical & Experimental Immunology 2008; (151): 51-60.

Baxendale H E, Johnson M, Keating S M, Ashton L, Burbidge P, Woodgate S, Southern J, Miller E, Goldblatt D. Circulating pneumococcal specific plasma and memory B cells in the elderly two years after pneumococcal conjugate versus polysaccharide vaccination. Vaccine 2010a; (In Press, Uncorrected Proof).

Baxendale H E, Keating S M, Johnson M, Southern J, Miller E, Goldblatt D. The early kinetics of circulating pneumococcal-specific memory B cells following pneumococcal conjugate and plain polysaccharide vaccines in the elderly. Vaccine 2010b; (28): 4763-4770.

Beiter K, Wartha F, Hurwitz R, Normark S, Zychlinsky A, Henriques-Normark B. The Capsule Sensitizes *Streptococcus pneumoniae* to {alpha}-Defensins Human Neutrophil Proteins 1 to 3. Infect Immun 2008; (76): 3710-3716.

Berger M, Norvell T M, Tosi M F, Emancipator S N, Konstan M W, Schreiber J R. Tissue-specific Fc gamma and complement receptor expression by alveolar macrophages determines relative importance of IgG and complement in promoting phagocytosis of Pseudomonas aeruginosa. Pediatr Res 1994; (35): 68-77.

Bergeron Y, Ouellet N, Deslauriers A M, Simard M, Olivier M, Bergeron M G. Cytokine Kinetics and Other Host Factors in Response to Pneumococcal Pulmonary Infection in Mice. Infect Immun 1998; (66): 912-922.

Bergmann S, Hammerschmidt S. Versatility of pneumococcal surface proteins. Microbiology 2006; (152): 295-303.

Bernatoniene J, Finn A. Advances in pneumococcal vaccines: advantages for infants and children. Drugs 2005; (65): 229-255.

Bernstein J M. Mucosal immunology of the upper respiratory tract. Respiration 1992; (59 Suppl 3:3-13.): 3-13.

Biffl W L M, Moore E E M, Moore F A M, Barnett C C J. Interleukin-6 Delays Neutrophil Apoptosis via a Mechanism Involving Platelet-Activating Factor. Journal of Trauma-Injury Infection & Critical Care 1996; (40): 575-579.

Black S, Shinefield H, Fireman B, Lewis E, Ray P, Hansen J R, Elvin L, Ensor K M, Hackell J, Siber G, Malinoski F, Madore D, Chang I, Kohberger R, Watson W, Austrian R, Edwards K. Efficacy, safety and immunogenicity of heptavalent

pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. Pediatr Infect Dis J 2000; (19): 187-195.

Bogaert D, de G R, Hermans P W. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. Lancet Infect Dis 2004a; (4): 144-154.

Bogaert D, Sluijter M, de Groot R, Hermans P W M. Multiplex opsonophagocytosis assay (MOPA): a useful tool for the monitoring of the 7-valent pneumococcal conjugate vaccine. Vaccine 2004b; (22): 4014-4020.

Bogaert D, Sluijter M, Toom N L, Mitchell T J, Goessens W H F, Clarke S C, de Groot R, Hermans P W M. Dynamics of pneumococcal colonization in healthy Dutch children. Microbiology 2006; (152): 377-385.

Bogaert D, Engelen M N, Timmers-Reker A J M, Elzenaar K P, Peerbooms P G H, Coutinho R A, de Groot R, Hermans P W M. Pneumococcal Carriage in Children in The Netherlands: a Molecular Epidemiological Study. J Clin Microbiol 2001; (39): 3316-3320.

Bogdan C, Vodovotz Y, Nathan C. Macrophage deactivation by interleukin 10. J Exp Med 1991; (174): 1549-1555.

Borthen L, Heimdahl A, Nord C E. Selective suppression of the anaerobic oropharyngeal microflora with local metronidazole. Br J Oral Maxillofac Surg 1987; (25): 49-56.

Branger J, Florquin S, Knapp S, Leemans J C, Pater J M, Speelman P, Golenbock D T, van der P T. LPS-binding protein-deficient mice have an impaired defense against Gram-negative but not Gram-positive pneumonia. Int Immunol 2004a; (16): 1605-1611.

Branger J, Knapp S, Weijer S, Leemans J C, Pater J M, Speelman P, Florquin S, van der Poll T. Role of Toll-Like Receptor 4 in Gram-Positive and Gram-Negative Pneumonia in Mice. Infect Immun 2004b; (72): 788-794.

Briles D E, Nahm M, Schroer K, Davie J, Baker P, Kearney J, Barletta R. Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 *Streptococcus pneumoniae*. J Exp Med 1981; (153): 694-705.

Briles D E, Ades E, Paton J C, Sampson J S, Carlone G M, Huebner R C, Virolainen A, Swiatlo E, Hollingshead S K. Intranasal Immunization of Mice with a Mixture of the Pneumococcal Proteins PsaA and PspA Is Highly Protective against Nasopharyngeal Carriage of *Streptococcus pneumoniae*. Infect Immun 2000a; (68): 796-800.

Briles D E, Novak L, Hotomi M, van Ginkel F W, King J. Nasal Colonization with *Streptococcus pneumoniae* Includes Subpopulations of Surface and Invasive Pneumococci. Infect Immun 2005; (73): 6945-6951.

Briles D, Hollingshead S, King J, Swift A, Braun P, Park M, Ferguson L, Nahm M, Nabors G. Immunization of Humans with Recombinant Pneumococcal Surface Protein A (rPspA) Elicits Antibodies That Passively Protect Mice from Fatal

Infection with *Streptococcus pneumoniae* Bearing Heterologous PspA. J Infect Dis 2000b; (182): 1694-1701.

Briles D, Hollingshead S, Paton J, Ades E, Novak L, van-áGinkel F, Benjamin á. Immunizations with Pneumococcal Surface Protein A and Pneumolysin Are Protective against Pneumonia in a Murine Model of Pulmonary Infection with *Streptococcus pneumoniae*. J Infect Dis 2003; (188): 339-348.

Brimblecombe F S, Cruickshank R, Masters P L, Reid D D, Stewart G T. Family studies of respiratory infections. Br Med J 1958; (1): 119-128.

Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss D S, Weinrauch Y, Zychlinsky A. Neutrophil extracellular traps kill bacteria. Science 2004; (303): 1532-1535.

Brouwer M C, de G J, Heckenberg S G, Zwinderman A H, van der P T, van de B D. Host genetic susceptibility to pneumococcal and meningococcal disease: a systematic review and meta-analysis. Lancet Infect Dis 2009; (9): 31-44.

Brown J S, Gilliland S M, Holden D W. A *Streptococcus pneumoniae* pathogenicity island encoding an ABC transporter involved in iron uptake and virulence. Mol Microbiol 2001a; (40): 572-585.

Brown J S, Hussell T, Gilliland S M, Holden D W, Paton J C, Ehrenstein M R, Walport M J, Botto M. The classical pathway is the dominant complement pathway required for innate immunity to *Streptococcus pneumoniae* infection in mice. Proc Natl Acad Sci U S A 2002; (99): 16969-16974.

Brown J S, Ogunniyi A D, Woodrow M C, Holden D W, Paton J C. Immunization with components of two iron uptake ABC transporters protects mice against systemic *Streptococcus pneumoniae* infection. Infect Immun 2001b; (69): 6702-6706.

Brown P D, Lerner S A. Community-acquired pneumonia. The Lancet 1998; (352): 1295-1302.

Brueggemann A, Griffiths D, Meats E, Peto T, Crook D, Spratt B. Clonal Relationships between Invasive and Carriage *Streptococcus pneumoniae* and SerotypeΓÇÉ and CloneΓÇÉSpecific Differences in Invasive Disease Potential. J Infect Dis 2003; (187): 1424-1432.

Brueggemann A, Peto T, Crook D, Butler J, Kristinsson K, Spratt B. Temporal and Geographic Stability of the SerogroupΓÇÉSpecific Invasive Disease Potential of *Streptococcus pneumoniae* in Children. J Infect Dis 2004; (190): 1203-1211.

Bryant K A, Block S L, Baker S A, Gruber W C, Scott D A. Safety and immunogenicity of a 13-valent pneumococcal conjugate vaccine. Pediatrics 2010; (125): 866-875.

Buchwald U K, Lees A, Steinitz M, Pirofski L A. A peptide mimotope of type 8 pneumococcal capsular polysaccharide induces a protective immune response in mice. Infect Immun 2005; (73): 325-333.

Buckley R H, Becker W G. Abnormalities in the regulation of human IgE synthesis. Immunol Rev 1978; (41:288-314.): 288-314.

Burman L A, Norrby R, Trollfors B. Invasive pneumococcal infections: incidence, predisposing factors, and prognosis. Rev Infect Dis 1985; (7): 133-142.

Butler J C, Breiman R F, Campbell J F, Lipman H B, Broome C V, Facklam R R. Pneumococcal polysaccharide vaccine efficacy. An evaluation of current recommendations. JAMA 1993; (270): 1826-1831.

Butler J C, Breiman R F, Lipman H B, Hofmann J, Facklam R R. Serotype distribution of *Streptococcus pneumoniae* infections among preschool children in the United States, 1978-1994: implications for development of a conjugate vaccine. J Infect Dis 1995; (171): 885-889.

Cadoz M, Armand J, Arminjon F, Michel J P, Michel M, Denis F, Schiffman G. A new 23 valent pneumococcal vaccine: immunogenicity and reactogenicity in adults. J Biol Stand 1985; (13): 261-265.

Calbo E, Garau J. Of mice and men: innate immunity in pneumococcal pneumonia. International Journal of Antimicrobial Agents 2010; (35): 107-113.

Carsetti R, Rosado M M, Donnanno S, Guazzi V, Soresina A, Meini A, Plebani A, Aiuti F, Quinti I. The loss of IgM memory B cells correlates with clinical disease in common variable immunodeficiency. J Allergy Clin Immunol 2005; (115): 412-417.

Cervantes-Barragan L, Gil-Cruz C, Pastelin-Palacios R, Lang K S, Isibasi A, Ludewig B, Lopez-Macias C. TLR2 and TLR4 signaling shapes specific antibody responses to Salmonella typhi antigens. Eur J Immunol 2009; (39): 126-135.

Chan YR, Liu JS, Pociask DA, Zheng M, Mietzner T, Berger T, Mak T, Clifton MC, Strong R, Ray P, Kolls J K. Lipocalin 2 is required for pulmonary host defense against Klebsiella infection. Journal of Immunology 2009.

Charkaluk M L, Kalach N, Mvogo H, Dehecq E, Magentie H, Raymond J, Gendrel D, Kremp O, Decoster A. Assessment of a rapid urinary antigen detection by an immunochromatographic test for diagnosis of pneumococcal infection in children. Diagnostic Microbiology and Infectious Disease 2006; (55): 89-94.

Chiang N, Serhan C N, Dahl+®n S E, Drazen J M, Hay D W P, Rovati G E, Shimizu T, Yokomizo T, Brink C. The Lipoxin Receptor ALX: Potent Ligand-Specific and Stereoselective Actions in Vivo. Pharmacological Reviews 2006; (58): 463-487.

Chiavolini D, Pozzi G, Ricci S. Animal Models of *Streptococcus pneumoniae* Disease. Clin Microbiol Rev 2008; (21): 666-685.

Cockeran R, Mitchell T J, Feldman C, Anderson R. Pneumolysin induces release of matrix metalloproteinase-8 and -9 from human neutrophils. Eur Respir J 2009; (34): 1167-1170.

Colombel J F, Loftus E V, Tremaine W J, Egan L J, Harmsen W S, Schleck C D, Zinsmeister A R, Sandborn W J. The safety profile of infliximab in patients with

Crohn's disease: The Mayo Clinic experience in 500 patients. Gastroenterology 2004; (126): 19-31.

Cowland J B, Sorensen O E, Sehested M, Borregaard N. Neutrophil Gelatinase-Associated Lipocalin Is Up-Regulated in Human Epithelial Cells by IL-1{beta}, but Not by TNF-{alpha}. J Immunol 2003; (171): 6630-6639.

Cripps A W, Otczyk D C, Kyd J M. Bacterial otitis media: a vaccine preventable disease? Vaccine 2005; (23): 2304-2310.

Cron L E, Bootsma H J, Noske N, Burghout P, Hammerschmidt S, Hermans P W M. Surface-associated lipoprotein PpmA of *Streptococcus pneumoniae* is involved in colonization in a strain-specific manner. Microbiology 2009; (155): 2401-2410.

Cruse G, Fernandes V E, de Salort J, Pankhania D, Marinas M S, Brewin H, Andrew P W, Bradding P, Kadioglu A. Human Lung Mast Cells Mediate Pneumococcal Cell Death in Response to Activation by Pneumolysin. J Immunol 2010; (184): 7108-7115.

Cua D J, Tato C M. Innate IL-17-producing cells: the sentinels of the immune system. Nat Rev Immunol 2010; (10): 479-489.

Dagan R, Givon-Lavi N, Greenberg D, Fritzell B, Siegrist C. Nasopharyngeal Carriage of *Streptococcus pneumoniae* Shortly before Vaccination with a Pneumococcal Conjugate Vaccine Causes SerotypeΓÇÉSpecific Hyporesponsiveness in Early Infancy. J Infect Dis 2010; (201): 1570-1579.

Dagan R, Givon-Lavi N, Zamir O, Sikuler-Cohen M, Guy L, Janco J, Yagupsky P, Fraser D. Reduction of Nasopharyngeal Carriage of *Streptococcus pneumoniae* after Administration of a 9-Valent Pneumococcal Conjugate Vaccine to Toddlers Attending Day Care Centers. J Infect Dis 2002; (185): 927-936.

Dallaire F, Ouellet N, Bergeron Y, Turmel V, Gauthier M C, Simard M, Bergeron M G. Microbiological and Inflammatory Factors Associated with the Development of Pneumococcal Pneumonia. J Infect Dis 2001; (184): 292-300.

Dave S, Brooks-Walter A, Pangburn M K, McDaniel L S. PspC, a Pneumococcal Surface Protein, Binds Human Factor H. Infect Immun 2001; (69): 3435-3437.

Dave S, Carmicle S, Hammerschmidt S, Pangburn M K, McDaniel L S. Dual Roles of PspC, a Surface Protein of *Streptococcus pneumoniae*, in Binding Human Secretory IgA and Factor H. J Immunol 2004; (173): 471-477.

Davidson M, Parkinson A J, Bulkow L R, Fitzgerald M A, Peters H V, Parks D J. The epidemiology of invasive pneumococcal disease in Alaska, 1986-1990--ethnic differences and opportunities for prevention. J Infect Dis 1994; (170): 368-376.

Davis K M, Akinbi H T, Standish A J, Weiser J N. Resistance to Mucosal Lysozyme Compensates for the Fitness Deficit of Peptidoglycan Modifications by *Streptococcus pneumoniae*. PLoS Pathog 2008; (4): e1000241.

Dawid S, Roche A M, Weiser J N. The blp Bacteriocins of *Streptococcus pneumoniae* Mediate Intraspecies Competition both In Vitro and In Vivo. Infect Immun 2007; (75): 443-451.

Dawid S, Sebert M E, Weiser J N. Bacteriocin Activity of *Streptococcus pneumoniae* Is Controlled by the Serine Protease HtrA via Posttranscriptional Regulation. J Bacteriol 2009; (191): 1509-1518.

de Roux A, Schmoele–Thoma B, Siber G, Hackell J, Kuhnke A, Ahlers N, Baker S, Razmpour A, Emini E, Fernsten P, Gruber W, Lockhart S, Burkhardt O, Welte T, Lode H. Comparison of Pneumococcal Conjugate Polysaccharide and Free Polysaccharide Vaccines in Elderly Adults: Conjugate Vaccine Elicits Improved Antibacterial Immune Responses and Immunological Memory. Clinical Infectious Diseases 2008; (46): 1015-1023.

Deban L, Bottazzi B, Garlanda C, de la Torre Y M, Mantovani A. Pentraxins: multifunctional proteins at the interface of innate immunity and inflammation. Biofactors 2009; (35): 138-145.

DeLyria E S, Redline R W, Blanchard T G. Vaccination of Mice Against H pylori Induces a Strong Th-17 Response and Immunity That Is Neutrophil Dependent. Gastroenterology 2009; (136): 247-256.

Didierlaurent A, Goulding J, Patel S, Snelgrove R, Low L, Bebien M, Lawrence T, van Rijt L S, Lambrecht B N, Sirard J C, Hussell T. Sustained desensitization to bacterial Toll-like receptor ligands after resolutionof respiratory influenza infection. J Exp Med 2008; (205): 323-329.

Duan J, Avci F Y, Kasper D L. Microbial carbohydrate depolymerization by antigen-presenting cells: Deamination prior to presentation by the MHCII pathway. PNAS 2008; (105): 5183-5188.

Dubin P J, Kolls J K. Th17 cytokines and mucosal immunity. Immunological Reviews 2008; (226): 160-171.

Echchannaoui H, Frei K, Schnell C, Leib S L, Zimmerli W, Landmann R. Toll-like receptor 2-deficient mice are highly susceptible to *Streptococcus pneumoniae* meningitis because of reduced bacterial clearing and enhanced inflammation. J Infect Dis 2002; (186): 798-806.

Ekdahl K, Ahlinder I, Hansson H B, Melander E, Molstad S, Soderstrom M, Persson K. Duration of nasopharyngeal carriage of penicillin-resistant *Streptococcus pneumoniae*: experiences from the South Swedish Pneumococcal Intervention Project. Clin Infect Dis 1997; (25): 1113-1117.

Endeman H, Cornips M C, Grutters J C, van den Bosch J M, Ruven H J, van Velzen-Blad H, Rijkers G T, Biesma D H. The Fcgamma receptor IIA-R/R131 genotype is associated with severe sepsis in community-acquired pneumonia. Clin Vaccine Immunol 2009; (16): 1087-1090.

Ertugrul N, Rodriguez-Barradas M C, Musher D M, Ryan M A, Agin C S, Murphy S J, Shayegani M, Watson D A. BOX-polymerase chain reaction-based DNA

analysis of nonserotypeable *Streptococcus pneumoniae* implicated in outbreaks of conjunctivitis. J Infect Dis 1997; (176): 1401-1405.

Faden H, Duffy L, Wasielewski R, Wolf J, Krystofik D, Tung Y. Relationship between nasopharyngeal colonization and the development of otitis media in children. Tonawanda/Williamsville Pediatrics. J Infect Dis 1997; (175): 1440-1445.

Faden H, Stanievich J, Brodsky L, Bernstein J, Ogra P L. Changes in nasopharyngeal flora during otitis media of childhood. The Pediatric Infectious Disease Journal 1990; (9): 623-626.

Fedson D S, Musher D M, Eskola J. Pneumococcal vaccine. In: Vaccines. (Eds.Plotkin S, Orenstein W). Philadelphia: WB Saunders Co., 1998; 3rd Edition: 553-607.

Fedson D S, Scott J A. The burden of pneumococcal disease among adults in developed and developing countries: what is and is not known. Vaccine 1999; (17 Suppl 1:S11-8.): S11-S18.

Feldman C, Anderson R, Cockeran R, Mitchell T, Cole P, Wilson R. The effects of pneumolysin and hydrogen peroxide, alone and in combination, on human ciliated epithelium in vitro. Respir Med 2002; (96): 580-585.

Feldman C, Cockeran R, Jedrzejas M J, Mitchell T J, Anderson R. Hyaluronidase augments pneumolysin-mediated injury to human ciliated epithelium. International Journal of Infectious Diseases 2007; (11): 11-15.

Ferreira D M, Darrieux M, Silva D A, Leite L C, Ferreira J M, Jr., Ho P L, Miyaji E N, Oliveira M L. Characterization of protective mucosal and systemic immune responses elicited by Pneumococcal Surface Proteins PspA and PspC nasal vaccines against a respiratory pneumococcal challenge in mice. Clin Vaccine Immunol 2009; (16): 636-45.

Fine M J, Smith M A, Carson C A, Meffe F, Sankey S S, Weissfeld L A, Detsky A S, Kapoor W N. Efficacy of Pneumococcal Vaccination in Adults: A Meta-analysis of Randomized Controlled Trials. Arch Intern Med 1994; (154): 2666-2677.

Fossiez F, Djossou O, Chomarat P, Flores-Romo L, it-Yahia S, Maat C, Pin J J, Garrone P, Garcia E, Saeland S, Blanchard D, Gaillard C, Das M B, Rouvier E, Golstein P, Banchereau J, Lebecque S. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. J Exp Med 1996; (183): 2593-2603.

Francis J P, Richmond P C, Pomat W S, Michael A, Keno H, Phuanukoonnon S, Nelson J B, Whinnen M, Heinrich T, Smith W A, Prescott S L, Holt P G, Siba P M, Lehmann D, van den Biggelaar A H J. Maternal Antibodies to Pneumolysin but Not to Pneumococcal Surface Protein A Delay Early Pneumococcal Carriage in High-Risk Papua New Guinean Infants. Clin Vaccine Immunol 2009; (16): 1633-1638.

French N, Gordon S B, Mwalukomo T, White S A, Mwafulirwa G, Longwe H, Mwaiponya M, Zijlstra E E, Molyneux M E, Gilks C F. A Trial of a 7-Valent Pneumococcal Conjugate Vaccine in HIV-Infected Adults. New England Journal of Medicine 2010; (362): 812-822.

Gallagher P M, Lowe G, Fitzgerald T, Bella A, Greene C M, McElvaney N G, O'Neill S J. Association of IL-10 polymorphism with severity of illness in community acquired pneumonia. Thorax 2003; (58): 154-156.

Ganley-Leal L M, Liu X, Wetzler L M. Toll-like receptor 2-mediated human B cell differentiation. Clinical Immunology 2006; (120): 272-284.

Garcia-Suarez M M, Cima-Cabal M D, Florez N, Garcia P, Cernuda-Cernuda R, Astudillo A, Vazquez F, De l T, Jr., Mendez F J. Protection against pneumococcal pneumonia in mice by monoclonal antibodies to pneumolysin. Infect Immun 2004; (72): 4534-4540.

Garmory H S, Titball R W. ATP-Binding Cassette Transporters Are Targets for the Development of Antibacterial Vaccines and Therapies. Infect Immun 2004; (72): 6757-6763.

Garred P, Honor C, Ma Y J, Munthe-Fog L, Hummelsh°j T. MBL2, FCN1, FCN2 and FCN3--The genes behind the initiation of the lectin pathway of complement. Molecular Immunology 2009; (46): 2737-2744.

Gauthier J F, Fortin A, Bergeron Y, Dumas M C, Champagne M E, Bergeron M G. Differential Contribution of Bacterial N-Formyl-Methionyl-Leucyl- Phenylalanine and Host-Derived CXC Chemokines to Neutrophil Infiltration into Pulmonary Alveoli during Murine Pneumococcal Pneumonia. Infect Immun 2007; (75): 5361-5367.

Geha R S, Leung D Y. Hyper immunoglobulin E syndrome. Immunodefic Rev 1989; (1): 155-172.

Gerosa F, Baldani-Guerra B, Lyakh L A, Batoni G, Esin S, Winkler-Pickett R T, Consolaro M R, De Marchi M, Giachino D, Robbiano A, Astegiano M, Sambataro A, Kastelein R A, Carra G, Trinchieri G. Differential regulation of interleukin 12 and interleukin 23 production in human dendritic cells. J Exp Med 2008; (205): 1447-61.

Ghaffar F, Muniz L, Katz K, Smith J, Shouse T, Davis P, McCracken G. Effects of Large dosages of amoxicillin/clavulanate or azithromycin on nasopharyngeal carriage of *Streptococcus pneumoniae*, *Haemophilus influenzae*, non-pneumococcal hemolytic streptococci, and *Staphylococcus aureus* in children with acute otitis media. Clinical Infectious Diseases 2002; (34): 1301-1309.

Ghaffar F M, Friedland I R M, Mccracken G H J. Dynamics of nasopharyngeal colonization by *Streptococcus pneumoniae*. Pediatric Infectious Disease Journal 1999; (18): 638-646.

Gianfaldoni C, Censini S, Hilleringmann M, Moschioni M, Facciotti C, Pansegrau W, Masignani V, Covacci A, Rappuoli R, Barocchi M A, Ruggiero P. *Streptococcus*

pneumoniae Pilus Subunits Protect Mice against Lethal Challenge. Infect Immun 2007; (75): 1059-1062.

Giefing C, Meinke A L, Hanner M, Henics T, Minh D B, Gelbmann D, Lundberg U, Senn B M, Schunn M, Habel A, Henriques-Normark B, Ortqvist A, Kalin M, von Gabain A, Nagy E. Discovery of a novel class of highly conserved vaccine antigens using genomic scale antigenic fingerprinting of pneumococcus with human antibodies. J Exp Med 2008; (205): 117-131.

Gill C, Mwanakasale V, Fox M, Chilengi R, Tembo M, Nsofwa M, Chalwe V, Mwananyanda L, Mukwamataba D, Malilwe B, Champo D, Macleod W, Thea D, Hamer D. Impact of Human Immunodeficiency Virus Infection on *Streptococcus pneumoniae* Colonization and Seroepidemiology among Zambian Women. J Infect Dis 2008; (197): 1000-1005.

Gingles N A, Alexander J E, Kadioglu A. Role of genetic resistance in invasive pneumococcal infection: identification and study of susceptibility and resistance in inbred mouse strains. Infect Immun 2001a; (69): 426-434.

Gingles N A, Alexander J E, Kadioglu A, Andrew P W, Kerr A, Mitchell T J, Hopes E, Denny P, Brown S, Jones H B, Little S, Booth G C, McPheat W L. Role of Genetic Resistance in Invasive Pneumococcal Infection: Identification and Study of Susceptibility and Resistance in Inbred Mouse Strains. Infect Immun 2001b; (69): 426-434.

Girardin S E, Boneca I G, Viala J, Chamaillard M, Labigne A s, Thomas G, Philpott D J, Sansonetti P J. Nod2 Is a General Sensor of Peptidoglycan through Muramyl Dipeptide (MDP) Detection. J Biol Chem 2003; (278): 8869-8872.

Givon-Lavi N, Fraser D, Dagan R. Vaccination of day-care center attendees reduces carriage of *Streptococcus pneumoniae* among their younger siblings. Pediatr Infect Dis J 2003; (22): 524-532.

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. Antibody responses to nasopharyngeal carriage of *Streptococcus pneumoniae* in adults: a longitudinal household study. J Infect Dis 2005; (192): 387-393.

Goldenberg H, McCool T, Weiser J. Cross-Reactivity of Human Immunoglobulin G2 Recognizing Phosphorylcholine and Evidence for Protection against Major Bacterial Pathogens of the Human Respiratory Tract. J Infect Dis 2004; (190): 1254-1263.

Gor D O, Ding X, Briles D E, Jacobs M R, Greenspan N S. Relationship between Surface Accessibility for PpmA, PsaA, and PspA and Antibody-Mediated Immunity to Systemic Infection by *Streptococcus pneumoniae*. Infect Immun 2005; (73): 1304-1312.

Gordon S B, Jarman E R, Kanyanda S, French N, Pridmore A C, Zijlstra E E, Molyneux M E, Read R C. Reduced interleukin-8 response to *Streptococcus pneumoniae* by alveolar macrophages from adults with HIV/AIDS. AIDS 2005; (19): 1197-1200.

Granat S M, Ollgren J, Herva E, Mia Z, Auranen K, Makela P H. Epidemiological evidence for serotype-independent acquired immunity to pneumococcal carriage. J Infect Dis 2009; (200): 99-106.

Granat S M M, Mia Z P, Ollgren J M, Herva E M, Das M M, Piirainen L M, Auranen K P, Makela P H M. Longitudinal Study on Pneumococcal Carriage During the First Year of Life in Bangladesh. [Article]. Pediatric Infectious Disease Journal 2007; (26): 319-324.

Gray B M, Dillon H C, Jr. Epidemiological studies of *Streptococcus pneumoniae* in infants: antibody to types 3, 6, 14, and 23 in the first two years of life. J Infect Dis 1988; (158): 948-955.

Griffith F. The Significance of Pneumococcal Types. J Hyg (Lond) 1928; (27): 113-159.

Grimbacher B, Holland S M, Gallin J I, Greenberg F, Hill S C, Malech H L, Miller J A, O'Connell A C, Puck J M. Hyper-IgE Syndrome with Recurrent Infections: An Autosomal Dominant Multisystem Disorder. New England Journal of Medicine 2008; (340): 692-702.

Hamel J, Charland N, Pineau I, Ouellet C, Rioux S, Martin D, Brodeur B R. Prevention of Pneumococcal Disease in Mice Immunized with Conserved Surface-Accessible Proteins. Infect Immun 2004; (72): 2659-2670.

Hammerschmidt S. Adherence molecules of pathogenic pneumococci. Current Opinion in Microbiology 2006; (9): 12-20.

Hammerschmidt S, Bethe G, Remane H, Chhatwal G S. Identification of Pneumococcal Surface Protein A as a Lactoferrin-Binding Protein of *Streptococcus pneumoniae*. Infect Immun 1999; (67): 1683-1687.

Hanage W P. Serotype-specific problems associated with pneumococcal conjugate vaccination. Future Microbiology 2008; (3): 23-30.

Hausdorff W, Bryant J, Kloek C, Paradiso P, Siber G. The Contribution of Specific Pneumococcal Serogroups to Different Disease Manifestations: Implications for Conjugate Vaccine Formulation and Use, Part II. Clinical Infectious Diseases 2000a; (30): 122-140.

Hausdorff W, Bryant J, Paradiso P, Siber G. Which Pneumococcal Serogroups Cause the Most Invasive Disease: Implications for Conjugate Vaccine Formulation and Use, Part I. Clinical Infectious Diseases 2000b; (30): 100-121.

Herbold W, Maus R, Hahn I, Ding N, Srivastava M, Christman J W, Mack M, Reutershan J, Briles D E, Paton J C, Winter C, Welte T, Maus U A. Importance of CXC Chemokine Receptor 2 in Alveolar Neutrophil and Exudate Macrophage Recruitment in Response to Pneumococcal Lung Infection. Infect Immun 2010; (78): 2620-2630.

Heritage P H I L, Underdown B R I A, Arsenault A Á, Snider D E N I, Mcdermott M A R K. Comparison of Murine Nasal-associated Lymphoid Tissue and Peyer's Patches. Am J Respir Crit Care Med 1997; (156): 1256-1262.

Hermans P W M, Adrian P V, Albert C, Estevúo S, Hoogenboezem T, Luijendijk I H T, Kamphausen T, Hammerschmidt S. The Streptococcal Lipoprotein Rotamase A (SlrA) Is a Functional Peptidyl-prolyl Isomerase Involved in Pneumococcal Colonization. J Biol Chem 2006; (281): 968-976.

Heyzer-Williams L J, Pelletier N, Mark L, Fazilleau N, Heyzer-Williams M G. Follicular helper T cells as cognate regulators of B cell immunity. Current Opinion in Immunology 2009; (21): 266-273.

Higgins S C, Jarnicki A G, Lavelle E C, Mills K H G. TLR4 Mediates Vaccine-Induced Protective Cellular Immunity to Bordetella pertussis: Role of IL-17-Producing T Cells. J Immunol 2006; (177): 7980-7989.

Hill P, Cheung Y, Akisanya A, Sankareh K, Lahai G, Greenwood B, Adegbola R. Nasopharyngeal Carriage of *Streptococcus pneumoniae* in Gambian Infants: A Longitudinal Study. Clinical Infectious Diseases 2008; (46): 807-814.

Hogberg L, Geli P, Ringberg H, Melander E, Lipsitch M, Ekdahl K. Age- and Serogroup-Related Differences in Observed Durations of Nasopharyngeal Carriage of Penicillin-Resistant Pneumococci. J Clin Microbiol 2007; (45): 948-952.

Holdsworth R J, Neill G D, Irving A D, Cuschieri A. Blood clearance and tissue distribution of 99Tc-labelled pneumococci following splenectomy in rabbits. Br J Exp Pathol 1989; (70): 669-677.

Hollingshead S K, Becker R, Briles D E. Diversity of PspA: Mosaic Genes and Evidence for Past Recombination in *Streptococcus pneumoniae*. Infect Immun 2000; (68): 5889-5900.

Holmes W E, Lee J, Kuang W J, Rice G C, Wood W I. Structure and functional expression of a human interleukin-8 receptor. Science 1991; (253): 1278-1280.

Holmlund E, Quiambao B, Ollgren J, Jaakkola T, Neyt C, Poolman J, Nohynek H, Kayhty H. Antibodies to Pneumococcal Proteins PhtD, CbpA, and LytC in Filipino Pregnant Women and Their Infants in Relation to Pneumococcal Carriage. Clin Vaccine Immunol 2009; (16): 916-923.

Houghton A M, Hartzell W O, Robbins C S, Gomis-Ruth F X, Shapiro S D. Macrophage elastase kills bacteria within murine macrophages. Nature 2009; (460): 637-641.

Hussell T, Goulding J. Structured regulation of inflammation during respiratory viral infection. The Lancet Infectious Diseases 2010; (10): 360-366.

Hyams C, Camberlein E, Cohen J M, Bax K, Brown J S. The *Streptococcus pneumoniae* Capsule Inhibits Complement Activity and Neutrophil Phagocytosis by Multiple Mechanisms. Infect Immun 2010a; (78): 704-715.

Hyams C, Yuste J, Bax K, Camberlein E, Weiser J N, Brown J S. *Streptococcus pneumoniae* Resistance to Complement-Mediated Immunity Is Dependent on the Capsular Serotype. Infect Immun 2010b; (78): 716-725.

Ivarsson M, Lundberg C, Quiding-Jerbrink M. Antibody production directed against pneumococci by immunocytes in the adenoid surface secretion. International Journal of Pediatric Otorhinolaryngology 2004; (68): 537-543.

Iwakura Y, Nakae S, Saijo S, Ishigame H. The roles of IL-17A in inflammatory immune responses and host defense against pathogens. Immunol Rev 2008; (226:57-79.): 57-79.

Jackson L A, Neuzil K M, Yu O, Benson P, Barlow W E, Adams A L, Hanson C A, Mahoney L D, Shay D K, Thompson W W. Effectiveness of Pneumococcal Polysaccharide Vaccine in Older Adults. New England Journal of Medicine 2003; (348): 1747-1755.

Jaffar Z, Ferrini M E, Herritt L A, Roberts K. Cutting Edge: Lung Mucosal Th17-Mediated Responses Induce Polymeric Ig Receptor Expression by the Airway Epithelium and Elevate Secretory IgA Levels. J Immunol 2009; (182): 4507-4511.

Janoff E N, Breiman R F, Daley C L, Hopewell P C. Pneumococcal disease during HIV infection. Epidemiologic, clinical, and immunologic perspectives. Ann Intern Med 1992; (117): 314-324.

Jedrzejas M J. Extracellular virulence factors of *Streptococcus pneumoniae*. Front Biosci 2004; (9:891-914.): 891-914.

Jefferies J, Nieminen L, Kirkham L A, Johnston C, Smith A, Mitchell T J. Identification of a secreted cholesterol-dependent cytolysin (mitilysin) from *Streptococcus mitis*. J Bacteriol 2007; (189): 627-632.

Jennings H J, Lugowski C, Young N M. Structure of the complex polysaccharide C-substance from *Streptococcus pneumoniae* type 1. Biochemistry 1980; (19): 4712-4719.

Joffe M D M, Alpern E R M. Occult Pneumococcal Bacteremia: A Review. Pediatric Emergency Care 2010; (26): 448-454.

Johnson S, Dykes J, Jue D, Sampson J, Carlone G, Ades E. Inhibition of Pneumococcal Carriage in Mice by Subcutaneous Immunization with Peptides from the Common Surface Protein Pneumococcal Surface Adhesin A. J Infect Dis 2002; (185): 489-496.

Jokinen J, Ahman H, Kilpi T, Meñkel P, Kayhty M. Concentration of Antipneumococcal Antibodies as a Serological Correlate of Protection: An Application to Acute Otitis Media. J Infect Dis 2004; (190): 545-550.

Jomaa M, Yuste J, Paton J C, Jones C, Dougan G, Brown J S. Antibodies to the Iron Uptake ABC Transporter Lipoproteins PiaA and PiuA Promote Opsonophagocytosis of *Streptococcus pneumoniae*. Infect Immun 2005; (73): 6852-6859.

Jomaa M, Terry S, Hale C, Jones C, Dougan G, Brown J. Immunization with the iron uptake ABC transporter proteins PiaA and PiuA prevents respiratory infection with *Streptococcus pneumoniae*. Vaccine 2006; (24): 5133-5139.

Jones H E, Taylor P R, McGreal E, Zamze S, Wong S Y C. The contribution of naturally occurring IgM antibodies, IgM cross-reactivity and complement dependency in murine humoral responses to pneumococcal capsular polysaccharides. Vaccine 2009; (27): 5806-5815.

Jones M R, Simms B T, Lupa M M, Kogan M S, Mizgerd J P. Lung NF-{kappa}B Activation and Neutrophil Recruitment Require IL-1 and TNF Receptor Signaling during Pneumococcal Pneumonia. J Immunol 2005; (175): 7530-7535.

Jonsson G M, Truedsson L M, Sturfelt G M, Oxelius V-A M P, Braconier J H M P, Sjoholm A G M. Hereditary C2 Deficiency in Sweden: Frequent Occurrence of Invasive Infection, Atherosclerosis, and Rheumatic Disease. Medicine 2005; (84): 23-34.

Jounblat R, Clark H, Eggleton P, Hawgood S, Andrew P W, Kadioglu A. The role of surfactant protein D in the colonisation of the respiratory tract and onset of bacteraemia during pneumococcal pneumonia. Respiratory Research 2005; (6): 126-138.

Jovanovic D V, Di Battista J A, Martel-Pelletier J, Jolicoeur F C, He Y, Zhang M, Mineau F, Pelletier J P. IL-17 Stimulates the Production and Expression of Proinflammatory Cytokines, IL-{beta} and TNF-{alpha}, by Human Macrophages. J Immunol 1998; (160): 3513-3521.

Joyce E, Popper S, Falkow S. *Streptococcus pneumoniae* nasopharyngeal colonization induces type I interferons and interferon-induced gene expression. BMC Genomics 2009; (10): 404.

Kadioglu A, Gingles N A, Grattan K. Host cellular immune response to pneumococcal lung infection in mice. Infect Immun 2000; (68): 1557-1562.

Kadioglu A, Coward W, Colston M J, Hewitt C R A, Andrew P W. CD4-T-Lymphocyte Interactions with Pneumolysin and Pneumococci Suggest a Crucial Protective Role in the Host Response to Pneumococcal Infection. Infect Immun 2004; (72): 2689-2697.

Kaltoft, Zeuthen N, Konradsen H B. Epidemiology of invasive pneumococcal infections in children aged 0-6 years in Denmark: a 19-year nationwide surveillance study. Acta Paediatr Suppl 2000; (89): 3-10.

Kang JY, Nan X, Jin MS, Youn SJ, Ryu YH, Mah S, Han SH, Lee H, Paik SG, Lee JO. Recognition of Lipopeptide Patterns by Toll-like Receptor 2-Toll-like Receptor 6 Heterodimer. Immunity 2009; (31): 873-884.

Kang Y S, Do Y, Lee H K, Park S H, Cheong C, Lynch R M, Loeffler J M, Steinman R M, Park C G. A Dominant Complement Fixation Pathway for Pneumococcal Polysaccharides Initiated by SIGN-R1 Interacting with C1q. Cell 2006; (125): 47-58.

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. The C-type lectin SIGN-R1 mediates uptake of the capsular polysaccharide of *Streptococcus pneumoniae* in the marginal

zone of mouse spleen. Proceedings of the National Academy of Sciences of the United States of America 2004; (101): 215-220.

Kao C Y, Chen Y, Thai P, Wachi S, Huang F, Kim C, Harper R W, Wu R. IL-17 Markedly Up-Regulates {beta}-Defensin-2 Expression in Human Airway Epithelium via JAK and NF-{kappa}B Signaling Pathways. J Immunol 2004; (173): 3482-3491.

Kaplan E L, Laxdal T, Quie P G. Studies of polymorphonuclear leukocytes from patients with chronic granulomatous disease of childhood: bactericidal capacity for streptococci. Pediatrics 1968; (41): 591-599.

Kawakami K, Yamamoto N, Kinjo Y, Miyagi K, Nakasone C, Uezu K, Kinjo T, Nakayama T, Taniguchi M, Saito A. Critical role of Valpha14+ natural killer T cells in the innate phase of host protection against *Streptococcus pneumoniae* infection. Eur J Immunol 2003; (33): 3322-3330.

Kaye P, Malkani R, Martin S, Slack MP, Trotter CL, Jit M, George R, Miller E. Invasive Pneumococcal Disease (IPD) in England & Wales after 7-valent conjugate vaccine (PCV7); potential impact of 10 and 13-valent vaccines. http://www.hpa.org.uk/web/HPAwebFile/HPAweb_C/1245581527892 . 2010. UK Health Protection Agency.

Kayhty H P, Ahman H P, Eriksson K, Sorberg M M, Nilsson L M. Immunogenicity and Tolerability of a Heptavalent Pneumococcal Conjugate Vaccine Administered at 3, 5 and 12 Months of Age. Pediatric Infectious Disease Journal 2005; (24): 108-114.

Kerr A R, Adrian P V, Estevao S, de Groot R, Alloing G, Claverys J P, Mitchell T J, Hermans P W M. The Ami-AliA/AliB Permease of *Streptococcus pneumoniae* Is Involved in Nasopharyngeal Colonization but Not in Invasive Disease. Infect Immun 2004; (72): 3902-3906.

Kett K, Brandtzaeg P, Radl J, Haaijman J J. Different subclass distribution of IgA-producing cells in human lymphoid organs and various secretory tissues. J Immunol 1986; (136): 3631-3635.

Khader S A, Bell G K, Pearl J E, Fountain J J, Rangel-Moreno J, Cilley G E, Shen F, Eaton S M, Gaffen S L, Swain S L, Locksley R M, Haynes L, Randall T D, Cooper A M. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. Nat Immunol 2007; (8): 369-377.

Khandavilli S, Homer K A, Yuste J, Basavanna S, Mitchell T, Brown J S. Maturation of *Streptococcus pneumoniae* lipoproteins by a type II signal peptidase is required for ABC transporter function and full virulence. Mol Microbiol 2008; (67): 541-557.

Kharat A S, Tomasz A. Inactivation of the srtA Gene Affects Localization of Surface Proteins and Decreases Adhesion of *Streptococcus pneumoniae* to Human Pharyngeal Cells In Vitro. Infect Immun 2003; (71): 2758-2765.

Kieber E T, Luo P, Qiu J, Agadjanyan M, Carey L, Hutchins W, Westerink M A, Steplewski Z. Peptide mimicry of adenocarcinoma-associated carbohydrate antigens. Hybridoma 1997; (16): 3-10.

Kirby A C, Newton D J, Carding S R, Kaye P M. Evidence for the involvement of lung-specific gammadelta T cell subsets in local responses to *Streptococcus pneumoniae* infection. Eur J Immunol 2007; (37): 3404-3413.

Kirby A C, Coles M C, Kaye P M. Alveolar Macrophages Transport Pathogens to Lung Draining Lymph Nodes. J Immunol 2009; (183): 1983-1989.

Kirby A, Raynes J, Kaye P. The Role Played by Tumor Necrosis Factor during Localized and Systemic Infection with *Streptococcus pneumoniae*. J Infect Dis 2005; (191): 1538-1547.

Kirby A, Raynes J, Kaye P. CD11b Regulates Recruitment of Alveolar Macrophages but Not Pulmonary Dendritic Cells after Pneumococcal Challenge. J Infect Dis 2006; (193): 205-213.

Kirkpatrick B, Reeves D S, MacGowan A P. A review of the clinical presentation, laboratory features, antimicrobial therapy and outcome of 77 episodes of pneumococcal meningitis occurring in children and adults. Journal of Infection 1994; (29): 171-182.

Kitamura D, Roes J, Kuhn R, Rajewsky K. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. Nature 1991; (350): 423-426.

Knapp S, Leemans J C, Florquin S, Branger J, Maris N A, Pater J, Van Rooijen N, van der Poll T. Alveolar Macrophages Have a Protective Antiinflammatory Role during Murine Pneumococcal Pneumonia. Am J Respir Crit Care Med 2003; (167): 171-179.

Knapp S, Wieland C W, van 't Veer C, Takeuchi O, Akira S, Florquin S, van der Poll T. Toll-Like Receptor 2 Plays a Role in the Early Inflammatory Response to Murine Pneumococcal Pneumonia but Does Not Contribute to Antibacterial Defense. J Immunol 2004; (172): 3132-3138.

Koedel U, Angele B, Rupprecht T, Wagner H, Roggenkamp A, Pfister H W, Kirschning C J. Toll-Like Receptor 2 Participates in Mediation of Immune Response in Experimental Pneumococcal Meningitis. J Immunol 2003; (170): 438-444.

Koh A Y, Priebe G P, Ray C, Van Rooijen N, Pier G B. Inescapable Need for Neutrophils as Mediators of Cellular Innate Immunity to Acute Pseudomonas aeruginosa Pneumonia. Infect Immun 2009; (77): 5300-5310.

Kolberg J, H°iby E A, Jantzen E. Detection of the phosphorylcholine epitope in streptococci,Haemophilusand pathogenicNeisseriaeby immunoblotting. Microbial Pathogenesis 1997; (22): 321-329.

Kolls J K, McCray P B, Chan Y R. Cytokine-mediated regulation of antimicrobial proteins. Nat Rev Immunol 2008; (8): 829-835.

Koppel E A, Wieland C W, van d B, V, Litjens M, Florquin S, van K Y, van der P T, Geijtenbeek T B. Specific ICAM-3 grabbing nonintegrin-related 1 (SIGNR1) expressed by marginal zone macrophages is essential for defense against pulmonary *Streptococcus pneumoniae* infection. Eur J Immunol 2005; (35): 2962-2969.

Kumashi P M, Girgawy E M, Tarrand J J M, Rolston K V M, Raad I I M, Safdar A M. *Streptococcus pneumoniae* Bacteremia in Patients With Cancer: Disease Characteristics and Outcomes in the Era of Escalating Drug Resistance (1998-2002). Medicine 2005; (84): 303-312.

Kuronuma K, Sano H, Kato K, Kudo K, Hyakushima N, Yokota S i, Takahashi H, Fujii N, Suzuki H, Kodama T, Abe S, Kuroki Y. Pulmonary Surfactant Protein A Augments the Phagocytosis of *Streptococcus pneumoniae* by Alveolar Macrophages through a Casein Kinase 2-dependent Increase of Cell Surface Localization of Scavenger Receptor A. J Biol Chem 2004; (279): 21421-21430.

Kyaw M H, Lynfield R, Schaffner W, Craig A S, Hadler J, Reingold A, Thomas A R, Harrison L H, Bennett N M, Farley M M, Facklam R R, Jorgensen J H, Besser J, Zell E R, Schuchat A, Whitney C G. Effect of Introduction of the Pneumococcal Conjugate Vaccine on Drug-Resistant *Streptococcus pneumoniae*. New England Journal of Medicine 2009; (354): 1455-1463.

Laan M, Cui Z H, Hoshino H, Lotvall J, Sjostrand M, Gruenert D C, Skoogh B E, Linden A. Neutrophil Recruitment by Human IL-17 Via C-X-C Chemokine Release in the Airways. J Immunol 1999; (162): 2347-2352.

Laine C, Mwangi T, Thompson C M, Obiero J, Lipsitch M, Scott J A. Age-specific immunoglobulin G (IgG) and IgA to pneumococcal protein antigens in a population in coastal Kenya. Infect Immun 2004; (72): 3331-3335.

Lanie J A, Ng W L, Kazmierczak K M, Andrzejewski T M, Davidsen T M, Wayne K J, Tettelin H, Glass J I, Winkler M E. Genome Sequence of Avery's Virulent Serotype 2 Strain D39 of *Streptococcus pneumoniae* and Comparison with That of Unencapsulated Laboratory Strain R6. J Bacteriol 2007; (189): 38-51.

Lau G W, Haataja S, Lonetto M, Kensit S E, Marra A, Bryant A P, McDevitt D, Morrison D A, Holden D W. A functional genomic analysis of type 3 *Streptococcus pneumoniae* virulence. Mol Microbiol 2001; (40): 555-571.

Lawrence M C, Pilling P A, Epa V C, Berry A M, Ogunniyi A D, Paton J C. The crystal structure of pneumococcal surface antigen PsaA reveals a metal-binding site and a novel structure for a putative ABC-type binding protein. Structure 1998; (6): 1553-1561.

Lee C J, Wang T R, Frasch C E. Immunogenicity in mice of pneumococcal glycoconjugate vaccines using pneumococcal protein carriers. Vaccine 2001; (19): 3216-3225.

Lee H J, Park J Y, Jang S H, Kim J H, Kim E C, Choi K W. High incidence of resistance to multiple antimicrobials in clinical isolates of *Streptococcus*

pneumoniae from a university hospital in Korea. Clin Infect Dis 1995; (20): 826-835.

Lee H Y, Andalibi A, Webster P, Moon S K, Teufert K, Kang S H, Li J D, Nagura M, Ganz T, Lim D. Antimicrobial activity of innate immune molecules against *Streptococcus pneumoniae*, Moraxella catarrhalis and nontypeable Haemophilus influenzae. BMC Infectious Diseases 2004; (4): 12.

Lee M S, Morrison D A. Identification of a New Regulator in *Streptococcus pneumoniae* Linking Quorum Sensing to Competence for Genetic Transformation. J Bacteriol 1999; (181): 5004-5016.

Lehrer R I, Jung G, Ruchala P, Wang W, Micewicz E D, Waring A J, Gillespie E J, Bradley K A, Ratner A J, Rest R F, Lu W. Human {alpha}-Defensins Inhibit Hemolysis Mediated by Cholesterol-Dependent Cytolysins. Infect Immun 2009; (77): 4028-4040.

LeMessurier K S, Ogunniyi A D, Paton J C. Differential expression of key pneumococcal virulence genes in vivo. Microbiology 2006; (152): 305-311.

Levy B D, Clish C B, Schmidt B, Gronert K, Serhan C N. Lipid mediator class switching during acute inflammation: signals in resolution. Nat Immunol 2001; (2): 612-619.

Lin Y, Slight S, Khader S. Th17 cytokines and vaccine-induced immunity. Seminars in Immunopathology 2010; (32): 79-90.

Linder A, Soehnlein O, Akesson P. Roles of Heparin-Binding Protein in Bacterial Infections. J Innate Immun 2010; (2): 431-8.

Lipsitch M. Interpreting results from trials of pneumococcal conjugate vaccines: a statistical test for detecting vaccine-induced increases in carriage of nonvaccine serotypes. Am J Epidemiol 2001; (154): 85-92.

Lipsitch M, Dykes J K, Johnson S E, Ades E W, King J, Briles D E, Carlone G M. Competition among *Streptococcus pneumoniae* for intranasal colonization in a mouse model. Vaccine 2000; (18): 2895-2901.

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

Littmann M, Albiger B, Frentzen A, Normark S, Henriques-Normark B, Plant L. *Streptococcus pneumoniae* evades human dendritic cell surveillance by pneumolysin expression. EMBO Mol Med 2009; (1): 211-222.

Llobet E, Tomas J M, Bengoechea J A. Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. Microbiology 2008; (154): 3877-3886.

Loda F A, Collier A M, Glezen W P, Strangert K, Clyde W A, Jr., Denny F W. Occurrence of Diplococcus pneumoniae in the upper respiratory tract of children. J Pediatr 1975; (87): 1087-1093.

Long J P, Tong H H, DeMaria T F. Immunization with Native or Recombinant *Streptococcus pneumoniae* Neuraminidase Affords Protection in the Chinchilla Otitis Media Model. Infect Immun 2004; (72): 4309-4313.

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. Interleukin-17A mediates acquired immunity to pneumococcal colonization. PLoS Pathog 2008; (4): e1000159.

Lubberts E. Th17 cytokines and arthritis. Seminars in Immunopathology 2010; (32): 43-53.

Lynch J M, Briles D E, Metzger D W. Increased Protection against Pneumococcal Disease by Mucosal Administration of Conjugate Vaccine plus Interleukin-12. Infect Immun 2003; (71): 4780-4788.

Ma J, Wang J, Wan J, Charboneau R, Chang Y, Barke R A, Roy S. Morphine Disrupts the IL-23/IL-17 Mediated Pulmonary Mucosal Host Defense Against *S. pneumoniae* Infection. Infect Immun 2009; (78): 830-7.

Madhi S, Adrian P, Kuwanda L, Cutland C, Albrich W, Klugman K. Long-Term Effect of Pneumococcal Conjugate Vaccine on Nasopharyngeal Colonization by *Streptococcus pneumoniae* and Associated Interactions with *Staphylococcus aureus* and *Haemophilus influenzae* Colonization in HIV–Infected and HIV–Uninfected Children. J Infect Dis 2007; (196): 1662-1666.

Magee A D, Yother J. Requirement for Capsule in Colonization by *Streptococcus pneumoniae*. Infect Immun 2001; (69): 3755-3761.

Malley R. Antibody and cell-mediated immunity to *Streptococcus pneumoniae*: implications for vaccine development. J Mol Med 2010; (88): 135-142.

Malley R, Lipsitch M, Stack A, Saladino R, Fleisher G, Pelton S, Thompson C, Briles D, Anderson P. Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci. Infect Immun 2001; (69): 4870-4873.

Malley R, Srivastava A, Lipsitch M, Thompson C M, Watkins C, Tzianabos A, Anderson P W. Antibody-independent, interleukin-17A-mediated, cross-serotype immunity to pneumococci in mice immunized intranasally with the cell wall polysaccharide. Infect Immun 2006; (74): 2187-2195.

Malley R, Henneke P, Morse S C, Cieslewicz M J, Lipsitch M, Thompson C M, Kurt-Jones E, Paton J C, Wessels M R, Golenbock D T. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. Proceedings of the National Academy of Sciences of the United States of America 2003; (100): 1966-1971.

Malley R, Trzcinski K, Srivastava A, Thompson C M, Anderson P W, Lipsitch M. CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. PNAS 2005; (102): 4848-4853.

Mangtani P, Cutts F, Hall A J. Efficacy of polysaccharide pneumococcal vaccine in adults in more developed countries: the state of the evidence. The Lancet Infectious Diseases 2003; (3): 71-78.

Marriott H M, Dockrell D H. The role of the macrophage in lung disease mediated by bacteria. Exp Lung Res 2007; (33): 493-505.

Marriott H M, Hellewell P G, Cross S S, Ince P G, Whyte M K B, Dockrell D H. Decreased Alveolar Macrophage Apoptosis Is Associated with Increased Pulmonary Inflammation in a Murine Model of Pneumococcal Pneumonia. J Immunol 2006; (177): 6480-6488.

Martin B, Hirota K, Cua D J, Stockinger B, Veldhoen M. Interleukin-17-Producing [gamma][delta] T Cells Selectively Expand in Response to Pathogen Products and Environmental Signals. Immunity 2009; (31): 321-330.

Matsuzaki G, Umemura M. Interleukin-17 as an effector molecule of innate and acquired immunity against infections. Microbiol Immunol 2007; (51): 1139-1147.

Matthias K A, Roche A M, Standish A J, Shchepetov M, Weiser J N. Neutrophil-Toxin Interactions Promote Antigen Delivery and Mucosal Clearance of *Streptococcus pneumoniae*. J Immunol 2008; (180): 6246-6254.

Mbelle N, Huebner R, Wasas A, Kimura A, Chang I, Klugman K. Immunogenicity and Impact on Nasopharyngeal Carriage of a Nonavalent Pneumococcal Conjugate Vaccine. J Infect Dis 1999; (180): 1171-1176.

McCool T L, Cate T R, Moy G, Weiser J N. The Immune Response to Pneumococcal Proteins during Experimental Human Carriage. J Exp Med 2002; (195): 359-365.

McCool T L, Cate T R, Tuomanen E I, Adrian P, Mitchell T J, Weiser J N. Serum immunoglobulin G response to candidate vaccine antigens during experimental human pneumococcal colonization. Infect Immun 2003; (71): 5724-5732.

McCool T L, Weiser J N. Limited Role of Antibody in Clearance of *Streptococcus pneumoniae* in a Murine Model of Colonization. Infect Immun 2004; (72): 5807-5813.

Meisel R, Kuypers L, Dirksen U, Schubert R, Gruhn B, Strauss G, Beutel K, Groll A H, Duffner U, Blutters-Sawatzki R, Holter W, Feuchtinger T, Gruttner H P, Schroten H, Zielen S, Ohmann C, Laws H J, Dilloo D, for the Impfung von Kindern nach allogener Stammzelltransplantation (IKAST) Study Group. Pneumococcal conjugate vaccine provides early protective antibody responses in children after related and unrelated allogeneic hematopoietic stem cell transplantation. Blood 2007; (109): 2322-2326.

Melegaro A, Choi Y, George R, Edmunds W J, Miller E, Gay N. Dynamic models of pneumococcal carriage and the impact of the Heptavalent Pneumococcal Conjugate Vaccine on invasive pneumococcal disease. BMC Infectious Diseases 2010; (10): 90.

Melin M, Di Paolo E, Tikkanen L, Jarva H, Neyt C, Kayhty H, Meri S, Poolman J, Vakevainen M. Interaction of Pneumococcal Histidine Triad Proteins with Human Complement. Infect Immun 2010; (78): 2089-2098.

Melin M M, Hollingshead S K, Briles D E, Lahdenkari M I, Kilpi T M, Kayhty H M. Development of Antibodies to PspA Families 1 and 2 in Children after Exposure to *Streptococcus pneumoniae*. Clin Vaccine Immunol 2008; (15): 1529-1535.

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, 'Shea J, Holland S M, Paul W E, Douek D C. Impaired TH17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. Nature 2008; (452): 773-776.

Minegishi Y, Saito M, Tsuchiya S, Tsuge I, Takada H, Hara T, Kawamura N, Ariga T, Pasic S, Stojkovic O, Metin A, Karasuyama H. Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome. Nature 2007; (448): 1058-1062.

Mitchell T J. The pathogenesis of streptococcal infections: from tooth decay to meningitis. Nat Rev Microbiol 2003; (1): 219-230.

Mitchell T J, Andrew P W, Saunders F K, Smith A N, Boulnois G J. Complement activation and antibody binding by pneumolysin via a region of the toxin homologous to a human acute-phase protein. Mol Microbiol 1991; (5): 1883-1888.

Mogensen T H, Paludan S R, Kilian M, Ostergaard L. 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 2006; (80): 267-277.

Mold C, Du Clos T W, Nakayama S, Edwards K M, Gewurz H. C-reactive protein reactivity with complement and effects on phagocytosis. Ann N Y Acad Sci 1982; (389:251-62.): 251-262.

Mold C, Rodic-Polic B, Du Clos T W. Protection from *Streptococcus pneumoniae* Infection by C-Reactive Protein and Natural Antibody Requires Complement But Not Fc{gamma} Receptors. J Immunol 2002; (168): 6375-6381.

Molrine D C, Antin J H, Guinan E C, Soiffer R J, MacDonald K, Malley R, Malinoski F, Trocciola S, Wilson M, Ambrosino D M. Donor immunization with pneumococcal conjugate vaccine and early protective antibody responses following allogeneic hematopoietic cell transplantation. Blood 2003; (101): 831-836.

Moore L J, Pridmore A C, Lee M E, Read R C. Induction of pro-inflammatory cytokine release by human macrophages during exposure of *Streptococcus pneumoniae* to penicillin is influenced by minimum inhibitory concentration ratio. International Journal of Antimicrobial Agents 2005; (26): 188-196.

Moore Q C, Bosarge J R, Quin L R, McDaniel L S. Enhanced protective immunity against pneumococcal infection with PspA DNA and protein. Vaccine 2006; (24): 5755-5761.

Morona J K, Morona R, Paton J C. Molecular and genetic characterization of the capsule biosynthesis locus of *Streptococcus pneumoniae* type 19B. J Bacteriol 1997; (179): 4953-4958.

Morona J, Miller D, Morona R, Paton J. The effect that mutations in the conserved capsular polysaccharide biosynthesis genes cpsA, cpsB, and cpsD have on virulence of *Streptococcus pneumoniae*. J Infect Dis 2004; (189): 1905-1913.

Morrison V A. An overview of the management of infection and febrile neutropenia in patients with cancer. Support Cancer Ther 2005; (2): 88-94.

Mosmann T R, Cherwinski H, Bond M W, Giedlin M A, Coffman R L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986; (136): 2348-2357.

Mureithi M, Finn A, Ota M, Zhang Q, Davenport V, Mitchell T, Williams N, Adegbola R, Heyderman R. T Cell Memory Response to Pneumococcal Protein Antigens in an Area of High Pneumococcal Carriage and Disease. J Infect Dis 2009; (200): 783-793.

Musher D M, Chapman A J, Goree A, Jonsson S, Briles D, Baughn R E. Natural and vaccine-related immunity to *Streptococcus pneumoniae*. J Infect Dis 1986; (154): 245-256.

Musher D M, Groover J E, Reichler M R, Riedo F X, Schwartz B, Watson D A, Baughn R E, Breiman R F. Emergence of antibody to capsular polysaccharides of *Streptococcus pneumoniae* during outbreaks of pneumonia: association with nasopharyngeal colonization. Clin Infect Dis 1997; (24): 441-446.

Musher D, Phan H, Baughn R. Protection against Bacteremic Pneumococcal Infection by Antibody to Pneumolysin. J Infect Dis 2001; (183): 827-830.

Nakamatsu M, Yamamoto N, Hatta M, Nakasone C, Kinjo T, Miyagi K, Uezu K, Nakamura K, Nakayama T, Taniguchi M, Iwakura Y, Kaku M, Fujita J, Kawakami K. Role of interferon-[gamma] in V[alpha]14+ natural killer T cell-mediated host defense against *Streptococcus pneumoniae* infection in murine lungs. Microbes and Infection 2007; (9): 364-374.

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. Accumulation of gamma/delta T cells in the lungs and their roles in neutrophil-mediated host defense against pneumococcal infection. Microbes and Infection 2007; (9): 251-258.

Nelson A L, Roche A M, Gould J M, Chim K, Ratner A J, Weiser J N. Capsule Enhances Pneumococcal Colonization by Limiting Mucus-Mediated Clearance. Infect Immun 2007; (75): 83-90.

Nesin M, Ramirez M, Tomasz A. Capsular transformation of a multidrug-resistant *Streptococcus pneumoniae* in vivo. J Infect Dis 1998; (177): 707-713.

Neufeld F. *Ueber die Agglutination der Pneumokokken und uber die Theorien der Agglutination*. In: Zeitschrift fur Hygiene Infektionskrankheiten. 1902; 54-72.

Nieminen J, St-Pierre C, Bhaumik P, Poirier F, Sato S. Role of Galectin-3 in Leukocyte Recruitment in a Murine Model of Lung Infection by *Streptococcus pneumoniae*. J Immunol 2008; (180): 2466-2473.

O'Brien K L, Steinhoff M C, Edwards K, Keyserling H, Thoms M L, Madore D. Immunologic priming of young children by pneumococcal glycoprotein conjugate, but not polysaccharide, vaccines. Pediatr Infect Dis J 1996; (15): 425-430.

O'Brien K L, Wolfson L J, Watt J P, Henkle E, oria-Knoll M, McCall N, Lee E, Mulholland K, Levine O S, Cherian T. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. Lancet 2009; (374): 893-902.

O'Brien K, Walters M, Sellman J, Quinlisk P, Regnery H, Schwartz B, Dowell S. Severe Pneumococcal Pneumonia in Previously Healthy Children: The Role of Preceding Influenza Infection. Clinical Infectious Diseases 2000; (30): 784-789.

O'Brien K-L, Millar E, Zell E, Bronsdon M, Weatherholtz R, Reid R, Becenti J, Kvamme S, Whitney C, Santosham M. Effect of Pneumococcal Conjugate Vaccine on Nasopharyngeal Colonization among Immunized and Unimmunized Children in a Community-Randomized Trial. J Infect Dis 2007; (196): 1211-1220.

Ogunniyi A D, Grabowicz M, Briles D E, Cook J, Paton J C. Development of a Vaccine against Invasive Pneumococcal Disease Based on Combinations of Virulence Proteins of *Streptococcus pneumoniae*. Infect Immun 2007; (75): 350-357.

Ogunniyi A D, Woodrow M C, Poolman J T, Paton J C. Protection against *Streptococcus pneumoniae* Elicited by Immunization with Pneumolysin and CbpA. Infect Immun 2001; (69): 5997-6003.

Oldenburg K R, Loganathan D, Goldstein I J, Schultz P G, Gallop M A. Peptide ligands for a sugar-binding protein isolated from a random peptide library. Proc Natl Acad Sci U S A 1992; (89): 5393-5397.

Opitz B, Puschel A, Schmeck B, Hocke A C, Rosseau S, Hammerschmidt S, Schumann R R, Suttorp N, Hippenstiel S. Nucleotide-binding Oligomerization Domain Proteins Are Innate Immune Receptors for Internalized *Streptococcus pneumoniae*. J Biol Chem 2004; (279): 36426-36432.

Ouyang W, Kolls J K, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity 2008; (28): 454-467.

Palaniappan R, Singh S, Singh U P, Sakthivel S K, Ades E W, Briles D E, Hollingshead S K, Paton J C, Sampson J S, Lillard J W, Jr. Differential PsaA-, PspA-, PspC-, and PdB-Specific Immune Responses in a Mouse Model of Pneumococcal Carriage. Infect Immun 2005; (73): 1006-1013.

Park H S, Francis K P, Yu J, Cleary P P. Membranous Cells in Nasal-Associated Lymphoid Tissue: A Portal of Entry for the Respiratory Mucosal Pathogen Group A Streptococcus. J Immunol 2003; (171): 2532-2537.

Park I, Choi I H, Kim S J, Shin J S. Peptide mimotopes of Neisseria meningitidis group B capsular polysaccharide. Yonsei Med J 2004; (45): 755-758.

Park I H, Pritchard D G, Cartee R, Brandao A, Brandileone M C, Nahm M H. Discovery of a New Capsular Serotype (6C) within Serogroup 6 of *Streptococcus pneumoniae*. J Clin Microbiol 2007; (45): 1225-1233.

Park J Y, Choi H j, Prabagar M G V, Choi W S, Kim S J, Cheong C, Park C G, Chin C Y, Kang Y S. The C-type lectin CD209b is expressed on microglia and it mediates the uptake of capsular polysaccharides of *Streptococcus pneumoniae*. Neuroscience Letters 2009; (450): 246-251.

Patel I S, Seemungal T A R, Wilks M, Lloyd-Owen S J, Donaldson G C, Wedzicha J A. Relationship between bacterial colonisation and the frequency, character, and severity of COPD exacerbations. Thorax 2002; (57:): 759-764.

Paterson G K, Mitchell T J. Innate immunity and the pneumococcus. Microbiology 2006; (152): 285-293.

PATH. 7th International Symposium on Pneumococci and Pneumococcal Diseases: Special Report. http://sabin.org/files/uploads/pdfs/FINAL%20ISPPD7%20ProceedingsReport.pdf . 2010. PATH. 20-8-2010.
Ref Type: Electronic Citation

Paton J C. The contribution of pneumolysin to the pathogenicity of *Streptococcus pneumoniae*. Trends in Microbiology 1996; (4): 103-106.

Paust H J, Turner J E, Steinmetz O M, Peters A, Heymann F, Holscher C, Wolf G, Kurts C, Mittrucker H W, Stahl R A K, Panzer U. The IL-23/Th17 Axis Contributes to Renal Injury in Experimental Glomerulonephritis. J Am Soc Nephrol 2009; (20): 969-979.

Pavia M, Bianco A, Nobile C G A, Marinelli P, Angelillo I F. Efficacy of Pneumococcal Vaccination in Children Younger Than 24 Months: A Meta-Analysis. Pediatrics 2009; (123): e1103-e1110.

Pepper M, Linehan J L, Pagan A J, Zell T, Dileepan T, Cleary P P, Jenkins M K. Different routes of bacterial infection induce long-lived TH1 memory cells and short-lived TH17 cells. Nat Immunol 2010; (11): 83-9.

Picard C, Casanova J L. Inherited disorders of cytokines. Curr Opin Pediatr 2004; (16): 648-658.

Picard C, Puel A, Bustamante J, Ku C L, Casanova J L. Primary immunodeficiencies associated with pneumococcal disease. Current Opinion in Allergy and Clinical Immunology 2003; (3).

Pilishvili T, Lexau C, Farley M M, Hadler J, Harrison L H, Bennett N M, Reingold A, Thomas A, Schaffner W, Craig A S, Smith P J, Beall B W, Whitney C G, Moore M R. Sustained Reductions in Invasive Pneumococcal Disease in the Era of Conjugate Vaccine. Journal of Infectious Diseases 2010; (201): 32-41.

Pimenta F C, Miyaji E N, Areas A P M, Oliveira M L S, de Andrade A L S S, Ho P L, Hollingshead S K, Leite L C C. Intranasal Immunization with the Cholera Toxin B Subunit-Pneumococcal Surface Antigen A Fusion Protein Induces Protection against Colonization with *Streptococcus pneumoniae* and Has Negligible Impact on the Nasopharyngeal and Oral Microbiota of Mice. Infect Immun 2006; (74): 4939-4944.

Pollard A J, Perrett K P, Beverley P C. Maintaining protection against invasive bacteria with protein-polysaccharide conjugate vaccines. Nature Reviews Immunology 2009; (9): 212-220.

Poltorak A, He X, Smirnova I, Liu M Y, Van H C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998; (282): 2085-2088.

Pracht D, Elm C, Gerber J, Bergmann S, Rohde M, Seiler M, Kim K S, Jenkinson H F, Nau R, Hammerschmidt S. PavA of *Streptococcus pneumoniae* Modulates Adherence, Invasion, and Meningeal Inflammation. Infect Immun 2005; (73): 2680-2689.

Priebe G P, Walsh R L, Cederroth T A, Kamei A, Coutinho-Sledge Y S, Goldberg J B, Pier G B. IL-17 Is a Critical Component of Vaccine-Induced Protection against Lung Infection by Lipopolysaccharide-Heterologous Strains of Pseudomonas aeruginosa. J Immunol 2008; (181): 4965-4975.

Quinton L J, Jones M R, Simms B T, Kogan M S, Robson B E, Skerrett S J, Mizgerd J P. Functions and Regulation of NF-{kappa}B RelA during Pneumococcal Pneumonia. J Immunol 2007; (178): 1896-1903.

Rabquer B, Shriner A K, Smithson S L, Westerink M A J. B cell mediated priming following pneumococcal colonization. Vaccine 2007; (25): 2036-2042.

Rapola S, J+ñntti V, Haikala R, Syrjänen R, Carlone G, Sampson J, Briles D, Paton J, Takala A, Kilpi T, K+ñyhty H. Natural Development of Antibodies to Pneumococcal Surface Protein A, Pneumococcal Surface Adhesin A, and Pneumolysin in Relation to Pneumococcal Carriage and Acute Otitis Media. J Infect Dis 2000; (182): 1146-1152.

Raquil M A, Anceriz N, Rouleau P, Tessier P A. Blockade of Antimicrobial Proteins S100A8 and S100A9 Inhibits Phagocyte Migration to the Alveoli in Streptococcal Pneumonia. J Immunol 2008; (180): 3366-3374.

Raymond J, Le-áThomas I, Moulin F, Commeau A, Gendrel D, Berche P. Sequential Colonization by *Streptococcus pneumoniae* of Healthy Children Living in an Orphanage. J Infect Dis 2000; (181): 1983-1988.

Rennels M B, Edwards K M, Keyserling H L, Reisinger K S, Hogerman D A, Madore D V, Chang I, Paradiso P R, Malinoski F J, Kimura A. Safety and Immunogenicity of Heptavalent Pneumococcal Vaccine Conjugated to CRM197 in United States Infants. Pediatrics 1998; (101): 604-611.

Reynolds J M, Pappu B P, Peng J, Martinez G J, Zhang Y, Chung Y, Ma L, Yang X O, Nurieva R I, Tian Q, Dong C. Toll-like Receptor 2 Signaling in CD4+ T Lymphocytes Promotes T Helper 17 Responses and Regulates the Pathogenesis of Autoimmune Disease. Immunity 2010; (32): 692-702.

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

Rivera-Olivero I A, Blommaart M, Bogaert D, Hermans P W M, de Waard J H. Multiplex PCR reveals a high rate of nasopharyngeal pneumococcal 7-valent conjugate vaccine serotypes co-colonizing indigenous Warao children in Venezuela. J Med Microbiol 2009; (58): 584-587.

Roche A M, King S J, Weiser J N. Live attenuated *Streptococcus pneumoniae* strains induce serotype-independent mucosal and systemic protection in mice. Infect Immun 2007; (75): 2469-2475.

Roche A M, Weiser J N. Identification of the Targets of Cross-Reactive Antibodies Induced by *Streptococcus pneumoniae* Colonization. Infect Immun 2010; (78): 2231-9.

Rodgers G L, Arguedas A, Cohen R, Dagan R. Global serotype distribution among *Streptococcus pneumoniae* isolates causing otitis media in children: Potential implications for pneumococcal conjugate vaccines. Vaccine 2009; (27): 3802-3810.

Rolston K V I. The spectrum of pulmonary infections in cancer patients. Current Opinion in Oncology 2001; (13): 218-223.

Rose L, Shivshankar P, Hinojosa E, Rodriguez A, Sanchez C, Orihuela C. Antibodies against PsrP, a Novel *Streptococcus pneumoniae* Adhesin, Block Adhesion and Protect Mice against Pneumococcal Challenge. J Infect Dis 2008; (198): 375-383.

Rosenow C, Ryan P, Weiser J N, Johnson S, Fontan P, Ortqvist A, Masure H R. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. Mol Microbiol 1997; (25): 819-829.

Roses R E, Xu S, Xu M, Koldovsky U, Koski G, Czerniecki B J. Differential Production of IL-23 and IL-12 by Myeloid-Derived Dendritic Cells in Response to TLR Agonists. J Immunol 2008; (181): 5120-5127.

Rothwarf D M, Zandi E, Natoli G, Karin M. IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. Nature 1998; (395): 297-300.

Roussel L, Houle F, Chan C, Yao Y, Berube J, Olivenstein R, Martin J G, Huot J, Hamid Q, Ferri L, Rousseau S.
IL-17 Promotes p38 MAPK-Dependent Endothelial Activation Enhancing Neutrophil Recruitment to Sites of Inflammation. J Immunol 2010; (184): 4531-4537.

Roy S, Hill A V S, Knox K, Griffiths D, Crook D. Research pointers: Association of common genetic variant with susceptibility to invasive pneumococcal disease. BMJ 2002; (324): 1369.

Scannell M, Flanagan M B, deStefani A, Wynne K J, Cagney G, Godson C, Maderna P. Annexin-1 and Peptide Derivatives Are Released by Apoptotic Cells and Stimulate Phagocytosis of Apoptotic Neutrophils by Macrophages. J Immunol 2007; (178): 4595-4605.

Schaaf B, Rupp J, Muller-Steinhardt M, Kruse J, Boehmke F, Maass M, Zabel P, Dalhoff K. The interleukin-6 -174 promoter polymorphism is associated with extrapulmonary bacterial dissemination in *Streptococcus pneumoniae* infection. Cytokine 2005; (31): 324-328.

Schneider J J, Unholzer A, Schaller M, Schafer-Korting M, Korting H C. Human defensins. J Mol Med 2005; (83): 587-595.

Schwarzenberger P, Huang W, Ye P, Oliver P, Manuel M, Zhang Z, Bagby G, Nelson S, Kolls J K. Requirement of Endogenous Stem Cell Factor and Granulocyte-Colony-Stimulating Factor for IL-17-Mediated Granulopoiesis. J Immunol 2000; (164): 4783-4789.

Segal A W, Fortunato A, Herd T. A rapid single centrifugation step method for the separation of erythrocytes, granulocytes and mononuclear cells on continuous density gradients of Percoll. J Immunol Methods 1980; (32): 209-214.

Selva L, Viana D, Regev-Yochay G, Trzcinski K, Corpa J M, Lasa I, Novick R P, Penadas J R. Killing niche competitors by remote-control bacteriophage induction. PNAS 2009; (106): 1234-1238.

Selvatici R, Falzarano S, Mollica A, Spisani S. Signal transduction pathways triggered by selective formylpeptide analogues in human neutrophils. European Journal of Pharmacology 2006; (534): 1-11.

Sen G, Khan A Q, Chen Q, Snapper C M. In Vivo Humoral Immune Responses to Isolated Pneumococcal Polysaccharides Are Dependent on the Presence of Associated TLR Ligands. J Immunol 2005; (175): 3084-3091.

Shaper M, Hollingshead S K, Benjamin W H, Jr., Briles D E. PspA Protects *Streptococcus pneumoniae* from Killing by Apolactoferrin, and Antibody to PspA Enhances Killing of Pneumococci by Apolactoferrin. Infect Immun 2004; (72): 5031-5040.

Shimizu T, Nishitani C, Mitsuzawa H, Ariki S, Takahashi M, Ohtani K, Wakamiya N, Kuroki Y. Mannose binding lectin and lung collectins interact with Toll-like receptor 4 and MD-2 by different mechanisms. Biochimica et Biophysica Acta 2009; (1790): 1705-1710.

Shinefield H R, Black S. Efficacy of pneumococcal conjugate vaccines in large scale field trials. Pediatr Infect Dis J 2000; (19): 394-397.

Shinefield H R M, Black S M, Ray P M, Chang I P, Lewis N M, Fireman B M, Hackell J M, Paradiso P R P, Siber G M, Kohberger R P, Madore D V P,

Malinowski F J M, Kimura A M, Le C M, Landaw I M, Aguilar J M, Hansen J B. Safety and immunogenicity of heptavalent pneumococcal CRM197 conjugate vaccine in infants and toddlers. Pediatric Infectious Disease Journal 1999; (18): 757-763.

Siegrist C A, Aspinall R. B-cell responses to vaccination at the extremes of age. Nat Rev Immunol 2009; (9): 185-194.

Simell B, Korkeila M, Pursiainen H, Kilpi T M, Kayhty H. Pneumococcal carriage and otitis media induce salivary antibodies to pneumococcal surface adhesin a, pneumolysin, and pneumococcal surface protein a in children. J Infect Dis 2001; (183): 887-896.

Simell B, Lahdenkari M, Reunanen A, Kayhty H, Vakevainen M. Effects of ageing and gender on naturally acquired antibodies to pneumococcal capsular polysaccharides and virulence-associated proteins. Clin Vaccine Immunol 2008; (15): 1391-1397.

Simell B, Ahokas P, Lahdenkari M, Poolman J, Henckaerts I, Kilpi T M, Kayhty H. Pneumococcal carriage and acute otitis media induce serum antibodies to pneumococcal surface proteins CbpA and PhtD in children. Vaccine 2009; (27): 4615-4621.

Simell B, Kilpi T, Kayhty H. Pneumococcal Carriage and Otitis Media Induce Salivary Antibodies to Pneumococcal Capsular Polysaccharides in Children. J Infect Dis 2002; (186): 1106-1114.

Sjostrom K, Spindler C, Ortqvist A, Kalin M, Sandgren A, Kahlmann–Berenzon S, Henriques–Normark B. Clonal and Capsular Types Decide Whether Pneumococci Will Act as a Primary or Opportunistic Pathogen. Clinical Infectious Diseases 2006; (42): 451-459.

Sleeman K, Griffiths D, Shackley F, Diggle L, Gupta S, Maiden M, Moxon E, Crook D, Peto T. Capsular Serotype-Specific Attack Rates and Duration of Carriage of *Streptococcus pneumoniae* in a Population of Children. J Infect Dis 2006; (194): 682-688.

Smillie W G, Warnock G H, White H J. A Study of a Type I Pneumococcus Epidemic at the State Hospital at Worcester, Mass. Am J Public Health Nations Health 1938; (28): 293-302.

Smith C M, Lo P C, Scuderi A, Kolberg J, Baxendale H, Goldblatt D, Oggioni M R, Felici F, Andrew P W. Peptide mimics of two pneumococcal capsular polysaccharide serotypes (6B and 9V) protect mice from a lethal challenge with *Streptococcus pneumoniae*. Eur J Immunol 2009; (39): 1527-1535.

Soehnlein O, Lindbom L. Phagocyte partnership during the onset and resolution of inflammation. Nat Rev Immunol 2010; (10): 427-439.

Soininen A, Pursiainen H, Kilpi T, Kayhty H. Natural Development of Antibodies to Pneumococcal Capsular Polysaccharides Depends on the Serotype: Association

with Pneumococcal Carriage and Acute Otitis Media in Young Children. J Infect Dis 2001; (184): 569-576.

Standish A J, Weiser J N. Human Neutrophils Kill *Streptococcus pneumoniae* via Serine Proteases. J Immunol 2009;jimmunol.

Stark M A, Huo Y, Burcin T L, Morris M A, Olson T S, Ley K. Phagocytosis of Apoptotic Neutrophils Regulates Granulopoiesis via IL-23 and IL-17. Immunity 2005; (22): 285-294.

Sun K, Metzger D W. Inhibition of pulmonary antibacterial defense by interferon-[gamma] during recovery from influenza infection. Nat Med 2008; (14): 558-564.

Sutcliffe I C, Harrington D J. Pattern searches for the identification of putative lipoprotein genes in Gram-positive bacterial genomes. Microbiology 2002; (148): 2065-2077.

Suzuki N, Suzuki S, Duncan G S, Millar D G, Wada T, Mirtsos C, Takada H, Wakeham A, Itie A, Li S, Penninger J M, Wesche H, Ohashi P S, Mak T W, Yeh W C. Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. Nature 2002; (416): 750-756.

Swingler S, Zhou J, Swingler C, Dauphin A, Greenough T, Jolicoeur P, Stevenson M. Evidence for a Pathogenic Determinant in HIV-1 Nef Involved in B Cell Dysfunction in HIV/AIDS. Cell Host & Microbe 2008; (4): 63-76.

Syrjanen R, Kilpi T, Kaijalainen T, Herva E, Takala A. Nasopharyngeal Carriage of *Streptococcus pneumoniae* in Finnish Children Younger Than 2 Years Old. J Infect Dis 2001; (184): 451-459.

Takagaki Y, DeCloux A, Bonneville M, Tonegawa S. Diversity of gamma delta T-cell receptors on murine intestinal intra-epithelial lymphocytes. Nature 1989; (339): 712-714.

Takashima K, Tateda K, Matsumoto T, Iizawa Y, Nakao M, Yamaguchi K. Role of tumor necrosis factor alpha in pathogenesis of pneumococcal pneumonia in mice. Infect Immun 1997; (65): 257-260.

Talkington D F, Brown B G, Tharpe J A, Koenig A, Russell H. Protection of mice against fatal pneumococcal challenge by immunization with pneumococcal surface adhesin A (PsaA). Microbial Pathogenesis 1996; (21): 17-22.

Tanaka S, Yoshimoto T, Naka T, Nakae S, Iwakura Y i, Cua D, Kubo M. Natural Occurring IL-17 Producing T Cells Regulate the Initial Phase of Neutrophil Mediated Airway Responses. J Immunol 2009; (183): 7523-30.

Tesmer L A, Lundy S K, Sarkar S, Fox D A. Th17 cells in human disease. Immunological Reviews 2008; (223): 87-113.

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. Complete Genome Sequence of a Virulent Isolate of *Streptococcus pneumoniae*. Science 2001; (293): 498-506.

The Institute for Genomic Research. *Streptococcus pneumoniae* TIGR4 Genome Page. http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=bsp . 2010.

Tilley S J, Orlova E V, Gilbert R J C, Andrew P W, Saibil H R. Structural Basis of Pore Formation by the Bacterial Toxin Pneumolysin. Cell 2005; (121): 247-256.

Tomasz A, McDonnell M, Westphal M, Zanati E. Coordinated incorporation of nascent peptidoglycan and teichoic acid into pneumococcal cell walls and conservation of peptidoglycan during growth. J Biol Chem 1975; (250): 337-341.

Tomasz A. Model for the Mechanism Controlling the Expression of Competent State in Pneumococcus Cultures. J Bacteriol 1966; (91): 1050-1061.

Tong H H, Liu X, Chen Y, James M, Demaria T. Effect of neuraminidase on receptor-mediated adherence of *Streptococcus pneumoniae* to chinchilla tracheal epithelium. Acta Otolaryngol 2002; (122): 413-419.

Torzillo P J, Hanna J N, Morey F, Gratten M, Dixon J, Erlich J. Invasive pneumococcal disease in central Australia. Med J Aust 1995; (20): 182-186.

Travassos L H, Girardin S E, Philpott D J, Blanot D, Nahori M A, Werts C, Boneca I G. Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. EMBO Rep 2004; (5): 1000-1006.

Trotter C L, Waight P, Andrews N J, Slack M, Efstratiou A, George R, Miller E. Epidemiology of invasive pneumococcal disease in the pre-conjugate vaccine era: England and Wales, 1996-2006. Journal of Infection 2010; (60): 200-208.

Trzcinski K, Thompson C, Malley R, Lipsitch M. 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 2005; (73): 7043-7046.

Trzcinski K, Thompson C M, Lipsitch M. Construction of Otherwise Isogenic Serotype 6B, 7F, 14, and 19F Capsular Variants of *Streptococcus pneumoniae* Strain TIGR4. Appl Environ Microbiol 2003; (69): 7364-7370.

Trzcinski K, Thompson C M, Srivastava A, Basset A, Malley R, Lipsitch M. Protection against Nasopharyngeal Colonization by *Streptococcus pneumoniae* is Mediated by Antigen-Specific CD4+ T Cells. Infect Immun 2008; (76): 2678-84 .

Tu A H, Fulgham R L, McCrory M A, Briles D E, Szalai A J. Pneumococcal Surface Protein A Inhibits Complement Activation by *Streptococcus pneumoniae*. Infect Immun 1999; (67): 4720-4724.

Tuomanen E I. The Biology of Pneumococcal Infection. [Review]. Pediatric Research 1997; (42): 253-258.

Valadon P, Nussbaum G, Boyd L F, Margulies D H, Scharff M D. Peptide Libraries Define the Fine Specificity of Anti-polysaccharide Antibodies toCryptococcus neoformans. Journal of Molecular Biology 1996; (261): 11-22.

van Beelen A J, Zelinkova Z, Taanman-Kueter E W, Muller F J, Hommes D W, Zaat S A J, Kapsenberg M L, de Jong E C. Stimulation of the Intracellular Bacterial Sensor NOD2 Programs Dendritic Cells to Promote Interleukin-17 Production in Human Memory T Cells. Immunity 2007; (27): 660-669.

van der P T, Opal S M. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. Lancet 2009; (374): 1543-1556.

van Rossum A M, Lysenko E S, Weiser J N. Host and bacterial factors contributing to the clearance of colonization by *Streptococcus pneumoniae* in a murine model. Infect Immun 2005; (73): 7718-7726.

Vasilevsky S, Chattopadhyay G, Colino J, Yeh T J, Chen Q, Sen G, Snapper C M. B and CD4+ T-cell expression of TLR2 is critical for optimal induction of a T-cell-dependent humoral immune response to intact *Streptococcus pneumoniae*. Eur J Immunol 2008; (38): 3316-3326.

Verkaik N, de Vogel C, Boelens H +, Grumann D, Hoogenboezem T, Vink C, Hooijkaas H, Foster T, Verbrugh H, van Belkum A, van Wamel W. Anti-staphylococcal humoral immune response in persistent nasal carriers and noncarriers of *Staphylococcus aureus*. J Infect Dis 2009; (199): 625-632.

Vives M M, Garcia M E R, Saenz P M, De Los Angeles Mora M M, Mata L P, Sabharwal H M, Svanborg C M. Nasopharyngeal colonization in Costa Rican children during the first year of life. Pediatric Infectious Disease Journal 1997; (16): 852-858.

Walport M J. Complement- First of Two Parts. N Engl J Med 2001a; (344): 1058-1066.

Walport M J. Complement- Second of Two Parts. N Engl J Med 2001b; (344): 1140-1144.

Wands J M, Roark C L, Aydintug M K, Jin N, Hahn Y S, Cook L, Yin X, Dal Porto J, Lahn M, Hyde D M, Gelfand E W, Mason R J, O'Brien R L, Born W K. Distribution and leukocyte contacts of {gamma}{delta} T cells in the lung. J Leukoc Biol 2005; (78): 1086-1096.

Wang B, Dileepan T, Briscoe S, Hyland K A, Kang J, Khoruts A, Cleary P P. Induction of TGF-β1 and TGF- β1–dependent predominant Th17 differentiation by group A streptococcal infection. PNAS 2010a; (107): 5937-5942.

Wang S, Li Y, Shi H, Scarpellini G, Torres-Escobar A, Roland K L, Curtiss R, III. Immune Responses to Recombinant Pneumococcal PsaA Antigen Delivered by a Live Attenuated Salmonella Vaccine. Infect Immun 2010b; (78): 3258-3271.

Wani J H, Gilbert J V, Plaut A G, Weiser J N. Identification, cloning, and sequencing of the immunoglobulin A1 protease gene of *Streptococcus pneumoniae*. Infect Immun 1996; (64): 3967-3974.

Wartha F, Beiter K, Albiger B, Fernebro J, Zychlinsky A, Normark S, Henriques-Normark B. Capsule and D-alanylated lipoteichoic acids protect *Streptococcus pneumoniae* against neutrophil extracellular traps. Cell Microbiol 2007; (9): 1162-1171.

Watson D A, Musher D M, Jacobson J W, Verhoef J. A brief history of the pneumococcus in biomedical research: a panoply of scientific discovery. Clin Infect Dis 1993; (17): 913-924.

Weber J R, Freyer D, Alexander C, Schr÷der N W J, Reiss A, K³ster C, Pfeil D, Tuomanen E I, Schumann R R. Recognition of Pneumococcal Peptidoglycan: An Expanded, Pivotal Role for LPS Binding Protein. Immunity 2003; (19): 269-279.

Weinberger D, Dagan R, Givon-Lavi N, Regev-Yochay G, Malley R, Lipsitch M. Epidemiologic Evidence for Serotype-Specific Acquired Immunity to Pneumococcal Carriage. J Infect Dis 2008; (197): 1511-1518.

Weiser J N, Austrian R, Sreenivasan P K, Masure H R. Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. Infect Immun 1994; (62): 2582-2589.

Weiser J N, Bae D, Fasching C, Scamurra R W, Ratner A J, Janoff E N. Antibody-enhanced pneumococcal adherence requires IgA1 protease. Proceedings of the National Academy of Sciences of the United States of America 2003; (100): 4215-4220.

Wernette C M, Frasch C E, Madore D, Carlone G, Goldblatt D, Plikaytis B, Benjamin W, Quataert S A, Hildreth S, Sikkema D J, Kayhty H, Jonsdottir I, Nahm M H. Enzyme-linked immunosorbent assay for quantitation of human antibodies to pneumococcal polysaccharides. Clin Vaccine Immunol 2003; (10): 514-519.

Werno A, Murdoch D. Medical Microbiology: Laboratory Diagnosis of Invasive Pneumococcal Disease. Clinical Infectious Diseases 2008; (46): 926-932.

Westerink M A, Giardina P C, Apicella M A, Kieber E T. Peptide mimicry of the meningococcal group C capsular polysaccharide. Proc Natl Acad Sci U S A 1995; (92): 4021-4025.

Whalan R H, Funnell S G, Bowler L D, Hudson M J, Robinson A, Dowson C G. PiuA and PiaA, iron uptake lipoproteins of *Streptococcus pneumoniae*, elicit serotype independent antibody responses following human pneumococcal septicaemia. FEMS Immunol Med Microbiol 2005; (43): 73-80.

Whalan R H, Funnell S G P, Bowler L D, Hudson M J, Robinson A, Dowson C G. Distribution and Genetic Diversity of the ABC Transporter Lipoproteins PiuA and PiaA within *Streptococcus pneumoniae* and Related Streptococci. J Bacteriol 2006; (188): 1031-1038.

Whitney C G, Farley M M, Hadler J, Harrison L H, Bennett N M, Lynfield R, Reingold A, Cieslak P R, Pilishvili T, Jackson D, Facklam R R, Jorgensen J H, Schuchat A. Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. N Engl J Med 2003; (348): 1737-1746.

WHO. Pneumococcal vaccines. WHO position paper. Weekly Epidemiol Rec 1999; (74): 177-183.

WHO. 23-valent pneumococcal polysaccharide vaccine. WHO position paper. Wkly Epidemiol Rec 2008; (83): 373-384.

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. Use of a Whole Genome Approach To Identify Vaccine Molecules Affording Protection against *Streptococcus pneumoniae* Infection. Infect Immun 2001; (69): 1593-1598.

Wu H Y, Nahm M H, Guo Y, Russell M W, Briles D E. Intranasal immunization of mice with PspA (pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with *Streptococcus pneumoniae*. J Infect Dis 1997a; (175): 839-846.

Wu H Y, Virolainen A, Mathews B, King J, Russell M W, Briles D E. Establishment of a *Streptococcus pneumoniae* nasopharyngeal colonization model in adult mice. Microb Pathog 1997b; (23): 127-137.

Wu Y, Wu W, Wong W M, Ward E, Thrasher A J, Goldblatt D, Osman M, Digard P, Canaday D H, Gustafsson K. Human {gamma}{delta} T Cells: A Lymphoid Lineage Cell Capable of Professional Phagocytosis. J Immunol 2009; (183): 5622-5629.

Xin W, Li Y, Mo H, Roland K L, Curtiss R, III. PspA Family Fusion Proteins Delivered by Attenuated Salmonella enterica Serovar Typhimurium Extend and Enhance Protection against *Streptococcus pneumoniae*. Infect Immun 2009; (77): 4518-4528.

Xu F, Droemann D, Rupp J, Shen H, Wu X, Goldmann T, Hippenstiel S, Zabel P, Dalhoff K. Modulation of the Inflammatory Response to *Streptococcus pneumoniae* in a Model of Acute Lung Tissue Infection. Am J Respir Cell Mol Biol 2008; (39): 522-529.

Yamada T, Kataoka S, Ogasawara K, Ishimitsu R, Hashigucci K, Suzuki T, Kawauchi H. Mucosal immunity of nasopharynx: an experimental study in TCR-transgenic (OVA23-3) mice. Rhinology 2005.

Yeh S H, Zangwill K M, Lee H, Chang S J, Wong V I, Greenberg D P, Ward J I. Heptavalent pneumococcal vaccine conjugated to outer membrane protein of Neisseria meningitidis serogroup b and nasopharyngeal carriage of *Streptococcus pneumoniae* in infants. Vaccine 2003; (21): 2627-2631.

Yuste J, Botto M, Bottoms S E, Brown J S. Serum Amyloid P Aids Complement-Mediated Immunity to *Streptococcus pneumoniae*. PLoS Pathog 2007; (3): e120.

Yuste J, Botto M, Paton J C, Holden D W, Brown J S. Additive Inhibition of Complement Deposition by Pneumolysin and PspA Facilitates *Streptococcus pneumoniae* Septicemia. J Immunol 2005; (175): 1813-1819.

Yuste J, Sen A, Truedsson L, Jonsson G, Tay L S, Hyams C, Baxendale H E, Goldblatt F, Botto M, Brown J S. Impaired opsonization with C3b and phagocytosis of *Streptococcus pneumoniae* in sera from subjects with defects in the classical complement pathway. Infect Immun 2008; (76): 3761-3770.

Zanetti M. The role of cathelicidins in the innate host defenses of mammals. Curr Issues Mol Biol 2005; (7): 179-196.

Zelante T, De L A, Bonifazi P, Montagnoli C, Bozza S, Moretti S, Belladonna M L, Vacca C, Conte C, Mosci P, Bistoni F, Puccetti P, Kastelein R A, Kopf M, Romani L. IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. Eur J Immunol 2007; (37): 2695-2706.

Zhang Q, Bernatoniene J, Bagrade L, Pollard A J, Mitchell T J, Paton J C, Finn A. Serum and mucosal antibody responses to pneumococcal protein antigens in children: relationships with carriage status. Eur J Immunol 2006; (36): 46-57.

Zhang Q, Bagrade L, Bernatoniene J, Clarke E, Paton J, Mitchell T, Nunez D, Finn A. Low CD4 T Cell Immunity to Pneumolysin Is Associated with Nasopharyngeal Carriage of Pneumococci in Children. J Infect Dis 2007; (195): 1194-1202.

Zhang Q, Choo S, Finn A. Immune Responses to Novel Pneumococcal Proteins Pneumolysin, PspA, PsaA, and CbpA in Adenoidal B Cells from Children. Infect Immun 2002; (70): 5363-5369.

Zhang Z, Clarke T B, Weiser J N. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. J Clin Invest 2009; (119): 1899-1909.

Zhu J F, Paul W E. Heterogeneity and plasticity of T helper cells. Cell Research 2010; (20); 4-12.

Zygmunt B M, Rharbaoui F, Groebe L, Guzman C A. Intranasal Immunization Promotes Th17 Immune Responses. J Immunol 2009; (183): 6933-8).

APPENDIX: BUFFERS

DEA BUFFER (FOR ELISA)

1M diethanolamine, 0.5 mM $MgCl_2$ [1 litre]

Add 97 ml diethanolamine (Sigma) to 850 ml distilled water in a fume hood.
Mix well with magnetic stirrer.
Add 0.1 g $MgCl_2$.6H2O (Sigma) and allow to dissolve.
Adjust pH to 9.8 ± 0.05 slowly adding 6M HCl.
Store protected from light at RT for up to 6 months.

10X TRIS-BUFFERED SALINE

50mM Tris, 150 mM NaCl, pH 7.4 [1 litre]

Add 80g NaCl to 800 ml water.
Add 2g KCl.
Add 30 g of Tris base (Fisher).
Adjust to pH 7.4 with HCl.
Make to 1 litre with water.

COOMASSIE BRILLIANT BLUE SOLUTION

0.1% Coomassie blue R350, 20% methanol, 10% acetic acid [400 ml]

Add 80 ml methanol to 120 ml distilled water.
Dissolve 0.4 g Coomassie blue R350 and filter.
Add 200 ml 20% acetic acid in distilled water.

DESTAIN SOLUTION

50% methanol, 10% acetic acid [1 litre]

Add 500 mL methanol to 300 mL water
Then add 100 mL acetic acid, and bring to 1 litre with water

10X TRANSFER BUFFER

Glycine	36.3 g
Tris (base)	150 g
SDS	3.75 g

Make to 1 litre with distilled water.

TRANSFER BUFFER WITH METHANOL

Add 80 ml 10X Transfer Buffer to 80ml methanol.
Dilute to 1X with 240 ml distilled water.