Pneumococcal colonisation models of the nasopharynx: The role of virulence factors and host immunity during colonisation.

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Abstract

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The human nasopharynx is the most important ecological niche for *Streptococcus pneumoniae* and the establishment of nasopharyngeal carriage is an essential pre-requisite to invasive pneumococcal disease. Virulence factors which mediate long term carriage and the immunogenicity of individual bacterial components are of interest to the development of vaccines which currently fall short of protecting against >90 known serotypes of pneumococci. This thesis contains a long term mouse model of nasopharyngeal carriage conducted in outbred mice, using both wild type and attenuated isogenic mutant pneumococcal strains. Whilst serotype-2 (D39) and serotype-3 (A66) pneumococci were carried asymptomatically in the nasopharynx for at least 21 days, mutants that lacked neuraminidases, PspA and pneumolysin were cleared from the nasopharynx <14 days. Both the carriage of WT-D39 and the clearance of the pneumolysin negative ($PlnA^-$) were associated with the generation of serum IgM anti-capsular antibody, and IgG anti-PspA, which correlated to bacterial numbers in the nasopharynx. Carriage also stimulated anti-capsular IgA in the cervical lymph nodes, and a local macrophage cellular response. Using attenuated carriage it was possible to model the effects of subsequent exposure to pneumococci, in the form of future carriage events and invasive disease. Prior colonisation significantly shortened the duration of carriage from >28 days to <14 days within the same serotype however, both the polysaccharide capsule and conserved protein antigens contribute to protection. Colonisation could also protect mice from a normally fatal invasive challenge with both D39 and A66. Immune sera alone from previously colonised mice was able to delay onset of fatal pneumonia, however the presence of both PspA and capsule antigens were not indispensible to the protection in this instance. Taken together, the results in this thesis support existing data about the immunogenicity of capsule and conserved proteins which may inform the creation of novel and more efficacious pneumococcal vaccines.

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Chapter 1: Introduction

1.1 Preface

S. pneumoniae is a human pathogen which continues to be responsible for large numbers of deaths in both the developing and developed world, despite the emergence of preventative therapies and improving treatments. Although the organism is the causative agent of pathologies such as meningitis and pneumonia, the major manifestation of pneumococcal-host contact is not invasive disease, and more commonly a persistent colonisation of the nasopharynx may transpire. Carriage can be acquired repeatedly at an early age, and the organism normally remains innocuously in the upper respiratory tract for a period of a few weeks to months, and thus the pneumococcus could also be considered part of the normal flora of the nasopharynx. The reasons why a sometimes commensal bacterium turns invasive are not well documented, but it is recognised that the balance between these two possible outcomes is dependent on host-bacterial interactions. Invasive disease is not often associated with long term carriage and more commonly develops soon after the acquisition of a new pneumococcal strain in the nasopharynx, therefore the fitness of host immunity at the time of colonisation as well as the virulence of the particular colonising strain may be crucial. It is perhaps unfortunate that many of the virulence factors which are adapted for survival in the nasopharynx play an important role in the pathogenesis of invasive disease, and therefore; if the balance is disrupted then progression towards serious disease can be rapid. In poorer countries where pneumococcal disease is most common, vital early antibiotic treatment can often be delayed and therefore the best strategy for prevention against pneumococcal disease is thought to be with widespread vaccination. The shortcomings of current vaccination strategy is its reliance solely on capsular polysaccharide, which is uniquely represented by >90 different pneumococcal serotypes. Whilst vaccination has changed the composition of pneumococcal disease the overall incidence of pneumococcal disease is unchanged in the population, as non-vaccine types replace vaccine included types. Animal models of pneumococcal colonisation allow for the identification of precise correlates of immune protection to carriage and invasive disease, many of which are conserved proteins such as pneumococcal surface protein-A and pneumolysin. A vaccine which incorporates

conserved proteins could provide protection against all serotypes simultaneously, and furthermore, any immunisation which is specifically tailored to the prevention of pneumococcal carriage may serve to both decrease the incidence of pneumococcal disease in vulnerable populations and reduce the available pool of pneumococci which can be transferred between individuals, thereby providing so called 'herd immunity', a phenomenon which has already been observed with existing pneumococcal vaccines.

The following introduction will outline the virulence factors which are important to pathogenesis of pneumococcal disease with particular attention to facets of nasopharyngeal carriage, cover the current *in vivo* and *in vitro* data on host responses to pneumococci and discuss existing and novel strategies to prevent pneumococcal disease.

1.2 The pneumococcus

S. pneumoniae referred to commonly as 'the pneumococcus', was probably first isolated in 1875 by Edwin Klebs from infected sputum and lung tissue from individuals with pneumonia (Klebs, 1875). It was better described by George M. Sternberg and Louis Pasteur simultaneously in 1880 when both inoculated rabbits with saliva from presumed asymptomatic carriers of *S. pneumoniae* into rabbits, and observed rapid progression of a septicaemic disease. It was Pasteur who also first described pneumococci surrounded by "an aureole which corresponds perhaps to a material substance", which later was recognised as the polysaccharide capsule, but through his observations, in 1883 it was the scientist Anton Weichselbaum who suggested the description *Diplococcus pneumoniae* which became the accepted terminology for nearly 100 years, before its re-classification in 1974. Since its discovery, pneumococci have played a role in the discovery of many scientific principles which are worthy of recognition, before the introduction to pneumococcal disease. The earliest descriptions of humoral immunity were made through the discovery of passive immunity by Georg and Felix Klemperer, with sera from recovering pneumonia patients affording protection against disease in rabbits. Pneumococcal disease also provided the impetus for the development of Penicillin, one of the most influential drugs of the 20th century, and contributed hugely to the use of polysaccharides in vaccine technology. However, perhaps the most noteworthy use of pneumococci came in 1928 when it was observed that when heat killed encapsulated pneumococci and non-encapsulated live strains were

co-administered to mice, that the non-encapsulated strains acquired the capsular type of the heat killed strain, a "transforming principle" which some years later proved to be DNA (Griffith, 1928).

1.3 Pneumococcal disease

The pneumococcus is responsible for a plethora of human diseases, which collectively cause a huge burden of mortality and morbidity worldwide. It is the most frequent cause of bacterial meningitis and community acquired pneumonia (CAP) internationally, and the World Health Organisation (WHO) estimates that almost 1.6 million deaths annually can be attributed to what is collectively termed invasive pneumococcal disease (IPD) (Schuchat *et al*., 1997; Ortqvist *et al*., 2005; Anonymous, 2007). Invasive disease broadly describes the presence of pneumococcus inside a usually sterile site (blood, pleural fluid, cerebrospinal fluid etc), whereas non-invasive forms of pneumonia and conditions such as otitis media and sinusitis are not normally associated with a positive blood culture. The pneumococcus is the most regularly detected pathogen associated with pneumonia in the United Kingdom (Lim *et al*., 2001), however by far the largest incidence of IPD can be found in the developing world, with up to 4-million cases each year in Africa, where mortality rates from disease associated with bacteraemia can exceed 30% (Scott, 2007; Berkley *et al*., 2005). It is likely that incidence rates between the developing and developed world differ due to common socio-economic factors such as malnutrition, however the prevalence of sickle cell anaemia and human immuno-deficiency virus (HIV) also represent substantial risk factors for IPD (Greenwood, 1999). Disregarding other demographic factors, those most acutely at risk of developing IPD are children under 24 months (O'Dempsey *et al*., 1996; Mufson and Stanek, 1999), and adults >65 years (Whitney *et al*., 2001; Watanakunakorn *et al*., 1993; Sleeman *et al*., 2001), although the majority of deaths are associated with adult disease, where HIV is likely a contributing factor (Lynch and Zhanel, 2009b). The serotypes responsible for IPD also vary substantially according to geography and age of the subjects at the time of sampling, serotypes 1, 2, 4, 5, 7F, 9V, 14, and 16 are all predominately recovered from invasive disease only (Brueggemann *et al*., 2003; Kronenberg *et al*., 2006; Hausdorff *et al*., 2000), but inevitably surveillance is complicated by the overlap between carriage and invasive strains and the fact that more than one serotype can be found simultaneously in the nasopharynx. Certain strains such as serotype-3 are often correlated with a higher

mortality rate (Henriques *et al*., 2000), but it unclear whether this finding alone can indicate a propensity to cause more severe forms of disease, as specific strains can more commonly afflict risk groups such as those >65 years of age or those with pre-existing underlying conditions (Alanee *et al*., 2007).

Less severe than invasive disease but much more prevalent, is Acute Otitis Media (AOM), which in America yearly accounts for over 30 million visits to health care practitioners, predominantly in children, of which *S. pneumoniae* is implicated in approximately 25% of cases (Klein, 2000; Kilpi *et al*., 2001). AOM develops when pneumococci translocate from the nasal cavity to the middle ear via the Eustachian tube. In contrast to their abilities to cause invasive disease it was found that most serotypes had an equal propensity to cause AOM which equated to their prevalence in nasopharyngeal carriage (Hanage *et al*., 2004).

1.4 Pneumococcal carriage

Pneumococcal disease almost exclusively effects the human population, which will be the focus of this thesis, although the bacterium can also be isolated from the nasopharynx of both primate (Chi *et al*, 2007) and equine hosts (Whatmore *et al*, 1999). Pneumococcal carriage of the mucosal surfaces of the upper respiratory tract provides a gateway into all disease states, and is the platform for propagating the horizontal transmission of carriage through the population via direct person to person contact (Bogaert *et al*., 2004) (Fig 1.1), and the proximity of humans to animal species may attributed to carriage in those cases (Chi *et al*, 2007). As ultimately the development of invasive disease can lead to death of the host and this proves deleterious for pneumococcal survival and transmission it is therefore logical that by far the most common manifestation of *S. pneumoniae* within its human host is a largely undetected and asymptomatic colonisation of the naso/oro pharynx. In both the developing and developed world at least one episode of pneumococcal carriage is almost universal within the first few years of life, with upwards of 50% of children under 5 years of age being colonised at any one time (Hansman *et al*., 1985; Hill *et al*., 2008; Syrjanen *et al*., 2001). The appearance of the first incidence of carriage is usually dependent upon the existence of the pneumococcus in the immediate environment but can be affected by a number of factors including; number of siblings, day care attendance and passive smoking (Leino *et al*., 2001; Hoti *et al*., 2009; Greenberg *et al*., 2006). Carriage is

detected earliest in developing countries such as The Gambia, where the first incidence of carriage can be routinely found within the first month after birth, with carriage rates peaking at just 4 months (Hill *et al*., 2006). In more affluent countries the first carriage event occurs later and the peak incidence of carriage is not reached until after the age of 2 (Dagan *et al*., 1996). The prevalence of colonisation is however strongly linked to age in all populations, and after 5 years of age carriage rates decline significantly from >50% down to <20%, levels which are then maintained at a constant level throughout adulthood (Abdullahi *et al*., 2008; Hussain *et al*., 2005). This dramatic and early change in carriage rates is thought to be related to maturation of the immune system, as children under the age of 2 produce poor serum antibody responses to thymus independent antigens such as pneumococcal capsular polysaccharide (CPS) (Douglas *et al*., 1983), and produce significantly less anti-capsular IgG than their mothers after colonisation with a specific serotype (Soininen *et al*., 2001). Nevertheless, anti-PS antibody can be detected in children as young as 6 months of age, and both anti-PS (although serotype specific) (Soininen *et al*., 2001) and anti-protein IgG titres (Goldblatt *et al*., 2005b) have been shown to increase with age, although it is unclear the relative contributions of each to a protective immune response in early life, (the host immune responses carriage will be discussed in more detail later in this chapter). Competition for the space within the nasopharynx has also been suggested to affect the age related prevalence of pneumococci, as, whilst the level of pneumococcal colonisation falls, carriage of another resident of the nasopharynx, *Staphylococcus aureus* actually increases, from approximately 10% to a maximum of 50% at 10 years of age (Bogaert *et al*., 2004). A further explanation for age related decline in carriage is the sheer number of immunologically diverse serotypes (91 currently identified) which may be encountered. Colonisation of the nasopharynx is an intermittent state, with each colonising strain persisting for anything between a few days and a few months (Smith *et al*., 1993; Erasto *et al*., 2010) before being cleared or replaced by a subsequent colonisation, and this turnover (conceivably due to continued exposure to pneumococci through nasopharyngeal colonisation) occurs faster in adults (Goldblatt *et al*., 2005b; Rapola *et al*., 2000). In approximately a third of cases more than one serotype of pneumococci (up to as many as 6) can be isolated from the nasopharynx at one time (Meats *et al*., 2003; Hill *et al*., 2008; Hansman *et al*., 1985), and in such a situation normally one serotype predominates with the other strain/s present in smaller quantities (Gray *et al*., 1980). By far the most common serotypes associated with carriage in

children are serotypes; 6, 19, 14 and 23 and this is surprisingly true almost regardless of geographical restriction, whilst the serotypes related to adult colonisation are much broader (Meats *et al*., 2003; Soininen *et al*., 2001; Goldblatt *et al*., 2005b). The transition between asymptomatic carriage of the nasopharynx and invasive disease is not well understood. Epidemiological evidence suggests that the relationship between carriage and disease is a complex one, taking into account age, vaccination status, serotype and previous exposure to pneumococci. Although some of the serotypes associated with carriage are also capable of causing invasive disease (6, 9, 14, 23) (Pineda *et al*., 2002), serotypes such as 1 and 2, which commonly cause IPD are almost never associated with carriage (Dochez and Avery, 1915). Moreover, there exists an inverse relationship between the duration of carriage and incidence of invasive disease, whereby those serotypes which are known to cause invasive disease have short colonisations and those which rarely become invasive persist longer in the nasopharynx (Sleeman *et al*., 2006). It is however, perhaps naive to consider that differences in pneumococcal carriage are dependent on capsular serotype alone. In one study, a shorter lag period of *in vitro* growth in minimal medium was associated with an increased prevalence of carriage, indicating that under selective pressure the genetic background of the pneumococcal strains may also influence the outcome during carriage (Battig *et al*., 2006).

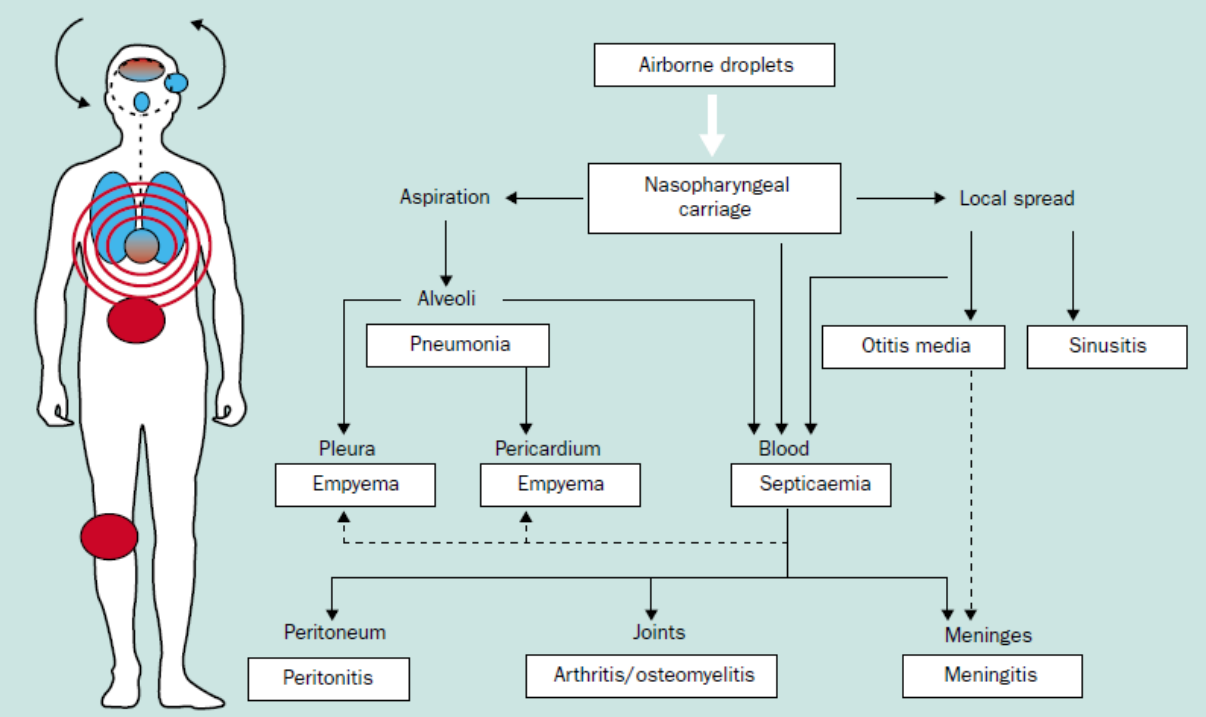

Figure 1.1 Pathogenesis of pneumococcal infection. This diagram shows the dissemination of pneumococci through the host. All subsequent routes start from respiratory intake of bacteria and nasopharyngeal carriage, which can then lead to otitis media, meningitis, pneumonia and septicaemia. Figure taken from Bogaert *et al*., 2004.

1.5 Pneumococcal virulence factors

The pneumococcus possesses a large arsenal of virulence factors which can be found both intracellularly (pneumolysin), bound to the cell wall surface (choline binding proteins) and surrounding the cell itself (the polysaccharide capsule). Many of these factors have multiple functions at different sites in the human body, some of which are involved nasopharyngeal carriage and some which contribute to invasive disease. Table 1.1 provides a summary of the major functions of the most common pneumococcal virulence factors, and the sites at which they exert their influence, followed by a more detailed introduction to pneumolysin. More detail on other protein factors and the polysaccharide capsule, is covered in the pathogenesis of pneumococci in the upper respiratory tract.

Pneumococcal virulence factors	Role in carriage
Polysaccharide capsule	Prevents entrapment in mucus secretion in the respiratory tract. Prevents opsonophagocytosis.
Pneumolysin (PLY)	Cytotoxic toxin which can divert complement components away from the cell. Can stimulate a wide range of stimulatory and inhibitory cytokines in sublytic concentrations.
Pneumococcal surface protein A (PspA)	Prevents binding of complement components on the surface of pneumococci. Neutralises apolactoferrin.
Pneumococcal surface protein C (PspC/CbpA)	Can attach to the host polymeric immune receptor (pIgR) to translocate across epithelial surfaces.
Phosphorylcholine (ChoP)	Can bind platelet activating factor receptor (rPAF) on host epithelium.
Neuraminidase A (NanA)	Can act with two additional exoglycosidases to cleave terminal sugar residues from glycoconjugates, revealing receptors for adherence, and yielding nutrients.
Neuraminidase B (NanB)	Can substitute the activity of NanA, but may act elsewhere in the respiratory tract.
Hyaluronidase (Hyl)	Breaks down hyaluronan in connective tissue.
Pneumococcal adhesion and virulence A (PavA)	Binds the extra-cellular matrix component fibronectin.
Enolase (Eno)	Binds the extra-cellular matrix component plasminogen.
IgA1 protease	Cleaves host IgA1, reducing antibody mediated clearance. Improves adherence to epithelial cells as cationic cleaved fragments of IgA counteract steric hindrance of capsule .
Pneumococcal surface antigen A (PsaA)	Uptake of zinc and manganese ions, which are essential for the expression of downstream adherence factors.

Table 1.1 Pneumococcal virulence factors in nasopharyngeal carriage. This list covers only the major virulence determinants during carriage, for full description see the main text below.

1.6 Pneumolysin

Pneumolysin (PLY) is a 53 kDa protein which belongs to a family of cholesterol dependent cytolysins (CDC's), expressed in a number of Gram positive organisms, including streptolysin O produced by *Streptococcus pyogenes* (Tweten, 2005). As the name suggests, the molecular target for PLY is cholesterol, and binding to host cells is initiated by the oligomerisation of up to 40 monomers, which first assemble a pre-pore on the cell membrane which is then injected through the lipid bilayer, a process which requires an exquisite conformational change of the molecule (Tilley *et al*., 2005). PLY consists of 4 domains that have specific roles in the creation of membrane pores, with

domain-4 being identified as crucial for both the binding to cholesterol, and haemolytic activity of the toxin (Baba *et al*., 2001). PLY differs from most CDC's as it is predominately located intracellularly, in the cytoplasm, and released only by the action of an autolysin which breaks down the peptidoglycan backbone of the bacterial cell wall. The autolysin of *S. pneumoniae* is N-acetylmuramoyl-L-alanine amidase or LytA (Ronda *et al*., 1987). LytA mutants lack full virulence in mice which may explain the importance of PLY and other cell wall components in inflammation induced dissemination of the bacterium (Berry and Paton, 2000). Whilst high concentrations of PLY lead to oligomerisation and the disruption of membrane integrity and cell lysis, there have been many described effects of PLY using sub-lytic doses. Pore formation of sub-lytic doses can occur in epithelial cells, which leads to cell signalling induced through p38 mitogen activated protein kinase, and the release of IL-8 or the murine equivalent MIP-2, that is a potent stimulator of neutrophils (Ratner *et al.*, 2006; Ratner *et al*., 2005). In accordance with the appearance of neutrophils, many pro-inflammatory cytokines have shown to be released by the action of PLY and PLY sufficient bacteria. IFN-γ, IL-17A, TNF-α, IL-1β and IL-6, are all released by the actions of PLY and are independent or dependent on the involvement of TLR-4 depending on the study (McNeela *et al*., 2010*;* Malley *et al*., 2003). Other properties of PLY are covered in the next section.

1.7 Pathogenesis of pneumococci in the upper respiratory tract

The establishment of long term pneumococcal carriage and the potential transmission to invasive disease requires the assistance of many pneumococcal virulence factors, and distinct requirements for those factors at different anatomical sites during the nasopharyngeal carriage and pneumococcal invasive disease have previously been observed (Orihuela *et al*., 2004a). Carriage can broadly be subdivided into 3 stages; initial attachment to the nasopharynx, deeper penetration of the epithelial barrier, and immune evasion which occurs constantly. Each stage will be covered below separately.

1.8 Initial attachment

Successful adherence to epithelial cells in the nasopharynx can be considered the first important first step in the pathogenesis of pneumococcal disease and asymptomatic carriage. The adhesion *S. pneumoniae* to the epithelial surface is enhanced by the modification of glycoconjugates on the surface of nasopharyngeal epithelial cells

(Andersson *et al*., 1983). This modification is facilitated by at least 3 distinct surface exoglycosidases; neuraminidase A (NanA) (Camara *et al*., 1994), β-galactosidase (BgaA) (Zahner and Hakenbeck, 2000) and β- N-acetylglucosaminidase (StrH) (Clarke *et al*., 1995). It is thought that these three enzymes work in concert to sequentially remove sialic acid, galactose and N-acetylglucosamine respectively from host glycoproteins, with each product acting as the substrate for the next enzyme in the pathway. This process ultimately exposes mannose residues, which provide contact to previously inaccessible adherence factors and also yields additional sources of carbon nutrients (King *et al*., 2006). In addition, these enzymes have been shown to cause the deglycosylation of human secretory component, IgA and lactoferrin, host factors which promote pneumococcal clearance, and thus their expression may aid the escape from components of innate immunity. Recently NanA, BgaA and StrH, were also identified to promote resistance to pneumococcal infection by preventing opsonophagocytosis by neutrophils, which are important components of an early response to nasopharyngeal carriage in mice (Matthias *et al*., 2008), a process which is likely facilitated by the deglycosylation of components of the alternative pathway of complement (Dalia *et al*., 2010). The importance of NanA is such that it is highly conserved and can be found in almost all clinical isolates of pneumococci (O'Toole *et al*., 1971). A second neuraminidase (NanB) can replace the actions of NanA *in vitro* (Burnaugh *et al*., 2008), although this enzyme appears to have a role later in carriage on the basis of having a different optimum pH (Berry *et al*., 1996) and the behaviour of a NanB mutant *in vivo* (Manco *et al*., 2006). Mutants lacking NanA have both reduced virulence *in vivo* by the intranasal route and are significantly less able to persist in the nasopharynx than wild type (WT) pneumococci (Tong *et al*., 2000; Manco *et al*., 2006), whereas the major effect of the NanB mutant was the inability to cause significant infection of the trachea and lung. A third neuraminidase, NanC has been described in approximately 50% of pneumococcal isolates tested (although it is absent in D39), and is associated predominately with invasive strains, however it is yet unclear as to the precise mechanism of its action and to what extent these 3 enzymes cooperate (Pettigrew *et al*., 2006).

In the naso/oro pharynx, typeable strains of the pneumococcus can be distinguished from other common-but non pathogenic streptococcal colonisers by the expression of a polysaccharide capsule (Whatmore *et al*., 2000). Soon after entering the nasopharynx

bacteria encounter secretions of mucus, which the largely anionic capsule helps to repel by electrostatic forces, allowing crucial transit through the luminal mucus to the underlying epithelial surface (Nelson *et al*., 2007). The expression of capsule is key to long term carriage in mice however the amount of capsular material required is not, as whilst at least 6% of capsule is indispensible to carriage *in vivo*, mutants with just 20% of capsule produce equivalent levels of carriage to their respective (WT) (Magee and Yother, 2001). The size of capsule does however determine resistance to non-opsonophagocytic killing by neutrophils, which may be the major mechanism of clearance in the absence of specific antibodies, and was shown to correlate with the density of pneumococcal colonisation (Weinberger *et al*., 2009). The amount of capsule required during different stages of carriage is diverse, and although a thick polysaccharide capsule is also advantageous in competing for space in the nasopharyngeal niche, both against additional pneumococci expressing different capsular types and another commensal coloniser, *Haemophilus influenza* (Weinberger *et al*., 2009; Lysenko *et al*., 2010), after the initial 'loose' attachment to the nasopharynx, a thick polysaccharide capsule then paradoxically limits the interactions between the anchorage surface proteins and the epithelium. Nevertheless, this is a problem that the pneumococcus is able to subvert, as in addition to expressing different amounts of polysaccharide capsule between serotypes, pneumococci also display a natural intra-serotype variance in CPS expression (Adamou *et al*., 1998). Pneumococcal isolates may exist in opaque and transparent variants, (a phenomenon known as phase variance) with opaque colonies more commonly associated with invasive disease and transparent colonies (which possess up to 5 times lesser amounts of capsule) being implicated in earlier stages of infection such as carriage (Kim and Weiser, 1998). However, even amongst transparent strains there is a serotype specific inverse relationship between increased expression of the first gene of the capsule operon, *cspA*, and the incidence of carriage with that strain in the population, (Hathaway *et al*., 2007). The expression of a thinner capsule has been shown to significantly enhance attachment to human epithelial cells *in vitro* and the ability to regulate capsule expression is essential, as after only 1 hour in contact with the epithelium pneumococci reduce the thickness of their capsule (Hammerschmidt *et al*., 2005), a process which is oxygen sensitive (Weiser *et al*., 2001). In contrast to nasopharyngeal carriage, in order to achieve invasion of the epithelium (the second stage of pathogenesis) where the pneumococcus will come into contact with phagocytes

(and lower oxygen concentrations), a subsequent switch to the opaque phenotype is beneficial (Briles *et al*., 2005). Similarly, during invasive disease the possession of a thick capsule is an important factor, and the poor survival of non-encapsulated bacteria in the bloodstream is symptomatic of enhanced complement mediated opsonophagocytosis to pneumococci without capsule (McCullers and Bartmess, 2003; Brown *et al*., 1983; Hyams *et al*., 2010a). In order to cause carriage or invasive disease the expression of more factors are required and the polysaccharide capsule is not the only virulence factor that the pneumococcus is able to change dependent on its environment (King *et al*., 2006). According to its role as a commensal bacterium it has been shown that in addition to the expression of capsular genes up to 81% of genes studied were upregulated in pneumococci in contact with epithelial cells (Orihuela *et al*., 2004b). Transparent colonies have greater expression of NanA, (King *et al*., 2004), as well as enhanced expression of CbpA (Rosenow *et al*., 1997) and phosphorylcholine (ChoP) (Orihuela *et al*., 2004b).

Long term survival in the nasopharynx must be dependent on the acquisition of nutrients from the host, and to date over 40 surface expressed lipoproteins have been identified in the pneumococcus, many of which are involved in these processes (Bergmann and Hammerschmidt, 2006). Two lipoproteins which have an important role during pneumococcal bacteraemia are pneumococcal iron acquisition A (PiaA) and pneumococcal surface antigen A (PsaA), which is important for the uptake of zinc and manganese ions (Lawrence *et al*., 1998). Manganese is important for resistance against oxidative stress which may be caused by reactive oxygen species (ROS) as part of the innate host response (Tseng *et al*., 2002). However, PsaA was originally thought to be an adhesion factor due do its effect on attachment to mammalian cells *in vitro* (Berry and Paton, 1996). The inability for a PsaA mutant to cause respiratory tract infection may therefore be a consequence of manganese acquisition on the expression other pneumococcal adhesion factors (Marra *et al*., 2002; Kadioglu *et al*., 2008).

1.9 Competition in the nasopharynx

Existing nasopharyngeal carriage, either with pneumococci, other species of commensal bacteria or viruses, may affect a subsequent pneumococcal carriage. It is estimated that many hundreds of species of diverse microflora can inhabit the human naso/oro pharynx (Aas *et al*., 2005). When the pneumococcus encounters pre-existing bacteria

of the same strain in the nasopharynx both populations may survive and even increase in density (Margolis *et al*., 2010). However the fact that serotypes which are equally able to colonise the nasopharynx only emerge in replacement of vaccine serotypes predicts an ability of the vaccine strains in unvaccinated populations to out compete others (Kadioglu *et al*., 2008). Pneumococci produce bacteriocins (or pneumocins), small antimicrobial peptides which are serotype specific and disrupt the growth of other pneumococcal serotypes during colonisation (Dawid *et al*., 2007). This may account for the reason why in multi-serotype carriage, as observed in humans, one particular serotype may dominate the others in numbers (Gray *et al*., 1980). Interspecies colonisation may be inhibited by hydrogen peroxidase (H_2O_2) produced by pneumococci when they undergo aerobic growth, which is effective at killing *H. influenza* and *Neisseria menigitidis* (Pericone *et al*., 2000) although it is not certain that the physiological levels of H_2O_2 are sufficient to antagonise co-colonisation (Margolis *et al*., 2010). In order to survive co-colonisation with a species such as *H. influenzae*, the pneumococcus must evade the immunity which its fellow coloniser creates. Whilst the pneumococcus possesses mechanisms to elude the innate immunity it itself may stimulate (capsule, IgA1 protease, PspA), co-inoculation of both *S. pneumoniae* and *H. influenzae* intranasally leads to the recruitment of significantly more neutrophils than seen with each organism separately, probably due to lipopolysaccharide (LPS), which leads to the preferential opsonophagocytosis of pneumococci, and allows *H. influenza* to prevail (Lysenko *et al*., 2005). Under this type of selective pressure pneumococci which have large polysaccharide capsules are better able to co-exist in the nasopharyngeal niche (Lysenko *et al*., 2010).

There is a long established link between pneumococcal pneumonia and the influenza virus (Watson *et al*., 2006). Increased pneumococcal carriage and transmission as well as the conversion between carriage and disease states in healthy individuals may require modification of the host tissues via an inflammatory response, which is similar to the response initiated by viral pathogens, that 'prepare' the respiratory tract for invasion (Diavatopoulos *et al*., 2010; Tuomanen, 1997). Endothelial and epithelial cells under the influence of tumour necrosis factor (TNF) and interleukin 1 (IL-1) upregulate expression of the platelet activating factor receptor (rPAF), and by binding to this receptor via the cell wall component ChoP, pneumococci can be uptaken, and thus migrate through the epithelium (Cundell *et al*., 1995a). In addition, rPAF may be

important in the entry of pneumococci into the blood as it is expressed on vascular endothelium (Cundell *et al*., 1995a). Enhanced PAF receptor expression and influenza infection both result in increased mortality in a mouse model (McCullers and Rehg, 2002), and nasopharyngeal carriage is also increased in these circumstances (Diavatopoulos *et al*., 2010). In fact, a chinchilla model of colonisation showed that the differences between opaque and transparent colonies ability to inhabit the upper respiratory tract were only apparent following influenza (Tong *et al*., 2001). Another consequence of pre-existing carriage is that sialic acid, released by the actions of neuraminidases (which is also expressed in viruses) has been shown to enhance biofilm formation, and increase the density of existing pneumococcal carriage to the extent that it even has the capacity to change asymptomatic carriage into invasive disease when given intranasally to mice (Trappetti *et al*., 2009). It is thought the action of sialic acid stimulates neuraminidase expression which then in turn yields more sialic acid in an amplification loop that ultimately leads to an increased carriage density; a side effect of which is the shedding of pneumococcus from the upper respiratory tract, leading to lower respiratory tract infection (Trappetti *et al*., 2009).

1.10 Penetration of the host epithelium

Deeper invasion of host tissues and indeed increased adherence may be a consequence of bacterial host interactions, with the initial contact between pneumococci and epithelial barriers acting as stimulus for the upregulation of factors which enhance subsequent attachment. As previously mentioned, cytokines TNF, and IL-1 have been shown to enhance later adhesion to epithelium by pneumococci (Cundell *et al.*, 1995a), and both TNF-α and IL-1α can also be released by dendritic cells in response to stimulation by sub-lytic concentrations of PLY (McNeela *et al*., 2010). Similarly, respiratory epithelial cells activated by pneumococcal contact with toll-like receptor two (TLR-2), release transforming growth factor beta (TGF-β) which increases the permeability of the epithelium and thus both the access of immune cells to the bacterial site, but also the invasive capacity of the bacteria (Beisswenger *et al*., 2009). PLY also causes inhibition of ciliary beating on respiratory epithelial cells *in vitro*, a vital early clearance mechanism, and induces damage to the epithelium, including the separation of tight junctions between cells (Rayner *et al*., 1995). As the attachment of pneumococci to undamaged epithelium is lower by comparison, the exposure of normally concealed residues by PLY facilitates stronger attachment to the

nasopharyngeal epithelium (Feldman *et al*., 1992). In addition to exploiting gaps between epithelial cells, in a similar fashion to the interactions with rPAF, pneumococci can exploit the polymeric immune receptor (pIgR) to initiate transcytosis directly through the mucosal barrier. The (pIgR) is normally used to transport antibody from the serum across epithelial barriers, to the mucosal compartments (Mostov and Blobel, 1982), however the pIgR has been shown to bind pneumococcal choline binding protein A (CbpA) also known as pneumococcal surface protein C (PspC), in a way that greatly enhances pneumococcal adherence and invasion of epithelial cells *in vitro* (Zhang *et al*., 2000). Bacteria lacking PspC are significantly attenuated at causing carriage in mice (Balachandran *et al*., 2002).

Beyond the epithelium, pneumococci must come into contact with the basement membrane and the extra-cellular matrix (ECM). *S. pneumoniae* express a surface hyaluronate lyase, part of a hyaluronidase family of enzymes which are capable of digesting the hyaluronan component of the ECM in order to mediate the invasion of the basement membrane (Jedrzejas *et al*., 2002). Another pneumococcal virulence factor, enolase (Eno) binds to plasminogen, enzymatically converting it to plasmin which then in turn helps to degrade the ECM components laminin and fibrin (Bergmann *et al*., 2001). Pneumococcal adhesion and virulence factor A (PavA) is known to bind to the fibronectin component of the ECM (Holmes *et al*., 2001). Recently is has been shown that PavA mutants were unable to cause long term carriage in mice, and also failed to translocate from the lungs into the blood after intranasal infection which highlights the importance of the invasion of host tissues (rather than simple attachment to them) in maintaining long term carriage (Kadioglu *et al*., 2010). Unusually for a Gram positive organism, the pneumococcus is also known to possess a pilus which has been shown to bind to various components of the ECM (Hilleringmann *et al*, 2008).

1.11 Immune evasion

The ability of capsule specific IgA antibodies to enhance mucosal defence against pneumococci has been shown *in vitro* (Janoff *et al*., 1999) and *in vivo* (Sun *et al*., 2004), and it has been suggested to contribute significantly to the 'herd immunity' against invasive disease, initiated by infant immunisation with the conjugate vaccine (Whitney *et al*., 2003). The secretory component of IgA can be bound and neutralised by CbpA/PspC in a manner which prevents the activation of complement system

(Hammerschmidt *et al*., 1997). Additionally, IgA1 protease expressed in many streptococcal species can cleave IgA1 in the hinge region, which leads to the creation of Fab and Fc fragments of IgA which are less able activate clearance of bacteria and agglutination of streptococci (Tyler and Cole, 1998). In addition to these effects, Fc and Fab fragments generated through IgA cleavage enhance the interaction between bacterial ChoP and the host platelet activating factor receptor (rPAF), either by changing the surface charge (Fab fragments are cationic), or simply allowing closer interaction between the bacteria and epithelial surface, thus negating the inhibitory effects of the negatively charged polysaccharide capsule on attachment (Weiser *et al*., 2003). The negatively charged capsule can help to repel complement components and phagocytes (Swiatlo *et al*., 2002). However, surface protruding choline binding proteins such as PspA and PspC are positively charged, and hydrophobic, resulting in improved adherence to host cells regardless of the presence of capsule (Swiatlo *et al*., 2002). As the capsule is the determinant of overall net negative charge in pneumococci the ability to change capsule expression and surface proteins may explain the prevalence of 'transparent' bacteria in carriage, and 'opaque' colonies in septicaemia where attachment to host surfaces is less important.

1.12 Animal models of carriage

Intrinsic limitations in human experimental studies have allowed for a greater focus to be placed on animal models of pneumococcal disease, where almost all aspects of pathogenesis, virulence, preventative therapies and treatments have been explored to some extent. The vast majority of carriage models in animals are carried out in mice, although some studies have employed an infant rat model to described important age related correlates of immunity against carriage (Malley *et al*., 1998), and a chinchilla model has been used to study the role of neuraminidases (Tong *et al*., 2000). Mice have proven to be relevant organisms in which to study pneumococcal carriage as they produce colonisation which is similar in duration to human experimental carriage (McCool *et al*., 2002; McCool and Weiser, 2004). Murine carriage is also limited to the region of the nasopharynx, is restricted by same minimum starting dose as humans ~10^4 CFU/ml (McCool *et al*., 2002; McCool and Weiser, 2004), and yet mice, like humans remain susceptible to invasive disease with the same serotype as the colonising strain (Wu *et al*., 1997b; Richards *et al*., 2010). True models of pneumococcal carriage in naive mice are a relatively new phenomenon. Perhaps the earliest and best described

carriage model is from 1997, where Wu *et al* observed stable long term carriage with capsular types 3, 4, 6A, 6B, 14, 19, and 23 for a periods of between 7><28 days *(*Wu *et al*., 1997b). They were also the first group to describe that carriage of the same density could be achieved in diverse genetic mouse strains (A/J, BALB/cJ, and CBA/N), despite different responses being observed in invasive pneumococcal challenges (Gingles *et al.*, 2001). One important feature of these experiments however, was that carriage without progression towards invasive disease was only possible in virulent strains by using vastly different amounts of inoculating bacteria (10^4 CFU of serotype 3, compared to 10^8 CFU of serotype 23F) and thus clear phenotypic differences are evident between pneumococcal strains in this model (Wu *et al*., 1997b). Subsequent murine models of carriage have tended to focus on using comparatively higher doses of avirulent strains to establish carriage, such as those based on 23F (McCool and Weiser, 2004; van Rossum *et al*., 2005). It is important to make a distinction here between models of long term carriage and models of colonisation, which vastly outnumber those which represent true carriage, and where the quantification of bacteria in the nasopharynx may be measured after only 36 hours (Paterson and Mitchell, 2006). It is difficult to suggest at which point colonisation becomes long term carriage, and this is complicated by the invasive elements of carriage. Because the predominate method of sampling of pneumococcal colonisation is using a nasal wash or lavage, this may miss colonies which are better attached to the nasopharyngeal tissue (van Rossum *et al*., 2005; Wu *et al*., 1997b). Furthermore, it is likely that the relative populations of invasive and loosely attached pneumococci may vary over time, and thus carriage may be underestimated in some models.

1.13 Prevention of pneumococcal disease; past, present and future

One of the first demonstrations of humoral immunity against pneumococci came in 1913, where rabbit antiserum was successfully used to cure patients with type 1 pneumonia (Dochez and Gillespie, 1913), and the idea that capsular based immunity contributed to protection against pneumonia came in 1926 (Felton, and Bailey, 1926). An early 4-valent polysaccharide vaccine was tested in America during World War Two and proved effective in preventing pneumococcal pneumonia against those serotypes in the vaccine (Macleod *et al*., 1945), and thus, the first commercially available hexavalent polysaccharide vaccines were made available in 1946. Unfortunately the release of this vaccine coincided with emergence of new antibiotic

and antimicrobial treatments and the efficacy of the vaccine was largely overlooked and eventually removed from the market. When it became clear that the treatments for pneumococcal disease could not prevent almost one in four deaths attributed to the pneumococcus the idea of polysaccharide vaccines was revisited. In 1977, a 14-valent vaccine produced by Merck, called PNEUMOVAX was marketed, which contained purified polysaccharide from 14 of the most relevant serotypes; 1, 2, 3, 4, 6A, 7F, 8, 9N, 12F, 14, 18C, 19F, 23F and 25F. Six years later PNUEMOVAX-23 (which is still used today), added a further 11 serotypes; 5, 6B, 9V, 10A, 11A, 15B, 17F, 19A, 20, 22F, and 33, whilst 25F and 6A were dropped. Despite its coverage of almost 90% of serotypes commonly found in invasive disease the use of PNEUMOVAX-23 is limited outside of North America (Fedson, 1998). There have been many studies to assess the efficacy of the polysaccharide vaccine but these results have been weakened by their performance in those most at risk of pneumococcal disease (Fedson, 1998). Protection overall in adults has been shown to be approximately 57% but significantly less in immune-compromised individuals, and almost half as effective at 85 years of age compared to 55 (Butler *et al*., 1993; Shapiro *et al*., 1991). The efficacy of the polysaccharide in those vaccinated under 17 months of age was negligible against both respiratory disease (Riley *et al*., 1981), and otitis media (Karma *et al*., 1985). As pneumococcal disease is common in children under the age of two it was important to develop a vaccine which was effective in this population. In 2000, the first 7-valent pneumococcal conjugate vaccine (PCV), PREVNAR was licensed, which coupled polysaccharides from serotypes; 4, 6B, 9V, 14, 18C, 19F and 23F to a non-toxic protein carrier from a diphtheria toxin molecule (CRM_{197}). In early clinical trials this vaccine, produced by Wyeth, was 100% efficacious in preventing IPD in children <2 (Black *et al*., 2000), and in contrast to PNEUMOVAX also retained significant effectiveness in patients with HIV (Klugman *et al*., 2003). Protein conjugate vaccines also differ in their ability to prevent mucosal infections such as acute otitis media, where PCV was 56% efficacious (Eskola *et al*., 2001). PCV also reduces incidence of pneumococcal carriage of those serotypes contained on the vaccine, although overall carriage rates are unaffected as non-vaccine serotypes replace them in the population (Ghaffar *et al*., 2004). The fact that PCV can prevent carriage is significant, as nasopharyngeal colonisation is the major niche in which pneumococci reside and are transmitted; lowering the incidence of carriage thereby reduces a particular serotypes chance of being passed on. This so-called herd immunity has been identified as levels of carriage

in non-vaccinated individuals in a population of those who received PCV is lower than expected (Black *et al*., 2001).

As early as 1954, observations of polysaccharide immunisation led to the statement that; "Specific active immunisation of children against pneumococci is impractical because of the large number of types and the poor antigenic response of children- the group most in need of protection" (Wright and Washington, 1954). Although the conjugate vaccine has improved efficacy in children the number of serotypes present is small, and the conjugation process makes these vaccines expensive for those in the developing world. Furthermore, the emergence of disease caused by serotypes not present in the vaccine is a constant threat, and may require almost constant changes in the vaccines formulation (Centers for Disease Control and Prevention (CDC), 2005). Because of these factors a new approach has started to look for possible protein antigens, which are highly conserved, and common to all serotypes of pneumococci, which could provide adequate protection. Two of the front runners to become new vaccine candidates are covered in detail below.

1.14 Vaccine candidacy: PLY

Antibodies against PLY can be detected in pneumonia patients, and anti-Ply IgG from these patients was able to passively protect mice from pneumococcal challenges (Musher *et al*., 2001). PLY was one of the first highly purified proteins tested for its protective response against the pneumococcus, and it was able to significantly lengthen survival times in mice subsequently intranasally infected with serotype-2 pneumococci (Paton *et al*., 1983). However, native PLY would not be suitable to use in a vaccine due to its high level of haemolytic activity, which produces features that resemble the effects of live pneumococci in mice (Feldman *et al*., 1991). Small amino acid changes can significantly reduced these effects *in vivo*, and one such mutation; Trp_{433}-Phe, known as PdB has just 1% of the haemolytic activity of PLY and is protective against pneumonia challenges both intranasally (Alexander *et al*., 1994) and intraperitoneally (Ogunniyi *et al*., 2001). As PLY is thought to be released only upon autolysis (Mitchell *et al*., 1997) it was considered that the effects of anti-PLY antibody may be more effective in neutralising the invasive capabilities of the toxin, rather than directly initiating phagocytosis of antibody bound pneumococci. Recently however, it has been shown that PLY can also become localised with the cell wall, by an undefined

mechanism, which may renew interest for PLY derivatives as use in a vaccine (Price and Camilli, 2009). Whilst serum antibodies against PLY can be detected, the levels of mucosal IgA to PLY are significantly less than other protein antigens (Zhang *et al*., 2002). However, an additional property of PLY as a mucosal adjuvant has been identified, a PLY fusion protein boosted IgG and IgA antibody titres to another pneumococcal protein PsaA, an effect which was not dependent on the cytotoxic activity the toxin (Douce *et al*., 2010). It is believed that a Th1 response characterised by immunoglobulin subclasses IgG2a, IgG2b, and IgG3 is the preferred pathway for the clearance of encapsulated bacteria like the pneumococcus (Lefeber *et al*., 2003). The toxin PLY has been shown to enhance the secretion of IL-12, IFN-γ and IL-8 through interaction with TLR's, which cause the differentiation of T-cells, down the Th1 pathway (McNeela *et al*., 2010). PLY was also observed to stimulate immunity through IL-17A and other associated cytokines, (McNeela *et al.*, 2010), which has been shown to be indispensible for protection against both subsequent invasive challenges and pneumococcal carriage in mice (Malley *et al*., 2006). In line with these studies, PLY has been trialled as carrier for pneumococcal polysaccharide, as an alternative to the existing conjugate vaccine; this vaccine has the advantage of developing to antibody to both the protein and polysaccharide simultaneously with added adjuvant effect and is effective at inducing opsonophagocytosis of pneumococci *in vitro* (Michon *et al*., 1998). However, PLY fusion proteins with another pneumococcal antigen PsaA, have been shown to have limited efficacy in protecting mice from invasive challenges (Douce *et al*., 2010). Although Douce *et al* state that the mucosal adjuvant effect is preserved in their model, they make use of a non-haemolytic version of PLY, which has previously described to be less stimulatory than the haemolytic form (McNeela *et al*., 2010). Clearly the balance between toxicity and the adjuvant effect must be resolved before its inclusion in any future mixed protein vaccine.

1.15 Vaccine candidacy: PspA

PspA is a surface expressed choline binding protein which can bind lactoferrin (Shaper *et al*., 2004), interfere with complement deposition (Ren *et al*., 2004) and is known to be expressed *in vivo* in almost all clinical isolates of pneumococci (Swiatlo *et al.*, 2003). It has been suggested as a possible vaccine candidate because antibody against PspA can be found in both human and animal carriage models (McCool *et al.*, 2002; Richards *et al.*, 2010), and protective antibody responses to it can be initiated both

systemically or mucosally (Briles *et al*., 1996; Yamamoto *et al*., 1997; Briles *et al*., 2000a). Mucosal immunisation of purified PspA plus cholera toxin has been shown to prevent subsequent carriage and invasive disease and septicaemia in mice (Wu *et al*., 1997a). Crucial to the immunogenicity of PspA is its location on the surface. The surface region of PspA has a net negative charge, allowing it to protrude at a maximum distance to the negatively charged capsule, and unlike other surface proteins the polysaccharide capsule is unable to inhibit binding of anti-PspA antibody to epitopes on the molecule (Daniels *et al*., 2006). The N-terminal amino acid sequence of pneumococcal PspA is highly variable and pneumococci can be subdivided into three families; family one consisting of clades 1 and 2, family two consisting of clades 3, 4, and 5, and the rarer family 3 containing just clade 6 (Hollingshead *et al*., 2000). Although protection against invasive pneumococcal challenges in mice may be enhanced within the same clade (Miyaji *et al*., 2002), immunisation with recombinant PspA fragments can produce antibodies which are broadly cross reactive (Nabors *et al*., 2000), and thus full protection from all serotypes may be achieved with two or three fragments of PspA.

1.16 Vaccine candidacy: Whole cell vaccine

Immunisation with whole cell vaccines (WCV) predates vaccination with individually purified pneumococcal components with the first clinical trials dating back to 1911 (Austrian, 1978). It has been stated that the effects of vaccination with a combination of pneumococcal antigens is stronger than each antigen individually (Briles *et al*., 2000a), and in parallel to combinations of proteins this has led others back to whole cell approach. A WCV made from ethanol killed non-encapsulated pneumococci and cholera toxin as adjuvant, was genetically modified to be autolysin negative ($LytA^-$) in order to grow to a higher density, and further developed to express a less haemolytic form of PLY in order to increase the safety of the vaccine (Malley *et al*., 2001; Lu *et al*., 2010). Intranasal immunization of mice with this formula was shown to provide multi-serotype protection against subsequent carriage, bacteraemia and septicaemia (Malley *et al.*, 2001; Malley *et al*., 2004). It is thought that the importance of CD4+ T-cells in immunity to the WCV, in addition to antibodies, could enhance the protective response to this vaccine (Malley *et al*., 2005b). The other major advantage the WCV has over complicated protein conjugates is that it would simply be far cheaper, easier to store and administer to patients in the field (Lu *et al*., 2010).

1.17 Innate immunity to pneumococcal carriage

Studies of the host immune responses stimulated by pneumococcal carriage only are limited, which may be suggestive of reduced immunity to this asymptomatic event. A strong mucosal response to carriage in humans is thought to determine whether pneumococci are cleared quickly or a lengthy carriage may occur, as strains which produce poor mucosal responses in children such as serotype 6 and 23F tend to have the longest duration of carriage (Gray *et al*., 1980). In the first 3 days of colonisation in mice there is an inflammatory response, often referred to as a mild suppurative rhinitis, which is characterised by an infiltration of neutrophils, into the luminal spaces where bacteria reside, but this influx of cells is ineffectual at clearing carriage *(*van Rossum *et al*., 2005). The accumulation of neutrophils is associated with an increase in the neutrophil chemokine macrophage inflammatory protein-1 and 2 (MIP-1/2), which is also associated with PLY mediated pore formation and subsequent activation of p38 mitogen-activated protein kinase signalling pathway (Ratner *et al*., 2006). The involvement of PLY in this process is supported by *in vitro* data which suggest that neutrophils can migrate towards PLY (Moreland and Bailey, 2006). Even before the activity of neutrophils, inflammation of respiratory epithelial cells is controlled by the recognition of bacterial patterns through toll-like receptors (TLR's), which lead to the activation of TGF-β signalling (Beisswenger *et al*., 2009). Signals through TGF-β can lead inflammatory mediators such as neutrophils and complement to the luminal surface, which aid in bacterial clearance. Colonisation is more severe in the absence of both TLR-2 (van Rossum *et al*., 2005), TLR-4 and the universal TLR signalling molecule MyD88, with TLR-4 also being recognised as important in the PLY dependent stimulation of macrophages (Malley *et al*., 2003). The precise role of PLY in TLR signalling has been contentious however, and recently many of its immuno-stimulatory properties, such as the release of IL-17 and IFN-γ were shown to be independent of TLR-4 (McNeela *et al*., 2010). As the incidence of pneumococcal carriage declines rapidly after two years (Hussain *et al*., 2005), the impact of innate immunity has been studied in an infant rat model, which found that both the density and duration of pneumococcal carriage is increased in younger animals (Bogaert *et al*., 2009). Macrophages from neonatal mice produced less chemokines and cytokines under stimulation from either whole killed pneumococci or TLR-4 agonists.

Macrophages are important mediators of neutrophil function (Cailhier *et al*., 2006), and MIP-2, keratinocyte chemo-attractant (KC) and granulocyte colony stimulating factor (G-CSF) which are involved in neutrophil recruitment and activation were less present in the infant model. The immune response to pneumococcal infection as measured by the changes in gene expression is generally lower in the nasopharynx than either the lungs or blood (Mahdi *et al.*, 2008). In nasal infection specifically, colonisation was associated however with higher levels of IL-6, tumour necrosis factor-alpha (TNF-α), CCL2 (monocyte chemo-attractant protein-1, MCP-1), CD54 (intracellular adhesion molecule-1, ICAM-1) CXCL2 (macrophage inflammatory protein-2, MIP-2), and CCL5 mRNA, all of which have been previously linked to the pathogenesis of pneumococcal infection, but it is interesting to note that the 3 different pneumococcal strains with 3 differing invasive capacities elicited diverse responses (Mahdi *et al*., 2008). In fact, both IL-6; an activator of monocytes and neutrophils (Borish *et al*., 1989) and TNF-α, a potent stimulator of inflammation which can alter the outcome of pneumococcal infection when co-administered with virulent pneumococci (Dallaire *et al*., 2001), were not stimulated in the nasopharynx with virulent D39, but were hugely up-regulated in a non-lethal mutant (Mahdi *et al.*, 2008). These results concur with another study which suggests the evasion of immune surveillance by a virulent serotype-3 strain (by resisting complement activated opsonophagocytosis) (Jarva *et al.*, 2002) is the effect of an absence of TNF-α, IL-12 and IL-10 in the nasopharynx in response to serotype-3, which leads subsequently to invasive disease rather than asymptomatic carriage (Ling *et al*., 2003). IL-12 stimulates the production of Th-1 biased cytokines, which lead to the recruitment of neutrophils and in combination with pneumococci can function as a mucosal adjuvant which enhances the humoral immune response (Arulanandam *et al.*, 1999; Sabirov and Metzger, 2008).

Like TLRs, pattern recognition receptors (PRR's) recognise conserved motifs or pathogen-associated molecular patterns (PAMP's), which may be shared between diverse microbial species (Janeway and Medzhitov, 2002). The acute phase protein, C-reactive protein (CRP), is a soluble PRR which can interact with pneumococcal surface ChoP, increasing complement activation and also block uptake of pneumococci through rPAF (Gould and Weiser, 2002).. With the addition of anti-PC antibodies, CRP allows mice to survive an otherwise lethal challenge with serotype-3 pneumococci (Mold *et al*., 2002).

Through their various activities, complement proteins are considered integral to the immunity against the pneumococcus, linking together the innate and adaptive immune systems and contributing both to rapid killing of pneumococci and lasting memory responses. The first demonstration of complement in phagocytosis is more than 100 years old (Wright *et al*., 1989). Since then, three distinct arms of complement system have been described: the classical pathway, the alternative pathway and the lectin pathway. Although the initiation of these 3 pathways is diverse (summarised in figure 1.2) they converge in the formation of C3, which is cleaved by the various C3 convertases into C3b, the so called opsonic fragment which is deposited on the bacterial surface. The other products of the C3 convertase, C3a/C5a are anaphylatoxins which both attract and stimulate pro-inflammatory cytokine release from antigen presenting cells with cognate C3a and C5a receptors (Soruri *et al*., 2003; Ehrengruber *et al*., 1994). They also provide co-stimulation and signals which stabilise naive T-cells (Strainic *et al.*, 2008). C3b is further cleaved by the action of factor H and factor I (Cunnion *et al.*, 2004), to yield iC3b, C3c and C3d which respectively act as different targets for components of the innate and adaptive immune system. Complement receptor-1 (CR1), CR3 (CD11b) and CR4 (CD11c) present on neutrophils, monocytes and macrophages recognise C3b and its cleavage products and promote attachment and phagocytosis respectively, with CR3 being the most potent against pneumococci (Gordon *et al*., 1986). CR2 (CD21), the receptor for C3d is found predominantly on B-cells, but also many other cell types leading to B-cell activation and differentiation (Bohnsack and Cooper, 1988), and lowers the threshold of B-cell activation in cooperation with the B-cell receptor (CD19) (Lyubchenko *et al*., 2005). Much of what is known about the complement system has been identified though either natural or induced mutations in individual complement components. Mice depleted of C3 fail to clear pneumococcal infection (Nakajima *et al*., 1990), and natural human deficiency in C3 makes individuals vulnerable to infection with *S. pneumoniae* (Alper *et al.*, 1972). Immuno-deficiencies which specifically target the classical pathway (Alper *et al*., 2003) and the lectin pathway (Roy *et al*., 2002) also predispose humans to pneumococcal disease, although the classical pathway appears to be the dominant pathway for innate immunity to the pneumococcus in mice, as shown by the particular susceptibility of $C1q^{-/-}$ mutants (Brown *et al.*, 2002). The mutation of complement receptors CR2 (CD21) and CR1 (CD35), which in mice are capable of binding all complement fragments did not alter the progression of an invasive pneumonia in naive individuals compared to

controls, but the memory response was significantly decreased in these mutants (Haas *et al*., 2002), highlighting the need for a fully active complement system to provide longer lasting protection. The extent of complement binding to pneumococci has been shown vary between different capsular serotypes (Hostetter, 1986), but the polysaccharide alone cannot account for these differences (Hyams *et al.*, 2010b), as more C3 is deposited onto the surface of non-invasive strains of pneumococci compared to invasive strains of the same serotype (Sabharwal *et al*., 2009).

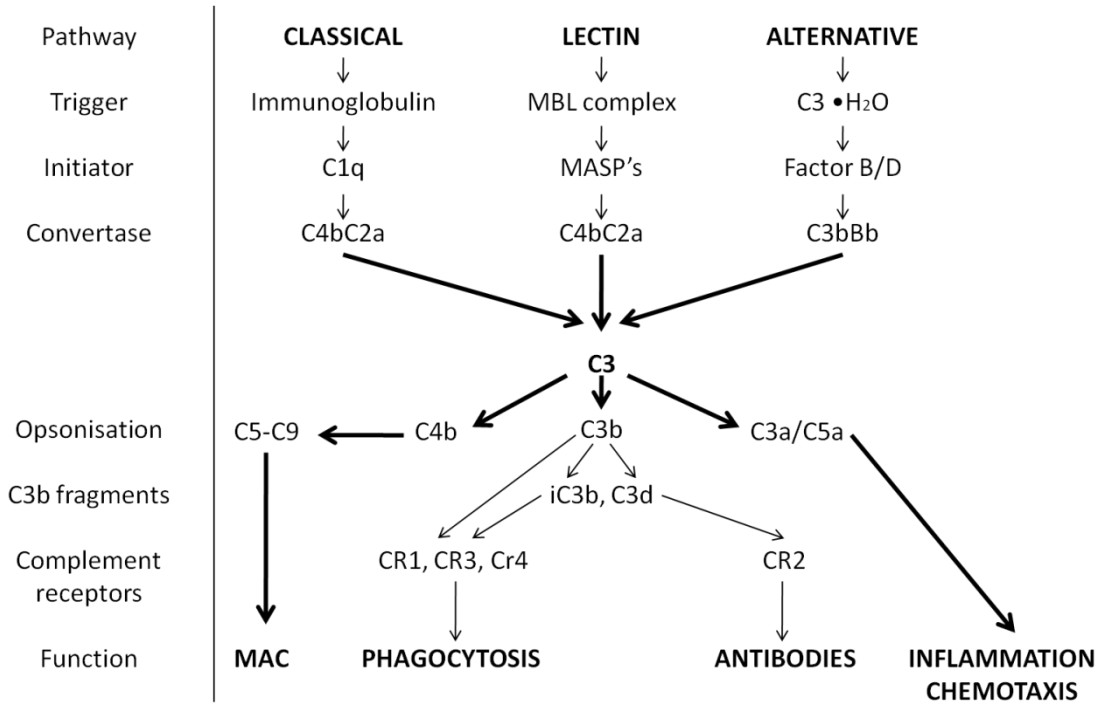

Figure 1.2 The complement pathway. Three diverse pathways converge on C3, and its cleavage products lead to the formation of the membrane attack complex (MAC), the anaphylatoxins C3a/C5a, and the opsonic fragment C3b. Adapted from Roozendaal and Carroll, 2006.

1.18 Adaptive immunity to pneumococcal infection

One of the hypotheses behind the age related decline in the incidence of pneumococcal carriage is the effect of natural exposure in the form of asymptomatic nasopharyngeal colonisation in generating a specific adaptive memory immune response which is then protective against subsequent carriage events. Increases in anti-capsular and anti-protein IgG, indicative of an adaptive immune response, can be generated through

natural carriage in humans, including young children (albeit at reduced concentrations) (Goldblatt *et al*., 2005bl; Rapola *et al*., 2000). Despite much evidence as to the relative incapacity of polysaccharide antigens in infancy, naturally acquired antibodies to certain serotypes (6A, 14, 23F) are predictive of protective responses against future carriage in children <5 years (Weinberger *et al.*, 2008), a result which agrees with the positive impact of the polysaccharide conjugate vaccine in reducing carriage rates in both immunised and non-immunised individuals (Huang *et al*., 2009). However in an experimental model of human nasopharyngeal carriage it was pre-existing antibodies to the pneumococcal protein PspA, and not capsule components that were the indicators of protection (McCool *et al*., 2002). In 2009, the first demonstration of serotype independent protection against colonisation was demonstrated in Bangladeshi children (Granat *et al*., 2009). These children were all below 1 years of age, and therefore may have been below the threshold of the generation of a robust polysaccharide response as seen in children above the age of 2. As epidemiological studies are inherently limited by the constraints of human observation, *i.e.* carriage events may be missed between visits, the spread of ages of subjects over a crucial period of immune development (1-5 years), analysis has been explored concurrently in mice. Murine pneumococcal carriage is also associated with the development of antibodies against polysaccharides (Richards *et al*., 2010) and surface proteins (McCool and Weiser, 2004). However, whilst antibody concentrations tend to correlate with the density of carriage (Trzcinski *et al*., 2005), they are not required for the clearance of pneumococci from the nasopharynx, as IgM transmembrane exon deficient (μMT) mice, which cannot produce mature B-cells or antibody, were colonised indistinguishably from WT mice (McCool and Weiser, 2004). There is also conflicting evidence to the impact of antibody on protection upon a second exposure to pneumococci, with some groups indicating that antibody is not required for protection against carriage (Trzcinski *et al*., 2005) whilst another demonstrating that they are indispensible; both in preventing subsequent carriage and invasive disease following attenuated colonisation (Roche *et al.*, 2007). In contrast to antibody deficient mice, MHCII$^{-/-}$ knockouts, which have functionally deficient and reduced numbers of CD4+ T-cells are impaired in the clearance of carriage (van Rossum *et al*., 2005) and have impaired protective responses against subsequent carriage and disease (Roche *et al*., 2007). CD4+, but not CD8+ T-cells play a crucial role in protection against carriage, as only mice deficient in MHCII or treated with an anti-CD4+ antibody were not protected after vaccination with WCV, when

compared to controls (Malley *et al.*, 2005b). MHCII$^{-/-}$ knockouts are also more susceptible to invasive pneumococcal infection in the lung, which is supported by an observed *in vitro* PLY dependent chemotaxis of T-cells (Kadioglu *et al*., 2004). CD4+ T-cells have been shown to be important in pneumococcal disease through the increased susceptibility of HIV patients to both invasive disease (Dworkin *et al*., 2001) and nasopharyngeal colonisation (Gill *et al.*, 2008). They provide the necessary costimulation to B-cells, which in turn induces class switching to IgG, somatic hypermutation, affinity maturation and the generation of long lasting memory responses (McHeyzer-Williams, 2005). A T-helper-1 response characterised by the production of IFN-γ and IL-2, has been shown to be protective in pneumococcal disease (Kemp *et al*., 2002), and pneumococcal carriage produced a profile of Th-1 cells as assessed by the production of IgG2b and IgG3 subclasses of immunoglobulin (van Rossum *et al*., 2005). Mice deficient in IL-18, a stimulator of Th-1 biased immunity through IFN-γ, were defective in clearing pneumococcal colonisation (Paterson *et al*., 2005). However the compensatory role of many cytokines is illustrated by the fact that mice which were lacking IL-12, a potent stimulator of Th-1 immunity, did not clear colonisation differently to Th-2 deficient mice (van Rossum *et al.*, 2005). Further evidence for the participation of the Th-1 response is the role of TLR-4 in protection against carriage (Malley *et al*., 2003) which has been shown to preferentially induce Th-1 like activities (Agrawal *et al*., 2003). Independently of T-helper profile a third mechanism of protection against pneumococcal carriage has emerged. It was identified that in response to intranasal immunisation with pneumococcal cell wall polysaccharides and cholera toxin the release of the cytokine IL-17A by CD4+ T-cells was increased, and the removal of IL-17A at the time of inoculation reduced the protective response of the immunisation (Malley *et al*., 2006). In addition, IL-17A production has been observed both *in vitro* and *in vivo* to be stimulated by the pneumococcal virulence factor PLY (McNeela *et al*., 2010). During naive nasopharyngeal carriage, the clearance of pneumococci from the nasopharynx is characterised by an influx of monocytes/macrophages to the luminal areas of the upper respiratory tract, whilst a second encounter of carriage results in infiltration of neutrophils to the nasal spaces (Zhang *et al*., 2009). Depletion of IL-17A in both instances was shown to abolish the infiltration of these cell types and thus reduce the clearance observed (Zhang *et al*., 2009). IL-17A is known to be released by a distinct subset of T-cells, known as Th-17 cells which have been shown to participate in the host defence against many

extracellular bacteria including *Klebsiella pneumoniae* (Happel *et al*., 2005) and *Bordetella pertussis* (Higgins *et al*., 2006) in addition to *S. pneumoniae*. Th-17 cells bridge the gap between innate and adaptive immune responses, stimulating the release of antimicrobial products and recruiting immune effector cells to the site of bacterial invasion, with the help of the mucosal surface, as described in figure 1.3. Neutrophils (which predominate later in naive carriage and are enhanced in secondary encounters with the pneumococcus) are not efficient in the early clearance pneumococcal carriage (Nelson *et al*., 2007), as this occurs before the appearance of specific antibody. It is therefore hypothesised that with the addition of opsonising antibody, neutrophils are better able to clear carriage (Zhang *et al*., 2009).

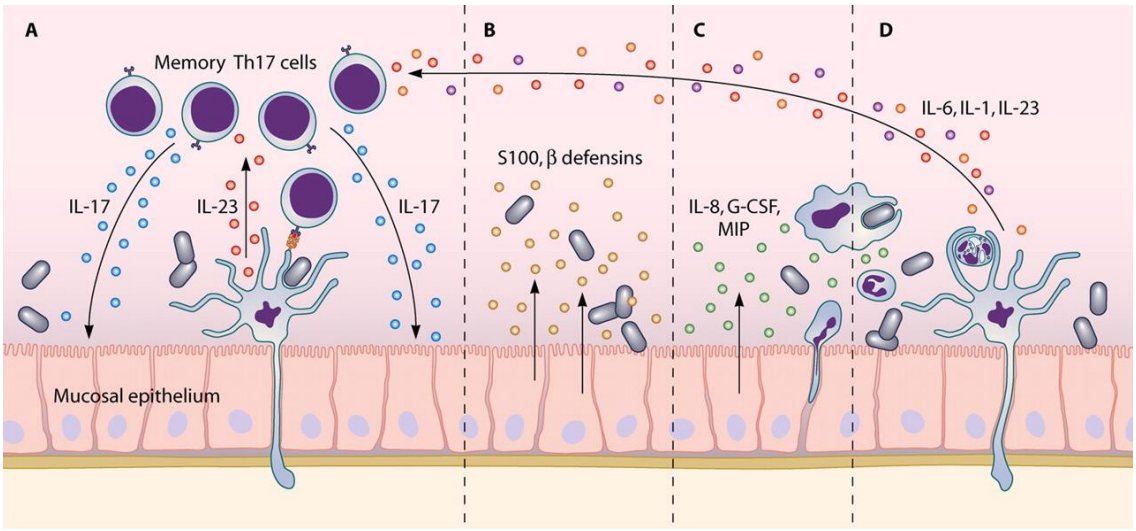

Figure 1.3 The mucosal Th-17 defence axis. (A) Peripheral dendritic cells release IL-17 which in turn stimulates the release of IL-17 from resident Th-17 cells. (B) IL-17A and IL-22 released from Th-17 cells stimulate the release of antimicrobial peptides such as β-defensin 2, lipocalin -2 and S100 family members from epithelial cells. (C) chemoattractants such as IL-8, MIP, and CXCL-1 released by epithelial cells recruit monocytes/macrophages, neutrophils and T-cells. (D) additional IL-6, IL-23 and IL-1 released by dendritic cells which have phagocytosed apoptotic neutrophils, then drives undifferentiated CD4+ cells towards the Th-17 phenotype, thus amplifying the response. Taken from Peck and Mellins, 2010.

1.19 Aims and objectives

My work had three major objectives; i) to establish a novel model of long term pneumococcal nasopharyngeal carriage which could be used to study the contribution of individual virulence factors to the persistence of bacteria in the nasopharynx, ii) analyse the host immune response to carriage in naive mice, and define specific bacterial targets of host immunity, iii) describe the protective response of primary carriage to subsequent encounters with the pneumococcus, both carriage and invasive, whilst exploring host cellular and humoral responses.

Chapter 2: Materials and methods

2.1 Bacteria

Pneumococcal isolates were routinely distinguished from other streptococcal species by positive Gram stain, characteristic beta-haemolysis on blood agar plates, optochin (Ethylhydrocupreine hydrochloride) sensitivity and by a negative catalase test result.

Serotype specificity was determined by Quellung reaction. Briefly; a sterile loop of pneumococci were mixed with 5 µl of type specific capsular anti-serum (Statens Serum Institute), with equal volumes of phosphate buffered saline (Oxoid) and methylene blue (BDH Laboratory Supplies). 5 µl of this mixture was added to a microscope slide (VWR International) and immediately covered with a coverslip (Scientific Laboratory Supplies LTD). Slides were examined by bright field microscopy under 1000x oil immersion and a positive reaction was identified by the appearance of the capsular material around the bacteria which are stained blue.

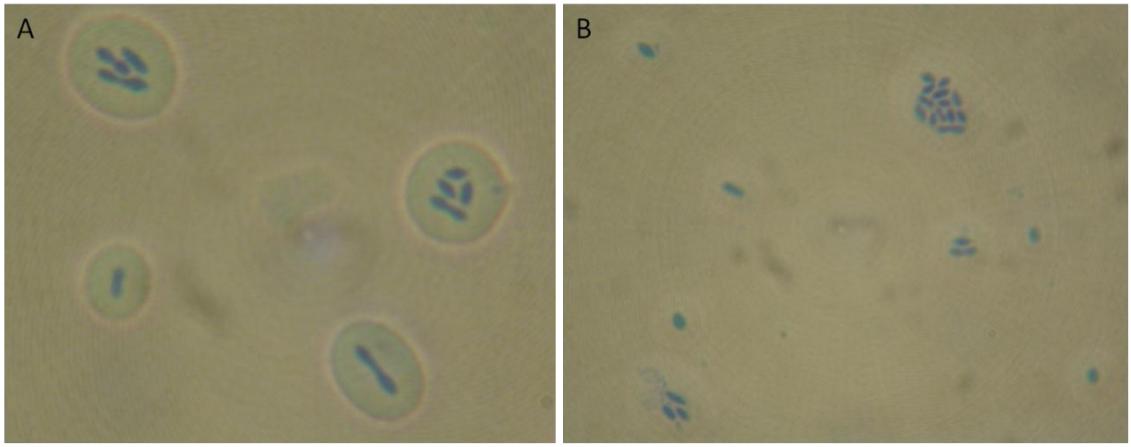

Figure 2.1 Quellung reaction. (A) serotype-2 D39, and (B) non-encapsulated strain 110.58, both incubated with type-2 antiserum. Light microscopy (x1000).

All pneumococcal isolates were stored long term in at -80 °C in brain-heart infusion (BHI) (Oxoid) supplemented with 15% glycerol (Sigma Aldrich). Additional frozen stocks were made from bead stocks in BHI + 20% Foetal Bovine Serum (FBS) (Gibco) as required, which could be stored >6 months without significant decline of viable bacteria.

Strain	Serotype	Strain background	Strain designation	Source	Antibiotic resistance
D39 (WT)	2	WT	7466	NCTC	-
A66 (WT)	3	WT	7978	NCTC	-
23F (WT)	23F	WT	11910	NCTC	-
$PlnA^-$ (pneumolysin mutant)	2	D39		Professor Peter W Andrew (University of Leicester)	Erythromycin 1mg/ml
Δ4 (Domain 4 pneumolysin negative mutant)	2	D39		Dr Madga Bortoni (University of Leicester)	Spectinomycin 0.1 mg/ml
$NanA^-$ (Neuraminidase A mutant)	2	D39		Dr James C Paton (University of Adelaide)	Erythromycin 1mg/ml
$NanB^-$ (Neuraminidase B mutant)	2	D39		Dr James C Paton (University of Adelaide)	-
$NanA^-/B^-$ (Neuraminidase A+B mutant)	2	D39		Dr Marco Oggioni (University of Sienna)	Erythromycin 1mg/ml
PspA- (Pneumococcal surface protein A mutant)	2	D39		Dr Marco Oggioni (University of Sienna)	
R6 (Capsule negative mutant)	-	D39	53568	ATCC	-
110.58 (Capsule negative mutant)	Non typeable	-		Dr Kathrin Mühlemann (University of Bern)	Penicillin 0.1 µl/ml

Table 2.1 Pneumococcal strains used in this thesis.

Strain 110.58 was chosen as in contrast to the other laboratory strains it represented a naturally occurring non-encapsulated strain which was recovered from the nasopharynx during a Swiss surveillance programme 1998-1999 (Muhlemann *et al*., 2003). In contrast to other non-encapsulated strains such as the laboratory generated R6, 110.58 has in place of capsular genes a sequence which is homologous to the *aliB* gene, which may have been acquired by horizontal transfer between *S. pneumoniae* and S*treptococcus mitis* (Hathaway *et al*., 2004).

2.2 Pneumococcal growth conditions

S. pneumoniae was grown either on agar plates or in liquid culture. Plated pneumococci were grown on blood agar base (Oxoid) supplemented with 5% v/v horse blood (Oxoid), with the addition of selective antibiotics as required for the different mutants (Table 2.1). Bacteria were streaked out from existing plates or bead stocks (a sweep of colonies or two beads respectively) under sterile conditions, and the fresh plates were inverted and incubated at 37 °C overnight in a sealed CO_2 jar.

Where it is described in this thesis, liquid culture refers to a sterile loop of pneumococci transferred from a plate (sweep of colonies) to 10 ml of BHI (Oxoid). Overnight cultures were grown for 16-18 hours, statically at 37 °C, in a tightly closed universal tube (Sterilin).

2.3 Viable counting

Using a round bottomed 96 well microtitre plate, serial dilutions (10^{-1}-10^{-6}) of pneumococci from liquid culture or homogenised mouse tissues were performed by adding 20 µl of the bacterial suspension to 180 µl of PBS, in duplicate. 3 x 20 µl of each dilution were dispensed onto blood agar plates in a marked section of blood agar plate (typically divided into 6 segments), and incubated as previously described. The next day, viable colonies were counted in sections where between 30 and 100 colonies could be observed, and bacterial numbers were expressed as colony forming units per ml, or per mg of tissue.

CFU/ml = (Total number of colonies counted in sector) ÷ (3) x **(**dilution factor) x (50)

CFU/mg of tissue = **(**CFU/ml) ÷ (weight in mg) x (volume of tissue homogenate)

2.4 Animal passage

In order to standardise their virulence, all strains (including mutant pneumococci) used for animal infections and *in vitro* studies unless otherwise stated, were subjected to animal passage as previously described (Canvin *et al*., 1995). Briefly, pneumococci were recovered from a 10 ml overnight culture by centrifugation at 3000 rpm (1734g) for 15 minutes in an Allegra x-22 centrifuge (Beckman Coulter), and resuspended in 5 ml of phosphate buffered saline (Oxoid). 100 µl of this culture was then administered

without delay into the peritoneal cavity of mice, using a 1 ml insulin syringe (Beckton Dickinson). Virulent pneumococci were recovered from the blood after 24 hours by cardiac puncture using a BD Microlance 21 G x 5/8" needle (Beckton Dickinson) and a 2 ml syringe (Terumo). 50 µl of blood per mouse were placed into 10 ml of BHI and the passaged bacteria were regrown overnight. The next day, approximately 9 ml of culture was aspirated and transferred to a fresh universal tube, taking care not to disturb the blood which had settled overnight. After centrifugation, the pellet was resuspended in 1 ml of BHI + 20% FBS (serum broth) which acts as a cryo-protectant. Approximately 700 µl of this culture was transferred to a 10 ml volume of serum broth, which was adjusted as required to a starting optical density (OD) of 0.7 (A_{500}). This culture is grown for approximately 5 hours, until OD 1.5 (mid-log phase) at which time the pneumococci are separated into 500 µl aliquots, and transferred to -80 °C.

2.5 Virulence testing

WT strains that were commonly used in this thesis were subjected to virulence testing before use in animal infection models (mutants had attenuated virulence as described elsewhere and were therefore not tested unless stated). Briefly, 50 µl of PBS containing 1 x 10^6 CFU of pneumococci was administered intranasally into 5 MF1 mice, which were monitored closely for the development of disease. Based on previous data from this laboratory it was established that a stock of serotype-2, WT-D39 (the most commonly used strain described in this work) of acceptable virulence should cause lethality in between 36-52 hours in at least 80% of mice. The development of signs of disease, and mortality was slower in serotype 3, A66 which was normally lethal in 72-120 hours. Stocks which fell outside of these parameters were rejected and re-passaged until the desired level of virulence was achieved.

2.6 Mice

For the animal work described in this thesis, outbred MF1 mice were selected to synergise with existing data from the laboratories of Professor P. W. Andrew and Dr A. Kadioglu. Due to observed differences in response to infectious disease (unpublished data), the age, sex and the source of the mice were kept constant between experiments. Female MF1 mice were purchased from Harlan Olac (UK) at 7 weeks of age and according to Home Office regulations were left for 1 week to acclimatise at the 'in house' animal facility, before use. Mice were used inside the range of 8–12 weeks of

age, at the start of each experiment. All procedures were approved by the UK Home Office and local ethics committee.

2.7 Preparation of infectious dose

Aliquots of frozen passaged pneumococci of known CFU/ml were thawed quickly in the palm of the hand and centrifuged at 12,000 rpm (10625 g) in a bench-top micro-centrifuge (Sigma). The supernatant was aspirated and the bacteria were resuspended in 400 µl of PBS. As required, a predetermined volume of this stock based on CFU was added to make 1 ml carriage dose in PBS, which contained 5 x 10^5 CFU per 10 µl. All mice in the same experiment were infected using the same aliquot to ensure consistency. Alternatively the dose was made in the same way but with 1 x 10^6 CFU per 50 µl, for invasive challenges and virulence tests.

2.8 Intranasal infection

Mice were lightly anaesthetised with 2.5% (v/v) Isofluorane USP (Isocare) over oxygen (1.4–1.6 litres/min), in an anaesthetic box. To aid infection mice were 'scruffed' and held vertically while 10 µl of PBS containing 5 x 10^5 CFU of *S. pneumoniae* were administered equally between both nostrils using a Gilson pipette, the mice were held vertically for an additional 10 seconds before they revived to prevent reflux of the dose. The animals were left on their backs to recover from the effects of the anaesthetic which further prevented the inoculum being discharged from the nostrils. The precise inoculum was determined by viable counting after each infection, and the experiment was repeated if the dose was deemed to be outside of acceptable range, 2-6 x 10^5 CFU per 10 µl.

2.9 Determination of carriage or infection

Animals were housed in an individually ventilated micro-isolator Cage *(*IVC*)* rack and monitored daily for external signs of disease. 'Pain score' sheets were completed daily, which use a tick system to describe signs of disease in an increasing scale which are summarised in table 2.2. In order to determine asymptomatic colonisation, at predetermined intervals (days 0, 1, 3, 7, 14, 21, 28) groups of 5 animals per time point were terminally anaesthetised and the total volume of blood was collected via cardiac puncture, in order to detect both the presence of septicaemia by serial plating, and to isolate serum for later analysis. The nasopharyngeal tissue, consisting predominantly of

the dorsal nasal turbinates and the ventral maxilloturbinates and also part of the back of the tongue, were then dissected and placed immediately in 5 ml of sterile PBS as described previously (Kadioglu *et al*., 2002). This tissue was weighed, and disrupted using an Ultra-Turrax T8 homogeniser (IKA) and numbers of pneumococci determined by colony counting so that they could be expressed as CFU/mg nasopharyngeal tissue. For plating nasopharyngeal isolates, blood agar plates were supplemented with 2 µg/ml gentamicin to remove non-pneumococcal contaminants. CFU was also determined in the lungs during colonisation, lungs were dissected and placed into 10 ml of PBS and CFU enumerated as described above. For *in vivo* studies it was necessary to plate the undiluted bacterial suspension *e.g.* blood, or homogenised nasopharynx/lungs, in order to detect low levels of pneumococcal infection. The lower limit of detection in the nasopharynx was 10 CFU per mg of tissue.

Sign of disease	Severity	Severity scale
Normal	N/A	0
Starey coat	+	1
Hunched appearance	+	1
Starey	++	2
Hunched	++	2
Starey and hunched	+/+	2
Starey and hunched	+/++	3
Starey and hunched	++/+	3
Starey and hunched	++/++	4
Lethargic	+	5
Lethargic	++	6

Table 2.2 Disease score of mice. It was not possible to discern the difference in severity between a starey coat and hunched appearance, and therefore they were ascribed the same score. At the maximum score (6), in addition to lethargy mice were usually starey and hunched (++/++) at which time, under the guidance of our project license, the mice were immediately culled.

2.10 Re-colonisation studies

Mice that had cleared the colonisation with an attenuated pneumococcus (PlnA$^-$, PspA$^-$, or NanA$^-$/B$^-$), by day 14, were re-administered with 5 x 10^5 CFU (in 10 µl) of WT pneumococci intranasally on day 28 of the experiment. Where possible, the presence of the original mutant strains were checked for by differentially plating day one nasopharyngeal samples on plates supplemented with the relevant antibiotics. Mice were typically sampled for carriage on day 1, 3, 7 and 14 days of the second colonisation.

2.11 Acute pneumonia challenge following colonisation

Mice that had cleared attenuated colonisation and age matched naive control mice were challenged with a 50 µl volume of PBS containing 1 x 10^6 CFU of WT pneumococci 28 days after the initial colonisation. Both sets of mice were monitored for signs of disease over a one week period post infection and were typically culled to determine the density of infection after 0, 24, and 48 hours.

2.12 Passive immunisation

Immune serum was collected by cardiac puncture from 5 mice which had survived both colonisation and subsequent invasive pneumococcal challenge (taken 7 days after acute challenge with D39). The serum was pooled, and 100 µl was administered intraperitoneally with a 1 ml insulin syringe into 5 naive MF1 mice, using PBS only for a further 4 control mice. 1 hour after immunisation a dose of WT-D39 prepared at 1 x 10^5 CFU/ml was and administered intraperitoneally into all mice with a 1 ml insulin syringe. All mice from each group were tail bled to assess the extent of infection at 24 hours and then were left for 1 week post infection to monitor survival.

2.13 Tail bleed

15 minutes prior to the procedure mice were warmed in their cages inside a static 37 °C incubator. Each mouse was then placed individually into a tail bleeding apparatus which contained the mice securely but left their tails exposed. The tails were briefly disinfected with industrial methylated spirits (IMS), and a 1 ml insulin syringe inserted parallel to the lateral tail vein was used to obtain approximately 30 µl of blood per

mouse. In order to prevent coagulation, 20 µl of blood was mixed without delay into 180 µl of in order to determine CFU.

2.14 Enzyme-Linked Immunosorbent Assay (ELISA)

Blood was collected by cardiac puncture, and left at 37 °C in a static incubator for 30 minutes to coagulate. The coagulum was removed using a sterile wooden toothpick and the remaining serum centrifuged at 300 g for 10 minutes, the supernatant aspirated, and stored at –20 °C until needed. Using this method approximately 500 µl of serum could be obtained from each mouse.

For anti-polysaccharide ELISA, the methodology followed was recommended by the World Health Organisation (Wernette *et al*., 2003). 96-well plates (Greiner Bio-one,) were coated overnight with 10 µg/ml serotype-2 polysaccharide (LGC standards) in PBS, at 4 °C. The following day, plates were washed with 5 times PBS + 0.05% v/v Tween 20 (Sigma Aldrich), allowing the first wash to stand for 30 seconds in the wells before continuing with subsequent washes. Before adding to the plate, samples of serum were diluted (1:1) in PBS + CWPS/22F (Statens Serum Institute) for 30 minutes, to prevent non-specific binding. The samples were added in duplicate and serially diluted 8 times with the first dilution 1:1 (50 µl + 50 µl) with PBS, transferring 50 µl to each subsequent well, and left for 2 hours at room temperature. The plates were washed as before and secondary mouse anti IgG, IgM and IgA (Sigma Aldrich) conjugated to alkaline phosphatase was prepared at 1:10,000 in PBS + Tween 20 + 0.1% BSA and 100 µl added to each well. After a further 2 hours of incubation at room temperature and a final washing procedure, 100 µl of a phosphatase substrate was added to each well and the plates left in the dark for the colour to develop. After approximately 5 minutes the development was ceased by adding 50 µl of 3M NaOH (Sigma Aldrich). Log titres of antibody were calculated from wells where the lowest dilution gave an optical density of 0.1 at $A_{405nm.}$

Purified proteins PspA (clade 2) and PspC were a kind gift of Elaine N. Miyaji (Centro de Biotecnologia, Instituto Butantan, Sao Paulo, Brazil). Proteins were coated overnight at 10 µg/ml in PBS, and after washing as described previously, plates were blocked for 2 hours at room temperature in PBS + Tween 20 + 1% BSA (Sigma Aldrich), before adding serum samples to the plate. The remaining procedure was identical to that of pneumococcal polysaccharide ELISA.

2.15 ELISPOT

Ex vivo B-cell ELISPOT assays were performed on mixed cell suspensions from cervical lymph nodes. 96-well multiscreen plates (Milipore) were coated for 2 hours at 37 °C with goat anti-mouse whole Ig (Southern Biotech) at 20 µg/ml (200 µl/well). Before adding cells, the plates were washed under sterile conditions, 3 times with sterile PBS (Gibco). Mixed CLN cell suspensions were thawed quickly in a 37 °C water bath, and washed in 15 ml of RPMI, and the cell number in each sample was quantified using a haemocytometer (Weber Scientific International). Cells were resuspended in appropriate volumes of RPMI + 10% FBS + 100 U/ml pen/strep (Invitrogen) + 10 mM hepes (Gibco) + 50 µM 2-*mercaptoethano*l (Sigma Aldrich), so that they could be seeded into duplicate wells at 5 x 10^5 cells per well (in 100 µl volume) and incubated overnight at 37 °C, + 5% CO^2. The next day the plates were washed 5 times in PBS without the need for sterile conditions. Anti-IgG, IgA and IgM secondary antibodies conjugated to alkaline phosphatase (Sigma Aldrich) were added at 1:1000 dilutions (10 times lower than used for ELISA) for 2 hours at room temperature. Plates were washed a further 5 times before being developed using an alkaline phosphatase substrate kit (Bio-rad) to the manufacturer's instructions. The reaction ceased by running the plate under a cold water tap for 2 minutes, after the first spots could be seen appearing on the plate. Spots were enumerated using an ELISPOT reader (Immunospot, S4 analyzer) and Immunospot software (version 3.2).

2.16 Flow cytometry

Flow cytometry was performed on frozen mixed cell suspensions from mouse CLN and nasal mucosal tissue. The cells were collected in RPMI and manually passed through a BD Falcon cell strainer (Beckton Dickinson), which had a 40 µm pore diameter, with the aid of a syringe plunger (Terumo). The suspension was centrifuged at 300 g for 10 minutes and the pelleted cells were frozen at -80 °C in RPMI + 15% FCS and 10% dimethyl-sulphoxide (DMSO) (Sigma Aldrich), in a slow freezing jar containing isopropanol (ReAgent), for at least 24 hours before transferring to conventional freezer boxes. When required, cells were thawed quickly in a 37 °C water bath, and washed once in 15 ml volumes of RPMI to remove traces of DMSO. The sample was then resuspended in 50 µl aliquots (per stain) of PBS + 1% BSA containing optimal concentrations of each fluorochrome. Cells were stained for 20 minutes at 4 °C using

CD4 FITC (T helper cells) clone H129.19, CD8 PE (cytotoxic T cells) clone H35-17-2, CD19 APC (B cells) clone 1D3, CD44 PE (^{hi} in memory T-cells) clone IM7, CD62L APC (^{hi} in naive T-cells) clone Mel-14, from BD biosciences. F4/80 FITC (macrophage/monocyte) clone BM8 and Gr-1 FITC (granulocyte/neutrophil) clone RB6-8C5 from Bio-legend, and FR4 PE (T-regs) clone eBio12e5, from eBiosciences. The cells were finally resuspended in 300 µl of PBS and taken immediately for data collection on the FACSCalibur flow cytometer (BD). Using CellQuest Pro software (BD), a dot plot displaying the forward and side scatter (measurements of size and granularity respectively) was created and a gate made to tightly fit around the lymphocytes only, was designated G=1. 10,000 events in G=1 were collected per stain and compared the percentage of total cells. Cells in G=1 were then represented in a new dot plot and a histogram showing side scatter and the fluorescent intensity of each individual stain. For enumeration of granulocytes, a gate excluding lymphocytes on the basis of size and granularity was used.

2.17 Preparation of cell Cytospin

Lungs collected in 10 ml of PBS on ice were manually disrupted in a Petri dish (Sterilin) using two scalpel blades (Swann-Norton, Sheffield) until small enough to pass through the tip of a p1000 pipette (Gilson). The tissue was washed from the dish with PBS and transferred to a cell strainer, and the cells were passed though into a 50 ml Falcon tube (Corning) with the aid of a plunger from a 2 ml syringe. Alternatively, cervical lymph nodes and naso/mucosal tissue could be passed directly through the cell strainer with the help of a plunger. The single use strainers were washed with 3 times 1 ml volumes of PBS and the 50 ml Falcon tubes were made up to a volume of 10 ml, and centrifuged at 300 g for 10 minutes, with the centrifuge set at 4 °C. The pellets were resuspended in 1 ml red blood cell lysis buffer BD Pharmlyse (Becton Dickinson), which consisted of 10 x buffer reconstituted in nano-pure water (at room temperature), for 5 minutes in the dark. The volume was made back up to 10 ml with PBS and was re-centrifuged at 300 g for 10 minutes. Lung homogenates were typically reconstituted in 10 ml of PBS ready for the cytospin whereas cervical lymph node and naso/mucosal tissue was resuspended in 5 ml of PBS, to achieve an optimal density for enumeration. 50 µl of each sample in triplicate was transferred to the cytospin apparatus consisting of a Shandon Cytoslide, Shandon Cytoclip, Cytofunnel and filer cards (Thermo-

Scientific), and centrifuged at 1500 rpm for 3 minutes in a Cytospin 2 centrifuge (Shandon). Slides were left at room temperature overnight to dry.

2.18 Cytospin staining

Cells were stained with a Reastain Quick diff kit (Reagena) which contains a methanol based fixative, an eosin Y based red dye, and Azur II based blue dye. Each slide was submerged fully and sequentially into the fixative, followed by the red and the blue dyes. After the final stain the slides were rinsed briefly in distilled water and left to air dry. Once dry the slides were mounted with DPX (BDH Laboratory Supplies) and a coverslip.

2.19 Cell separation

In order to obtain a single cell suspension of CD4+ T-lymphocytes for use in transmigration, 20 ml of heparinised peripheral blood was taken from healthy donors, diluted 1:1 with PBS, and carefully layered onto Lymphoprep (density gradient of 1.077; Axis-Shield) at a ratio of 2:1 (blood to Lymphoprep). The layered blood and Lymphoprep was centrifuged at 800 g for 30 minutes, and the lymphocyte layer harvested by Pasteur pipette. The collected lymphocytes were then washed in RPMI and resuspended in 1 ml of RMPI to be counted by a haemocytometer. The known concentration of lymphocytes were washed and resuspended in PBS + 1% BSA + EDTA and incubated with anti-CD4 micro-beads (Miltenyi Biotec) (20 µl per 1 x 10^7 cells) for 20 minutes at 4 °C. Positive selection of CD4+ T-lymphocytes was achieved by passing the lymphocyte mixture through a MS column connected to a magnet (Miltenyi Biotec). Viability of cells was measured by trypan blue exclusion and found to be 92.8% ± 0.486% (n = 10), and CD4-T-cell purity was shown to be >95% as assessed by flow cytometry.

2.20 Transmigration assay

The transmigration methodology was adapted from (Kadioglu *et al*., 2004). Briefly, Transwell filters with a pore size of 5 µm (Costar, Corning Inc., New York, U.S.A.) were pre-wetted with 30 µl of RPMI, placed into a 6.5-mm-diameter tissue culture-treated polycarbonate membrane Transwell filter plates (Costar, Corning Inc) and incubated for 30 minutes at 37 °C. After 30 minutes, 100 µl of RPMI containing 5 x 10^5

CD4+ T-lymphocytes were added the top chamber of the Transwell apparatus, and 600 µl of whole frozen pneumococci was added to the lower chamber. The apparatus was incubated at 37 °C, + 5% CO_2 for 90 minutes. After incubation the upper wells were removed from the lower chamber, and the entire volume was removed with a Gilson pipette, without disturbing the bottom of the filter. 20 µl of the top chamber liquid was resuspended with an equal volume of trypan blue (Sigma Aldrich), and placed into a haemocytometer. To calculate the number of migrated cells, both chambers of the haemocytometer chamber were counted in duplicate, with the amount of cells being quantified by the calculation; (number of cells counted x 2 x 10^4) / 0.1. This number was subtracted from the number of T-cells per well at the start of the assay, and the remaining number was deemed to be the amount of migrated cells, which was then converted to a percentage of the starting cell number.

2.21 Transmission Electron Microscopy (TEM)

To assess the nature of capsular polysaccharide in the bacteria strains used in this thesis frozen aliquots of passaged and non passaged D39 and $PlnA^-$ were thawed, and centrifuged at 300 g for 10 minutes in a micro-centrifuge. After removing the majority of the supernatant the pellet was then washed 3 times in PBS at 12,000 rpm for 2 minutes and resuspended in 2.5% glutaraldehyde/0.05M HEPES (pH 7.2), and fixed for 3 hours.

The sample preparation for TEM was carried out by Miss Natalie S. Allcock of the Electron Microscopy Lab, Adrian building, University of Leicester. Briefly, the samples were washed 3 times for 20 minutes in 0.05M HEPES (555g), and then fixed in 1% osmium tetroxide in 0.05M HEPES for 60 minutes. The pellet was washed again in distilled de-ionised water, 3 times for 10 minutes, and embedded in 3% Agar (Agar Scientific). The pellet was dehydrated in 70% ethanol for 15 minutes, and then fresh 70% ethanol was added overnight. The next day the pellet was further dehydrated with 90% ethanol for 30 minutes, 100% absolute ethanol for 30 minutes, 100% analytical grade ethanol for 30 minutes and finally in propylene oxide for 2 x 15 minutes. The bacteria were then embedded in a number of changes of low viscosity resin (Marivac) overnight and then polymerised for 16 hours at 60 °C.

The embedded samples were sectioned to a thickness of 80 nm using a Reichert Ultracut S ultramicrotome, and were collected onto a copped mesh grid. Before

microscopy, the sections were counterstained with 2% Uranyl acetate (EMS) for 20 minutes, and then in Reynold's Lead citrate (EMS) for a further 2 minutes. The bacterial samples were viewed on a JEOL 1220 TEM with an accelerating voltage of 80 kV, and images were captured using a Mageview III digital camera with analysis software.

2.22 Histology

At preselected time points during *in vivo* studies various mouse tissues were collected and placed on ice. A mould of aluminium foil was created around a bijoux tube, submerged completely into dry ice and half filled with approximately 1 cm of OCT (Sakura, Finetek). As the OCT was nearly set, tissue was added to centre of the medium and immediately covered with an additional ~1 cm of OCT allowed to harden completely on dry ice, before being transferred to -80 °C.

2.23 Tissue sectioning

At least 24 hours before sectioning, tissue samples were removed from -80 °C and transferred to a -20 °C freezer. Tissue sections of between 7and 15 microns were cut using a Bright cryostat (Bright Instruments) and collected onto glass microscope slides. Slides were left to air dry for at least 1 hour and stained immediately with haematoxylin and eosin, or stored in a desiccated slide box at -80 °C for immunofluorescence staining.

2.24 Immunuhistochemistry

For immunofluorescence, tissue samples of CLN and nasal mucosal were frozen as described previously and 7 µm sections were cut. Immunofluorescence was performed by Dr Alun Kirby at the University of York, using the following primary antibodies: BM8 (anti-F4/80), N418 (anti-CD11c), M5/114 (anti-MHCII) RM4-5 (anti-CD4), 53-67 (anti-CD8), H57-597 (anti-TCRb) (eBioscience), ED3 (anti-CD169) (AbD Serotec), FA11 (anti-CD68) (Acris Antibodies, Germany), ERTR9 (anti-SIGNR1) (Bachem), (anti-CD31) biotin, (anti-CD19), (anti-Meca32) biotin 546, (anti-PNAg) biotin 647, (anti-CD206) (biotin) (BD biosciences).

Most of the antibodies were directly conjugated to fluorochromes but where indicated biotin antibodies were conjugated to secondary antibody Alexa-Fluor conjugates

(Invitrogen). Sections were mounted in ProLong Gold (Invitrogen) and images were taken using a Zeiss Axioplan LSM 510 confocal microscope as single optical slices of between 0.8 and 1.0 µm. The images were analysed using Zeiss LSM image browser software v4.

2.25 Cell culture

Detroit 562 cells (CCL-138) (ATCC) a human pharyngeal cell line, and A549 cells (CCL-185) (ATCC) a human lung epithelial cell line, were used to characterise the adhesion and invasion of pneumococci and isogenic mutants. Detroit cells were maintained in minimal essential medium (MEM) (Gibco), and A549's in Dulbecco's modification of Eagles medium (DMEM) (Sigma), both with the addition of 10% foetal bovine serum (Gibco) and 1% penicillin-Streptomycin (Sigma Aldrich).

To establish the colony, cells were removed from liquid nitrogen and warmed quickly in a 37 °C water bath, and once thawed, transferred to 10 ml of the respective media in a 15 ml Falcon tube (Corning). The cells were centrifuged at 300 g for 10 minutes and the pellet resuspended in 1 ml of fresh media, and added to 9 ml in a 75 cm^2 flask (Beckton Dickinson). Both cell types were grown at 37 °C in 5% CO_2 until they reached 80% confluence, when they were transferred to fresh flasks. All experiments in Detroit 562 and A549 cells were carried out between passage number 10 and 15.

2.26 Cell splitting/passaging

Once every 3-5 days both cell types reached the desired confluence and were split. In order to remove the cells from the base of the flasks, the old medium was aspirated off the cells and discarded, and the base of the flask was washed once carefully with sterile PBS (Gibco). 5 ml of warmed trypsin (5 mg/ml) + 0.02% EDTA was added to the flask and left to incubate at 37 °C for 5-10 minutes. The resulting volume which now contains all the cells is aspirated off and added to an equal volume of media, and centrifuged at 300 g for 10 minutes. The supernatant is discarded and the cells resuspended in 2 ml of fresh media, using 1 ml in 9 ml of fresh media to establish a new flask.

Alternatively, if after 3 days flasks had not reached confluence then the old media was aspirated, washed once as before, and 10 ml of fresh warmed media was added.

2.27 Adhesion/invasion assay

One day prior to transferring the cells to a 24 well plate, the media was changed in the flask with 10 ml of fresh media which contained no antibiotics, as this would be harmful to the pneumococcus. The next day, trypsinised cells were removed from the flask as for splitting, and resuspended in 2 ml of media. A 20 µl sample was mixed with 20 µl of trypan blue and placed into a haemocytometer to be counted under the microscope. Using the calculation; cells counted x 2 x 10^4, the numbers of cells/ml was determined so that 10^5 cells per well in 1 ml of media could be transferred to a tissue culture treated 24 well plate (Beckton Dickinson). After a further 1-2 days at 37 °C, + 5% CO_2 the cultures in the 24 well plates reached 100% confluence, which was optimal for the adhesion and invasion assays. On the day of the assay, the cell layers were washed 3 times with DPBS, and 1 ml of bacteria at 10^6 CFU/ml were added in duplicate to the 24 well plates (2 identical wells for adhesion, and 2 identical wells for invasion), and returned to the incubator for 2 hours. The pneumococci used for the adhesion/invasion assays were grown overnight in liquid culture, and adjusted to O.D 0.1 (500_{nm}) which had previously been shown to approximate to 10^6 viable cells per ml. The cell layers were then washed a further 5 times with 1 ml volumes of sterile PBS to removed unattached pneumococci. To quantify adhesion, the cells were removed from the 24 well plate with 100 µl of trypsin + 0.02% EDTA for 5-10 minutes at 37 °C. The D562 and A549 cells were lysed with the addition of 800 µl of ice cold distilled water for 5 minutes, which was then reconstituted with the addition of 100 µl of 10 x PBS. To quantify invasion, extracellular bacteria were killed by the addition of 5 µg/ml of penicillin over an additional 2 hours at 37 °C, before receiving 3 additional washes with sterile PBS and removing the cells from the wells as described above. The amount of adhered/invasive pneumococci was quantified by serial plating of dilution 10^{-1}-10^{-6} on blood agar plates as described previously. To control for the growth of bacteria during the assay, the number of bound bacteria was compared to control wells which consisted of bacteria alone incubated without cells, for 2 hours at 37 °C, + 5% CO_2.

Chapter 3: Establishment of a murine model of nasopharyngeal carriage and investigation into the effect of major pneumococcal virulence factors on duration and density of colonisation

3.1 Preface

The findings from mouse models of pneumococcal colonisation can be extrapolated to pneumococcal human nasopharyngeal carriage as they share many important characteristics. The duration of the carriage (from a few weeks to a few months), and the minimum dose required to initiate a persistent carriage are similar in mice and humans (McCool *et al*., 2002; McCool and Weiser, 2004). Additionally, many of the serotypes which have been identified to cause carriage in mice are also those strains most commonly detected in surveillance studies of human nasopharyngeal carriage (serotypes-4, 6B, 14, and 23F) (Wu *et al*., 1997b). The study by Wu *et al* also showed that mouse pneumococcal carriage models were robust enough to be replicated in genetically diverse strains of mice, such as the BALB/c and CBA, and thus could be representative of a human heterogeneous population. More recently however, a degree of variation in mouse strain-specific responses to pneumococcal intranasal infection has been identified, which questions key interpretations from this and other studies (Gingles *et al*., 2001). For example, BALB/c and C57BL/6 mice are naturally resistant to invasive pneumococcal disease with serotype-2 (strain-D39), often surviving beyond 7 days with an infectious dose that a 'susceptible' mouse from a CBA background may succumb to after just 24 hours. Perhaps unsurprisingly the density and duration of nasopharyngeal carriage between inbred mouse strains has also been shown to vary significantly (McCool and Weiser, 2004), and thus the mouse strain chosen for each experiment may be more critical to the outcome than previously understood, and must be considered carefully when establishing a new model of nasopharyngeal carriage. A further complication is that many of the bacterial strains commonly used in carriage models (serotypes-14, 19, 23F) are avirulent in mice when introduced either by the intranasal or intravenous route (Wallick *et al*., 1983). As there is significant crossover of genes required during nasopharyngeal colonisation and invasive disease, other factors may be evident in these strains, such as their ability to interact with host immune mechanisms, which could permit successful colonisation, and thus conclusions made

using less invasive serotypes may be misleading (Hava and Camilli, 2002). To this end I sought to develop a novel model of pneumococcal nasopharyngeal colonisation, which would better reflect carriage of a potentially invasive serotype in humans. The MF1 mouse was chosen as the model organism as it is outbred and therefore more closely representative of a heterogeneous human population than inbred strains. MF1 mice are moderately susceptible to pneumococcal infection with D39 pneumococci (the predominate strain of choice throughout this thesis), compared to other mouse strains when administered intranasally, and succumb to infection at around 48 hours (Alexander *et al*., 1998). D39 is a historically important pneumococcal strain as it was used in the seminal study, observing that DNA was the source of genetic material in experiments of bacterial transformation (Avery *et al*., 1979), however due to its virulence in mice; it has since been used to study much of the pathogenesis of pneumococcal disease. Isogenic mutants are readily available on a D39 background and this makes it the ideal choice to study the impact of individual virulence factors in carriage.

In this chapter, both WT bacteria and isogenic individual pneumococcal virulence factor mutants are assayed for their ability to influence survival in a long term mouse nasopharyngeal carriage model. The WT strains include the laboratory strain D39 (serotype-2), the more clinically relevant A66 (serotype-3) and a non-encapsulated strain derived from human nasopharyngeal carriage, 110.58. D39 pneumococcal mutants which lack the toxoid PLY, neuraminidases and pneumococcal surface protein-A (PspA), are also tested. Finally, the effects of different virulence determinants on *in vitro* adherence and invasion to human epithelial cell lines are assessed.

Results

3.2 Carriage of WT-D39

WT-D39 pneumococci were carried in the nasopharynx of outbred MF1 mice for at least 28 days (Fig 3.1) (the limit of observation in this study) without developing long term infection of the lung or septicaemia, and without developing any outward signs of the infection which are commonly associated with invasive pneumococcal disease. The percentage of mice which carried pneumococci on all days tested was 100%, with the exception of day 14 where carriage rates were still >90%. The density of the pneumococcal colonisation in the nasopharynx was maintained at a relatively stable level throughout the duration of the experiment (mean=2.563 (Log CFU/mg of nasopharynx) ± 0.1063 (S.E.M.)), and only on day 14 (mean=2.017 ± 0.1134 (p=0.0001), and day 28 (mean=2.362 ± 0.1294 (p=0.0246)) did the bacterial CFU differ significantly from the number observed 15 minutes after inoculation (time zero) (Fig 3.1). However, only the deviation in CFU observed at day 14, (below the level of both days 7 and 21) was consistently observed in 3 separate experiments. The level of bacteria in the nasopharynx is representative of a 'true carriage' as opposed to a mere contamination of the nasal surfaces, as although the CFUs at day 0 were reflective of the initial dose of pneumococci (which ranged from 3.84-6.01 $x10^5$ CFU/10 µl), by day 3 there was very little variation in nasopharyngeal bacterial numbers, regardless of starting inoculum between 3 separate experiments (mean=2.752 ± 0.1083).

Mice were assessed daily for signs of disease, such as starry coat or hunched appearance, none were observed for the entire duration of carriage (data not shown), however it was also necessary to monitor weight and temperature throughout carriage, as a lack of appetite and a drop in core body temperature can also indicate the presence of infection (Morton and Griffiths, 1985). Weight gain and temperature were not statistically different in mice colonised with WT-D39 and control naive mice during 14 days of observation (Fig 3.2), and the weights of both sets of animals were within the parameters issued by the mouse breeder Harlan UK.

(http://www.harlan.com/research_models_and_services/health_reports/by_strain/mf1_health_monitoring_reports.hl.)

In addition to the nasopharyngeal CFU, pneumococcal numbers were also assessed in the lungs and blood of all colonised mice. In every strain tested and at all time points, no pneumococci were found in blood (data not shown). However bacteria could be recovered from the lungs immediately following inoculation (mean=1.785 (Log CFU/mg of lung tissue) ± 0.2927 (S.E.M)), and this was the case for both WT-D39, and the isogenic mutant PlnA$^-$, although the level in both the mutant and WT was not significantly different (data pooled) (Fig 3.3). After 24 hours there was a significant reduction in CFU (p=0.0335) in the lungs, and after 3 days of colonisation no further colonies were detected (Fig 3.3). It is notable that in carriage events where the initial nasopharyngeal CFU was higher (Fig 3.4) this correlated with a higher starting inoculum, but did not lead either to sepsis or a prolonged (chronic) infection of the lung which was statistically different to lung infection after the standard carriage dose (data not shown).

3.3 Optimising the carriage dose

Whilst experimenting with different carriage doses it became evident that a starting inoculum which contained two-fold higher numbers of bacteria (1 x10^6 CFU) than the standard carriage dose, in the same volume (10 µl), led to a rapid clearance of WT-D39 pneumococci from the nasopharynx <14 days post infection (Fig 3.4). With this 'high' dose, significantly higher numbers of CFU were detected immediately after infection in the nasopharynx at time zero (p=0.0092), but at 2 days (p=0.0193), 3 days (p=0.0362) and 14 days (p=0.0018) post infection there were less pneumococci in the nasopharynx when compared to the 'standard' colonising dose of 5 x 10^5 CFU/10 µl at the corresponding time points, and no bacteria could be recovered from the nasopharynx at day 14 with the higher dose (p=0.0018) (Fig 3.4). Despite the higher starting inoculum in this challenge, which contained the same number of viable bacteria used in lethal invasive pneumococcal challenges, there was no prolonged evidence of pneumococcal colonies in the lungs, which were below the level of detection after just 24 hours (data not shown). There was also no septicaemia, and all mice remained asymptomatic throughout the duration of this experiment (data not shown).

3.4 The role of PLY in nasopharyngeal carriage

In order to observe the effect of individual pneumococcal virulence factors on nasopharyngeal colonisation, isogenic mutants of D39 were selected and assessed using

this model. PLY is a cholesterol dependent cytolysin which has known immuno-modulatory effects, and is essential for full virulence of pneumococcal disease *in vivo* (Berry *et al*., 1989). Although mutants lacking PLY have been shown to be attenuated at causing infection of the lower respiratory tract and sepsis (Kadioglu *et al*., 2002), the effect of the toxin in the nasopharynx is less well defined, and it has been speculated that the absence of PLY may actually lengthen nasopharyngeal colonisation in mice (Rubins *et al*., 1998; van Rossum *et al*., 2005).

Using this carriage model, MF1 mice were intranasally infected with a dose of the PLY negative mutant PlnA$^-$ which was identical to the WT-D39 dose used previously, and CFU's were monitored in the nasopharynx, lungs and blood over 28 days. As observed during WT-D39 carriage, there was no evidence of sepsis throughout the duration of the experiment, and colonies in the lungs fell sharply after 24 hours (Fig 3.3). However, in this instance, CFU's in the nasopharynx also fell consistently throughout the early stages of carriage, exhibiting significantly lower bacterial numbers than WT-D39 at day 3 ($p=0.0080$) and day 7 ($p=0.0054$) (Fig 3.5), although carriage rates up to and including day 7 were still 100%. Colonies of PlnA$^-$ were almost entirely cleared from the nasopharynx by day 14 (mean=1.149 ± 0.0842 ($p=0.0001$), where carriage could be detected in <30% of mice (0% in 1 out of 2 experiments), and all mice were completely below the level of detection at 28 days post infection ($p=0.0001$) (Fig 3.5). The consistent presence of colonies until day 7 of the experiment, independently of starting inoculum suggests that PlnA$^-$ is able to actively colonise in a similar fashion to WT-D39, and is not merely contaminating the surface of the nasopharynx.

In contrast to PlnA$^-$ which is a full length PLY deletant, Δ4 is a PLY negative mutant made on a D39 background which lacks only domain-4 of the toxin. Expression of domain-4, which is at the C-terminus of PLY, has been shown to be essential for the binding to erythrocytes and cholesterol *in vitro* (Baba *et al*., 2001). It is thought that only domain-4 actually penetrates the lipid bilayer (Tilley *et al*., 2005) and without it, PLY is non-haemolytic (Baba *et al*., 2001). This was confirmed by haemolysis test before commencing these experiments (data not shown). Point mutations in sites which affect the haemolytic capabilities of PLY alone have been shown to greatly reduce the virulence of pneumococci *in vivo* (Berry *et al*., 1995), but the effects of a domain-4 mutant have not yet been assessed in a model of long term nasopharyngeal carriage.

The Δ4 mutant was not statistically different to PlnA$^-$ colonisation, and CFU's in the nasopharynx were significantly lower than time zero at days 1, 3, and 7 post infection (p=0.0357), and also significantly lower than WT-D39 at day 14 (p=0.0018) (Fig 3.6). Pneumococcal colonies of Δ4 were maintained in the nasopharynx for 7 days post infection but no detectable CFU could be recovered from any of the mice at day 14 (p=0.0357 compared to day 0) (Fig 3.6).

3.5 The role of neuraminidases in nasopharyngeal carriage

Neuraminidases are LPXTG anchored enzymes present on most clinical isolates of *S. pneumoniae,* which although surface expressed; may be released by protease action (O'Toole *et al*., 1971; Camara *et al*., 1994). They contribute to the pathogenesis of pneumococcal disease by cleaving residues on host surfaces to allow greater attachment to the respiratory epithelium (King *et al*., 2006). The effect of a duplication insertion mutant of the *nanA* gene has been previously described in a model of chinchilla nasopharyngeal carriage, where the duration of deficient pneumococci in the upper respiratory tract was reduced compared to the WT by more than two weeks (Tong *et al*., 2000). NanA$^-$ mutants have also been tested in a mouse model of acute pneumococcal infection, where the mutant could not persist longer than 12 hours in the nasopharynx (Manco *et al*., 2006), however neuraminidase mutants have not yet been tested in a long term mouse model of nasopharyngeal carriage.

Using this model, a D39 isogenic mutant lacking neuraminidase-A was present immediately after inoculation (time zero) but was cleared entirely from the nasopharynx before 7 days (p=0.0357), which was significantly faster than the PLY negative mutant PlnA$^-$ (p=0.0022 at day 7) (Fig 3.7). At just 24 hours after inoculation, 60% of mice had no detectable CFU in the nasopharynx (mean=1.220 (Log CFU/mg of nasopharynx) ± 0.1320 (S.E.M.) (p=0.0357), and neither the number of bacteria, or the proportion of mice carrying pneumococci above the level of detection increased significantly at day 3. All mice tested were completely clear of pneumococci at day 7 and day 14 of the experiment (Fig 3.7). As observed with the PLY mutants there was a temporary infection of the lungs which lasted until 24 hours, and the blood was clear from pneumococci at all time points (data not shown).

It has been suggested that neuraminidase-A and neuraminidase-B may have distinct sites of action because of differences in host tissue specific activity and their optimum

pH for enzymatic activity (Berry *et al*., 1996). This idea was supported in a mouse model of acute pneumonia, where a NanB$^-$ mutant was able to persist for longer in the nasopharynx than NanA$^-$ (Manco *et al*., 2006). Like NanA$^-$, Nan B$^-$ has yet to be tested in a long term model of carriage.

In this model, colonies of the neuraminidase-B isogenic mutant of D39 had declined rapidly in the nasopharynx by 24 hours post infection (p=0.0357) (Fig 3.8). However, in contrast to NanA$^-$, the level of pneumococcal colonisation had recovered in the nasopharynx by day 3 (p=0.0117 compared to day 3 of NanA$^-$ colonisation), where 100% of mice had pneumococci in the nasopharynx that exceeded the levels observed at time zero (Fig 3.8). NanB$^-$ colonies also persisted longer in the nasopharynx than NanA$^-$ (p=0.0075) and were still present in 100% of mice tested at day 7, although in slightly reduced numbers compared to day 3. Ultimately there was no difference in the ability of the neuraminidase-B and the PLY mutant to cause long term carriage as no pneumococci could be detected in the nasopharynx at 14 days post infection (p=0.0357 compared to WT-D39) (Fig 3.8).

3.6 The role of PspA in nasopharyngeal carriage

Pneumococcal surface protein-A (PspA) is an important membrane associated virulence factor, which is required for full virulence (Briles *et al*., 1988). PspA may aid the survival of pneumococci by interfering with both complement mediated opsonophagocytosis and killing by host apolactoferrin (Tu *et al*., 1999; Shaper *et al*., 2004). However, due to the nature of its surface expression it is also a major target of protective immunity, and the suitability of PspA as a vaccine candidate in humans has been explored extensively (Briles *et al*., 1996). A PspA mutant based on a D39 background was cleared more rapidly from the blood infected mice (Briles *et al*., 1988), and in a mouse model of nasopharyngeal colonisation it showed a 30-fold reduction in carriage densities compared to WT–D39, although in comparison to our model the initial dose of bacteria was very high (10^7 CFU/ml) and the follow up time was short (<7 days) (Ogunniyi *et al*., 2007).

In the model presented here, CFU's of PspA$^-$ were reduced gradually in the nasopharynx compared to day zero levels during the first 7 days of the colonisation and carriage rates remained 100% until day 7 of the experiment (Fig 3.9). At day 1 and day 3 the levels of pneumococcal colonisation were not statistically different from WT-D39

colonisation, however at day 7, mice were colonised at significantly lower densities compared to WT-D39 ($p=0.0416$) at this time point (Fig 3.9). There was a further significant reduction in PspA$^-$ between day 7 and 14 ($p=0.0079$) as pneumococci were completely cleared from the nasopharyngeal tissue of all mice tested (Fig 3.9).

3.7 Diverse pneumococcal serotypes in nasopharyngeal carriage

In addition to the serotype-2 WT-D39, a serotype-3 WT strain-A66 was also tested in the nasopharyngeal carriage model. Serotype-3 is perhaps more clinically relevant than serotype-2, as it includes many more strains found to cause invasive pneumococcal disease in humans (Henriques *et al*., 2000). A66 is also highly virulent in mice and, although it has been previously described to grow less well than D39 in the nasopharynx, in a model of acute pneumonia, it was similarly able to cause long term carriage in BALB/cByJ mice (Kadioglu *et al*., 2002; Magee and Yother, 2001).

In this model the resultant carriage was not dissimilar to WT-D39 as A66 pneumococci were detected at all days assayed (mean=2.28 ± 0.189), and the carriage of A66 lasted for 21 days (limit of observation) with no significant deviation in CFU from day 0 (Fig 3.10). The density of bacteria in the nasopharynx was however significantly lower than D39 carriage at day 3 ($p=0.0142$) (Fig 3.10) which may correlate with the slower progression observed in invasive disease (Kadioglu *et al*., 2002). Interestingly, mice colonised with A66 exhibited a drop in CFU at day 14 (mean=1.585 ± 0.2385) (not significant) which coincides precisely with a similar drop in WT-D39 (Fig 3.1), which suggests there may be a host mediated protective effect at this time point which is acting to clear bacteria independently of capsule type.

3.8 Carriage with non-encapsulated pneumococci

The polysaccharide capsule is perhaps one of the most important virulence factors in the arsenal of the pneumococcus. Although pneumococci that express less amounts of polysaccharide capsule may have an selective advantage in attaching to the nasopharynx, non-encapsulated strains are severely impaired both in their ability to cause invasive disease and to colonise the nasopharynx (Magee and Yother, 2001; Roche *et al*., 2007; Nelson *et al*., 2007).

In order to test the effects of capsule in this model, a non-encapsulated natural clinical isolate was chosen, (110.58), which was derived from a patient nasopharyngeal swab

during a Swiss surveillance programme (Muhlemann *et al*., 2003). In contrast to previous studies using non-encapsulated pneumococci, long term of 110.58 was evident in 100% of mice for all days tested (day 14 was the limit of observation in this study), and CFU's did not differ significantly from either of the encapsulated WT strains in the first 7 days (Fig 3.11). However, the density of colonisation was highest at day 14 (mean=3.12 ± 0.05701), and was significantly more than was observed in WT-D39 carriage (p=0.0044) and in WT-A66 carriage at the corresponding time point (p=0.0286) (Fig 3.11), indicating a possible absence of host immune response, in this compared to WT strains. These results indicate that carriage would most likely have been maintained for at least 28 days (as observed with WT encapsulated strains).

3.9 The role of capsule in invasive pneumococcal disease

In line with previous studies, the finding that long term carriage could be achieved in mice with non-encapsulated pneumococci was unexpected (Magee and Yother, 2001). It is thought that many of the same factors which contribute to carriage have a dual role in the progression of invasive disease (Hava and Camilli, 2002), and therefore we sought to determine whether the non-capsulated 110.58 could cause invasive pneumococcal disease when administered intranasally. An equivalent infectious dose of 110.58 which is normally fatal in mice with D39 was prepared and administered to 8 MF1 mice, alongside WT-D39 as a control. At 24 hours post infection, all mice infected with D39 had developed bacteraemia of the lungs and septicaemia, and also maintained large amounts of pneumococci in the nasopharynx, and subsequently, survival rates with D39 infection after one week were less than 20% (Fig 3.12). In contrast, no pneumococci could be found in any sterile site infected with 110.58, (including the nasopharynx) and all mice infected survived to one week post infection without exhibiting outward signs of disease (p=0.0085) (Fig 3.12). This suggests there may be factors unique to these non-encapsulated carriage strains which compensate for the previously thought, indispensible role of polysaccharide capsule in permitting long term carriage, a requirement which is still evident in the ability to cause invasive disease in the host.

It has previously been reported that the pneumococcus can undergo a spontaneous transition between opaque and transparent phenotypes *in vivo*, and that the opaque phenotype which has a thick capsule is more commonly associated with invasive

disease than transparent colonies, which are associated with carriage and possess a thin polysaccharide capsule (Weiser *et al*., 1994). The majority of the pneumococcal stocks used in this thesis when plated on blood agar plates had opaque morphology, due to the animal passaging process, where colonies are collected from the blood following I.P. infection. A stock made from bacteria recovered from the nasopharynx of mice after 7 days of carriage was not visually distinguishable from passaged WT-D39 on blood agar plates (data not shown). However in order to assess whether colonising bacteria retained the ability to cause invasive disease (regardless of morphology), a comparison of virulence between the standard passaged D39, and a stock made during carriage was conducted. At time zero and 24 hours post infection there was no significant differences in either nasopharyngeal, lung or blood CFU between the two strains (Fig 3.13). The mortality rates were high in both conditions and were also not statistically different, 77.8% from the stock recovered from the nasopharynx and 88.9% in the 'conventionally' passaged D39 (Fig 3.13, D).

3.10 Pneumococcal adherence and invasion *in vitro*

In order to explore some of the observations from *in vivo* mouse data, an *in vitro* model of pneumococcal adherence and invasion of epithelial cells was used to compare selected WT and attenuated pneumococcal strains used in carriage experiments, in their ability to interact with Detroit 562 cells (a human nasopharyngeal cell line), and A549 cells (a human lung epithelial cell line). The first interesting finding was that adherence to nasopharyngeal and lung epithelial cells was not significantly different for any strain tested (Fig 3.14), and in all strains exhibited low percentages of adherence (>1% of total pneumococci adhered in all strains) was observed. Adherence to D562 and A549 cells was substantially lower (~100 fold lower) in WT-D39 than either WT-A66 ($p=<0.0001$) or WT-23F ($p=<0.0001$), and contrary to expectations there was no statistical difference between passaged and non-passaged WT-D39 strains (Fig 3.14, A, B). There was also no statistical differences in the adherence capacity of pneumococcal mutants $PlnA^-$ and Neuraminidase A/B^- compared to parental WT-D39, however R6, which is based on a D39 background but lacks a polysaccharide capsule, adhered significantly better than the parent D39 in both types of epithelial cells tested ($p=<0.0001$) (Fig 3.14, A, B). It was not possible to detect any colonies of WT-D39 or its encapsulated isogenic mutants which invaded epithelial cells, however both A66 and 23F pneumococci were visible inside both A549's ($p=0.0022$) ($p=0.0161$) and D562 ($p=0.0186$) ($p=0.0019$) cells

respectively (Fig 3.14, C, D) (Fig 3.15). Invasion of A549 ($p=0.0161$) and D562 cells ($p=0.0161$) was also evident for the non-encapsulated D39 mutant, R6 (Fig 3.14, C, D). As it was previously shown that pneumococci in contact with epithelial cells *in vitro* and *in vivo* can reduce the thickness of their capsule, a process which increased adherence (Hammerschmidt *et al*., 2005), WT-D39 pneumococci were recovered from the epithelial cells after incubation and grown overnight in BHI, and a stock was made from these bacteria. However, it was not possible to observe an enhanced amount of adherence or invasion of epithelial cells compared to standard D39 (data not shown).

Electron microscopy of the pneumococci used for the adherence and invasion assays showed that both animal passaged $PlnA^-$ and WT-D39 had thicker polysaccharide capsules than non-passaged WT-D39 (Fig 3.15). The capsule appears to be 'ruffled' on the surface of the passaged WT-D39, which is believed to be an artefact of the glutaraldehyde fixation process for scanning electron microscopy.

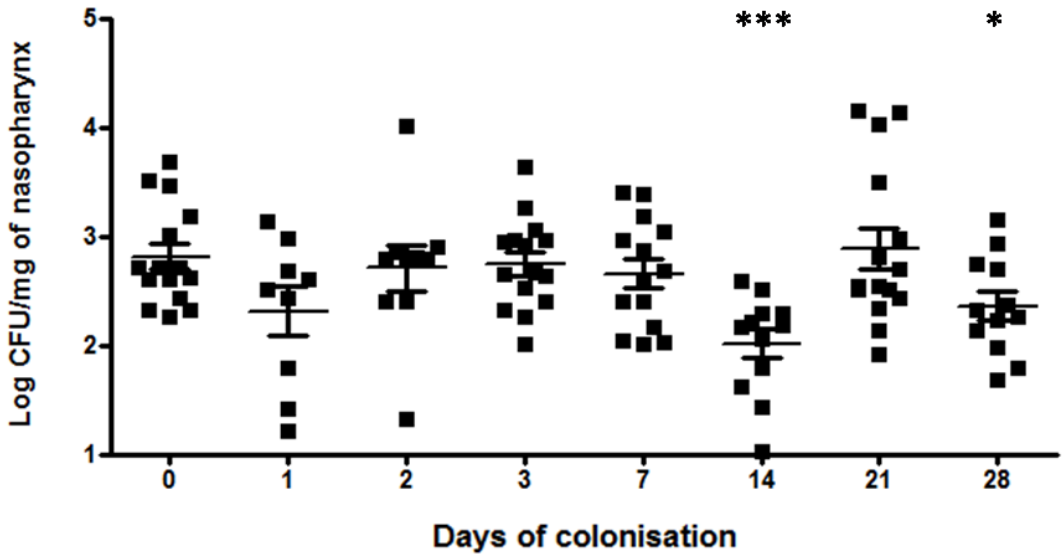

Figure 3.1 Nasopharyngeal carriage of WT-D39. The density of colonisation is expressed as mean Log CFU/mg of total homogenised nasopharynx ± S.E.M. Each closed square represents one individual mouse that was culled at that time point and data is based on a minimum of n=15 animals for each group at all time points, and 3 independent experiments where the starting doses were between 3.84-6.01 x 10^5 CFU/10 µl. Time point '0' represents 15 minutes post inoculation. The lower limit of detection was 1 bacteria per mg of tissue. Statistical significance of changes in CFU compared to day 0 were assessed using the Mann-Whitney test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001).

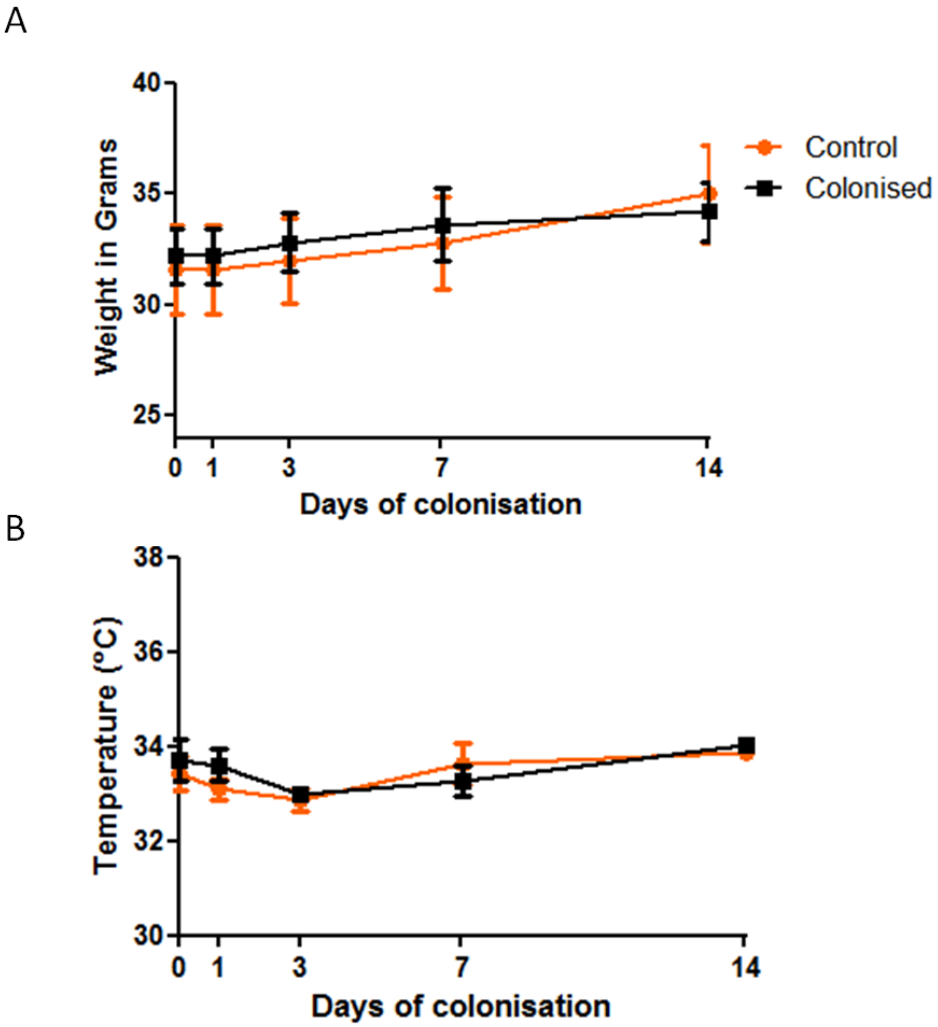

Figure 3.2 Signs of illness during carriage of WT-D39. (A) Weight gain in Grams, and (B) body temperature in degrees Celsius (°C), was measured in the same WT-D39 carrying and control mice over a two-week period. The presence of carriage in WT-D39 colonised mice was confirmed after the 14 days (data not shown). Data represented is the mean temperature/weight ± S.E.M., and is based on n=5 mice per time point. Statistical significant differences were assessed using the Mann-Whitney test.

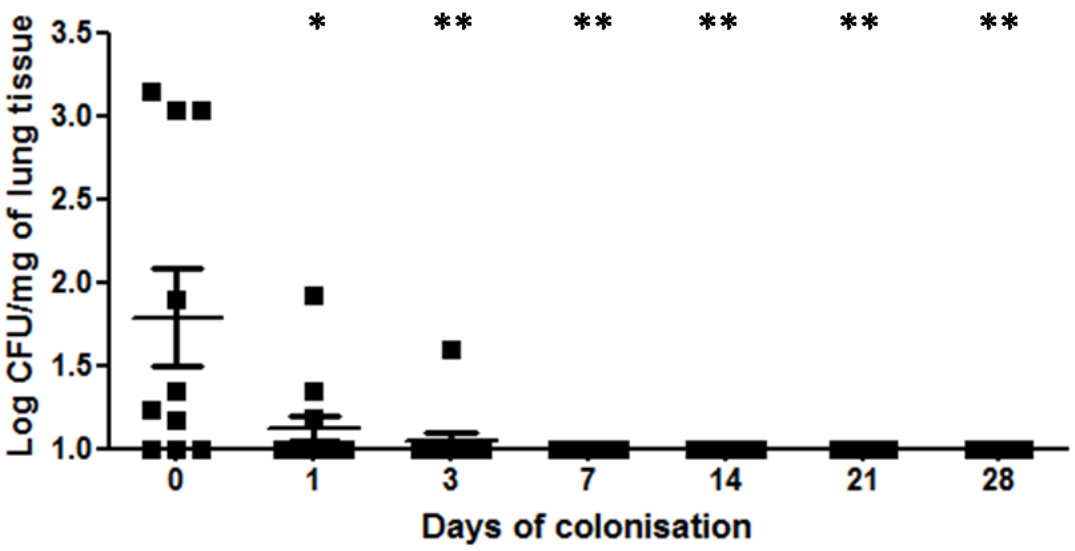

Figure 3.3 Infection of the lung during carriage of serotype-2 pneumococci. The density of lung infection in colonised mice is expressed as the mean Log CFU/mg of total homogenised lung tissue ± S.E.M. Each closed square represents one individual mouse that was culled at that time point. Data is representative of both WT and isogenic mutants of D39 (n=5 mice infected with WT-D39 and n=5 mice infected with PlnA$^-$ for each time point). There was no statistical difference in lung CFU between WT-D39 and PlnA$^-$ colonised mice. Statistical significance compared to day 0 was assessed using the Mann-Whitney test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001).

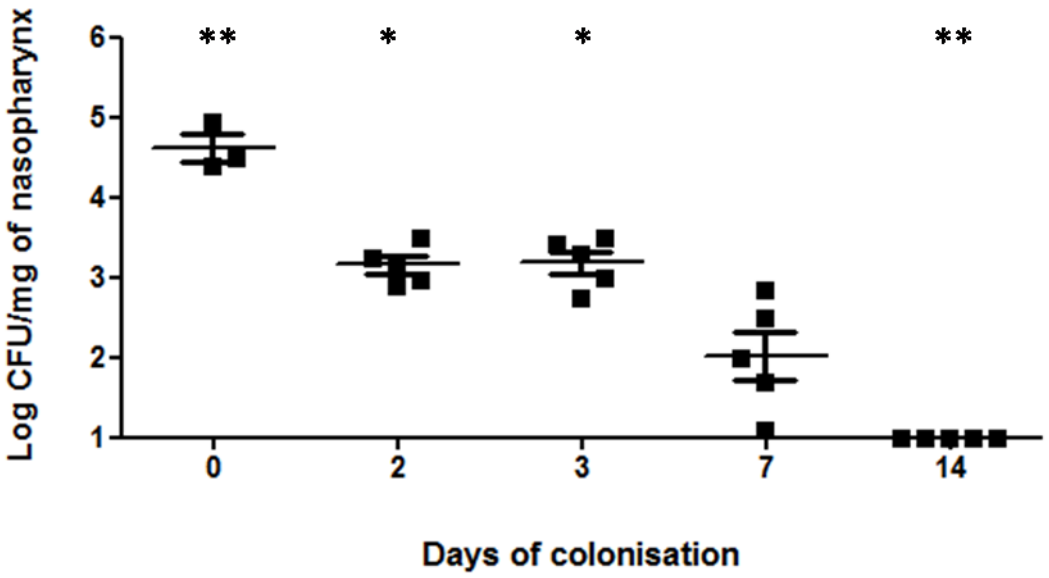

Figure 3.4 The effect of an increasing carriage dose. MF1 mice were intranasally infected with two-fold higher dose (compared to normal carriage dose) of WT-D39, which consisted of 1.06 x 10^6 CFU in 10 µl of PBS. The density of colonisation is expressed as mean Log CFU/mg of total homogenised nasopharynx ± S.E.M. Each closed square represents one individual mouse that was culled and the data represents n=5 mice per time point (n=3 at time zero). Statistical significance as compared to standard dose of WT-D39 (Fig 3.1) was assessed using the Mann-Whitney test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001).

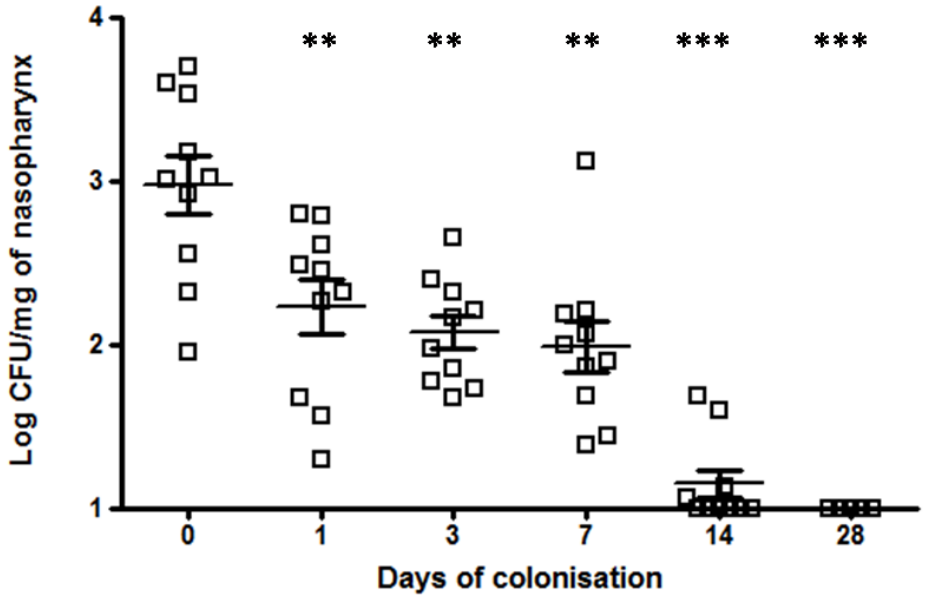

Figure 3.5 Nasopharyngeal carriage of the PLY negative pneumococcal mutant (PlnA$^-$). MF1 mice were intranasally infected with 5 x 10^5 CFU/10 µl of the D39 PLY deficient mutant strain. The density of colonisation is expressed as mean Log CFU/mg of total homogenised nasopharynx ± S.E.M. Each open square represents one individual mouse culled at that time point and data is n=10 animals for each group at all time points, and is representative of 2 independent experiments. The lower limit of detection was 1 bacteria per 10^{-1} (Log 1). Statistical significance of changes in CFU compared to day 0 were assessed using the Mann-Whitney test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001).

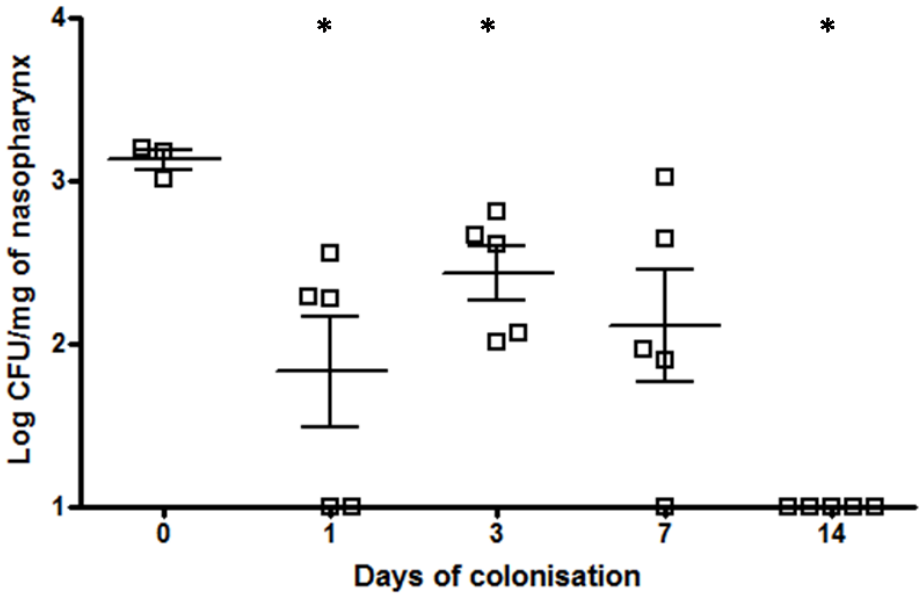

Figure 3.6 Colonisation of D39 Δ4. MF1 mice were intranasally infected with 5 x 10^5 CFU/10 µl of the D39 PLY deficient mutant strain Δ4. The density of colonisation is expressed as mean Log CFU/mg of total homogenised nasopharynx ± S.E.M. Each open square represents one individual mouse culled at that time point and data is n=5 mice for each group at all time points except time zero (n=3). The lower limit of detection was 1 bacteria per 10^{-1} (Log 1). Statistical significance of changes in CFU compared to day 0 were assessed using the Mann-Whitney test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001).

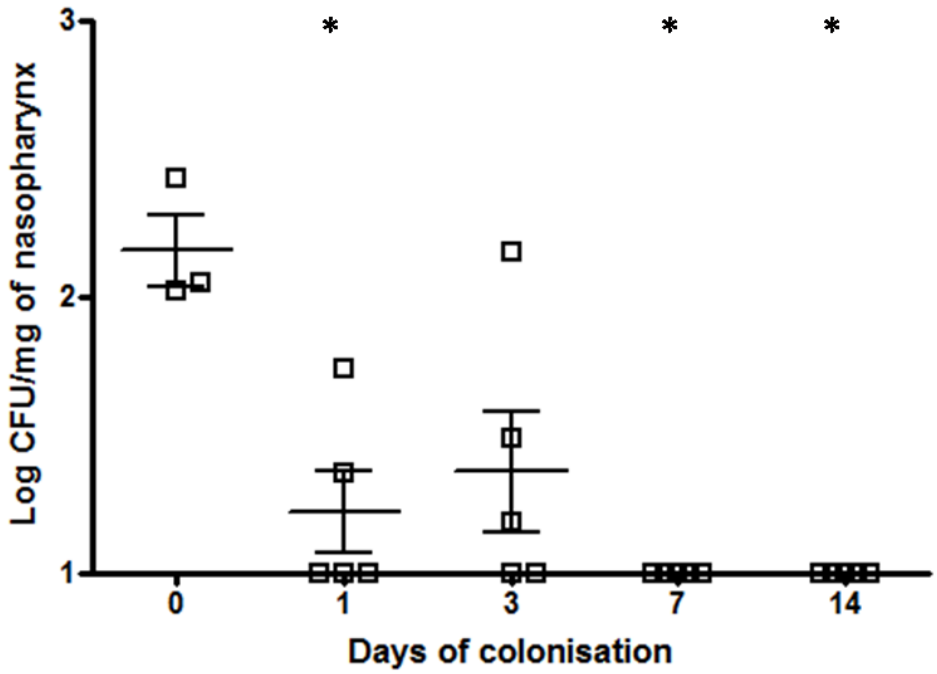

Figure 3.7 Colonisation of D39 NanA^{-}. MF1 mice were intranasally infected with 5 x 10^5 CFU/10 µl of the D39 NanA deficient mutant strain. The density of colonisation is expressed as mean Log CFU/mg of total homogenised nasopharynx ± S.E.M. Each open square represents one individual mouse culled at that time point and data is n=5 mice for each group at all time points except time zero (n=3). The lower limit of detection was 1 bacteria per 10^{-1} (Log 1). Statistical significance of changes in CFU compared to day 0 were assessed using the Mann-Whitney test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001).

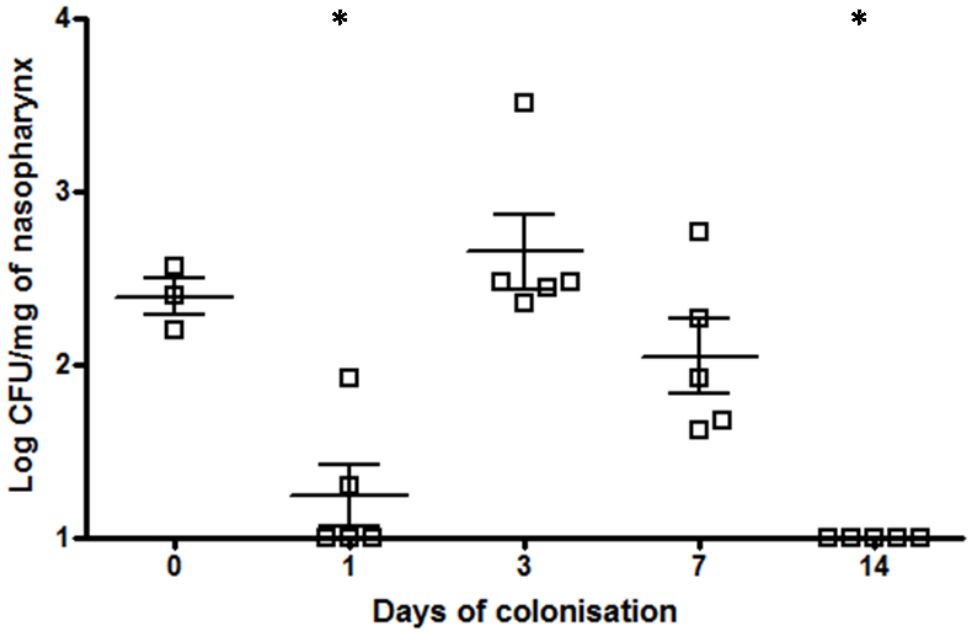

Figure 3.8 Colonisation of D39 NanB$^-$. MF1 mice were intranasally infected with 5 x 10^5 CFU/10 µl of the D39 NanB deficient mutant strain. The density of colonisation is expressed as mean Log CFU/mg of total homogenised nasopharynx ± S.E.M. Each open square represents one individual mouse culled at that time point and data is n=5 mice for each group at all time points except time zero (n=3). The lower limit of detection was 1 bacteria per 10^{-1} (Log 1). Statistical significance of changes in CFU compared to day 0 were assessed using the Mann-Whitney test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001).

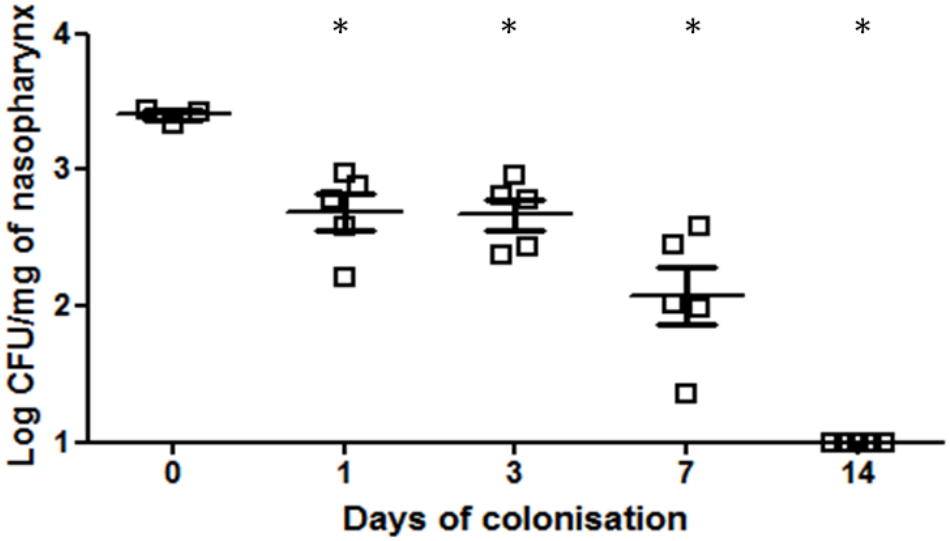

Figure 3.9 Colonisation of D39 PspA⁻. MF1 mice were intranasally infected with 5 x 10^5 CFU/10 µl of the D39 PspA deficient mutant strain. The density of colonisation is expressed as mean Log CFU/mg of total homogenised nasopharynx ± S.E.M. Each open square represents one individual mouse culled at that time point and data is n=5 mice for each group at all time points except time zero (n=3). The lower limit of detection was 1 bacteria per 10^{-1} (Log 1). Statistical significance of changes in CFU compared to day 0 were assessed using the Mann-Whitney test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001).

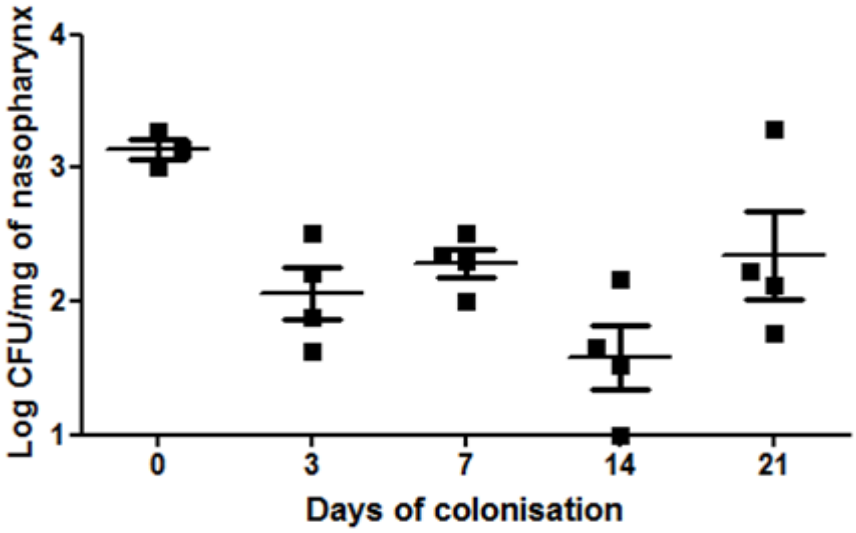

Figure 3.10 Colonisation of Serotype-3-A66. MF1 mice were intranasally infected with 5 x 10^5 CFU/10 µl of the WT-serotype-3 strain, A66. The density of colonisation is expressed as mean Log CFU/mg of total homogenised nasopharynx ± S.E.M. Each closed square represents one individual mouse culled at that time point and data is n=4 mice for each group at all time points except time zero (n=3). The lower limit of detection was 1 bacteria per 10^{-1} (Log 1). Statistical significance of changes in CFU compared to day 0 were assessed using the Mann-Whitney test.

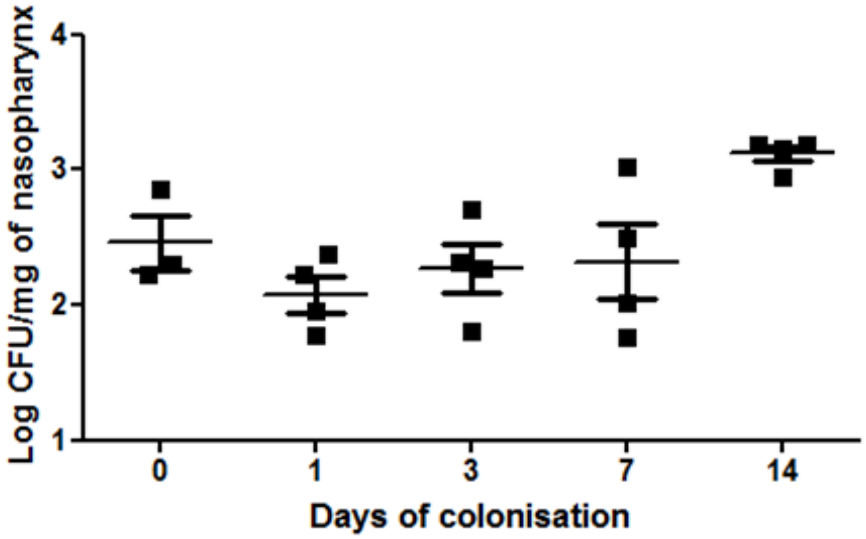

Figure 3.11 Colonisation of non-encapsulated pneumococci 110.58. MF1 mice were intranasally infected with 5 x 10^5 CFU/10 µl of the non-encapsulated naturally occurring 110.58. The density of colonisation is expressed as mean Log CFU/mg of total homogenised nasopharynx ± S.E.M. Each closed square represents one individual mouse culled at that time point and data is n=4 mice for each group at all time points except time zero (n=3). The lower limit of detection was 1 bacteria per 10^{-1} (Log 1). Statistical significance of changes in CFU compared to day 0 were assessed using the Mann-Whitney test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001).

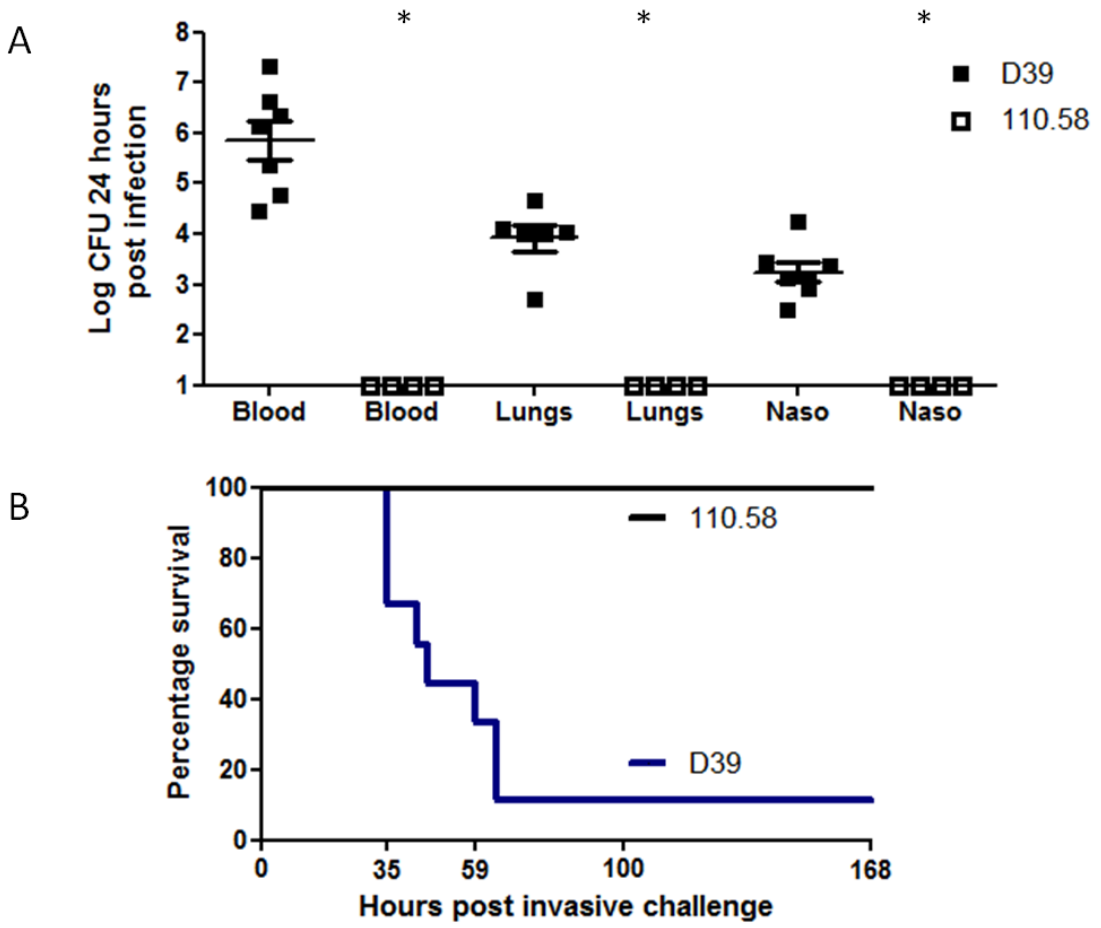

Figure 3.12 Virulence of non-encapsulated 110.58. MF1 mice were subjected to an invasive intranasal challenge dose (1 x 10^6 CFU/50 µl) of WT-D39 (closed squares) or 110.58 (open squares) and CFU's were measured at 24 hours in the nasopharynx, and blood of infected mice (A). The density of pneumococcal infection is expressed as mean Log CFU/mg of total homogenised tissue, or CFU/ml of blood ± S.E.M. n=4 mice for each strain tested. Additional mice were monitored for survival up to 7 days post infection (B). Statistical significance of the difference in mean CFU's compared to D39 was measured using the Mann-Whitney test. *(p=<0.05). Comparison of survival curves was calculated using the Log Rank (Mantel-Cox) test.

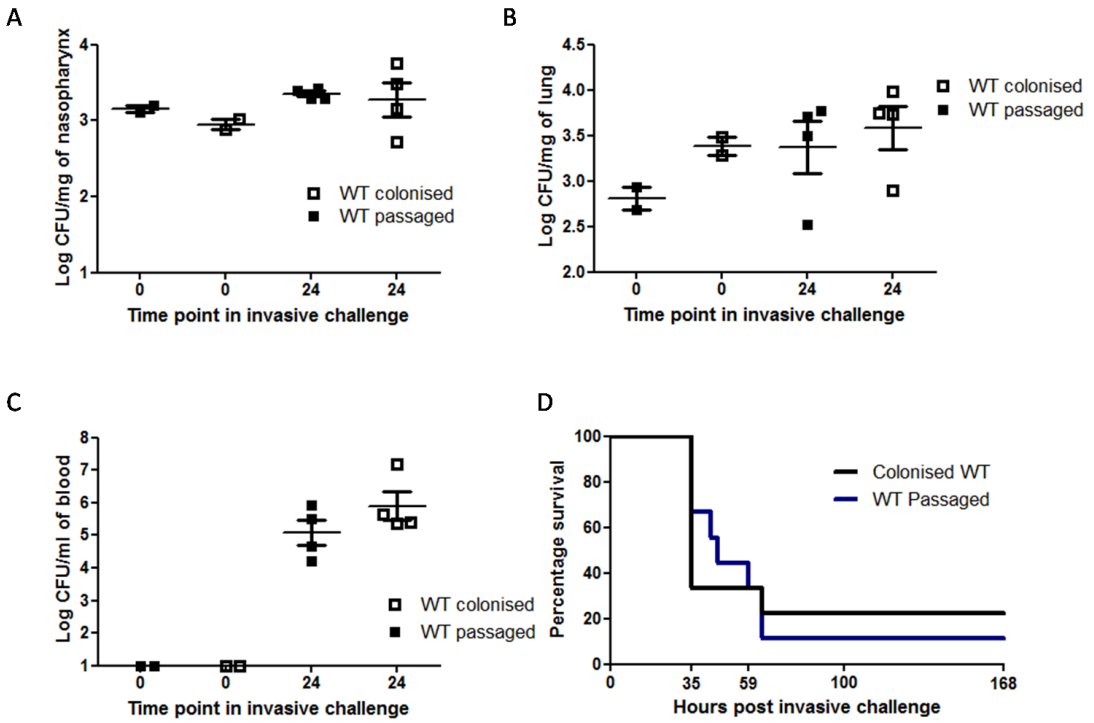

Figure 3.13 The effect of carriage on the virulence of D39. MF1 mice were subjected to an intranasal challenge with identical invasive challenge doses of standard passaged WT-D39 (closed squares) and WT bacteria that had been recovered from the nasopharynx after 7 days of carriage (open squares). CFU's were measured at time zero and 24 hours in the nasopharynx (A), lungs (B) and blood (C) of infected mice. The density of pneumococcal infection is expressed as mean Log CFU/mg of total homogenised tissue, or CFU/ml of blood ± S.E.M. n=4 mice for each strain tested (D). Additional mice were monitored for survival up to 7 days post infection (B). Statistical differences of mean CFU's were measured using the Mann-Whitney test and comparison of survival curves was calculated using the Log Rank (Mantel-Cox) test.

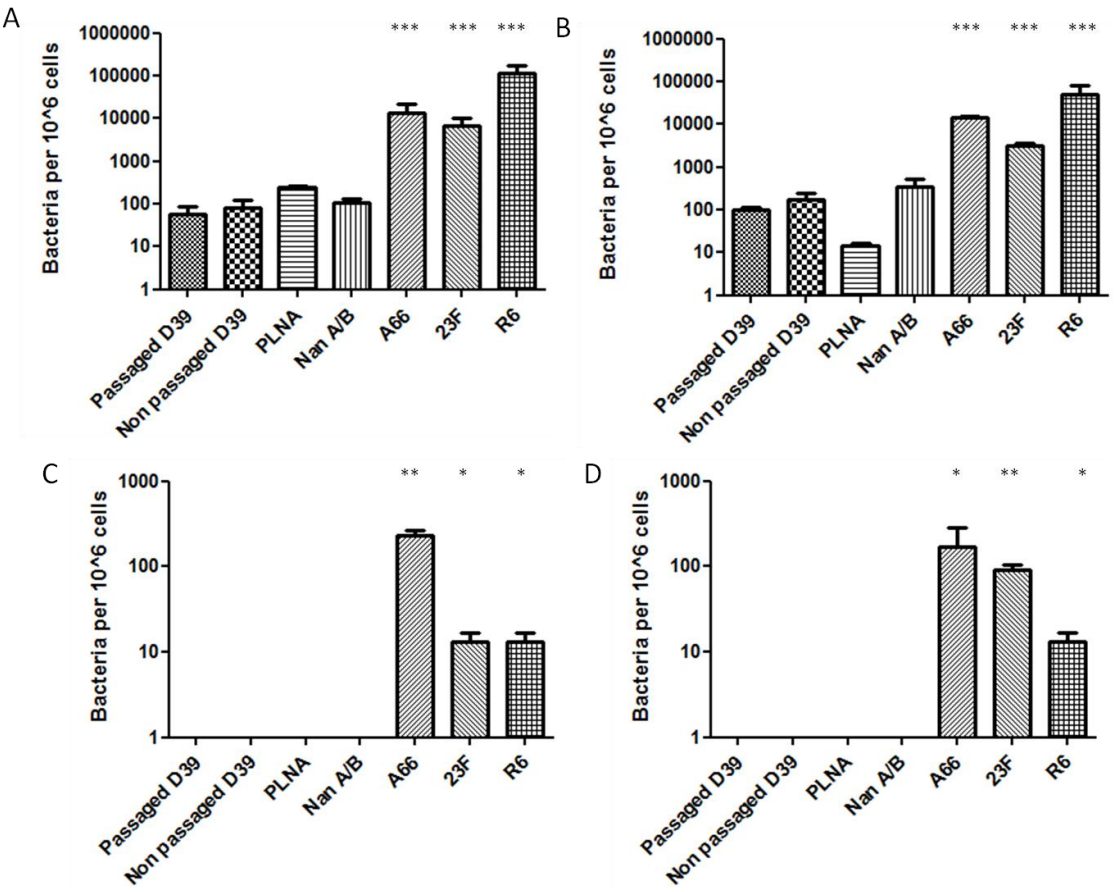

Figure 3.14 Adherence and invasion of the pneumococcus *in vitro*. Strains of WT and isogenic mutant pneumococci were assessed for their ability to adhere or invade to A459 cells (A, C respectively) and Detroit 562 cells (B, D respectively). 1 x 10^6 pneumococci from overnight cultures, were added to confluent monolayers of epithelial cells containing ~ 1 x 10^8 cells, (the precise number was calculated at the start of each assay). After 2 hours the cells were washed and the CFU/well calculated in triplicate wells by serial plating. For invasion, penicillin (5 µg/ml) was added to kill all extracellular bacteria and the CFU was reassessed in 2 hours after lysis of pneumococci with the addition of ice cold water. To control for the growth of bacteria during the assay, the number of pneumococci was compared to control wells which consisted of bacteria alone incubated without cells, for 2 hours at 37°C, 5% CO_2. Statistical significance was compared to passaged WT-D39 in all cases by students T test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001).

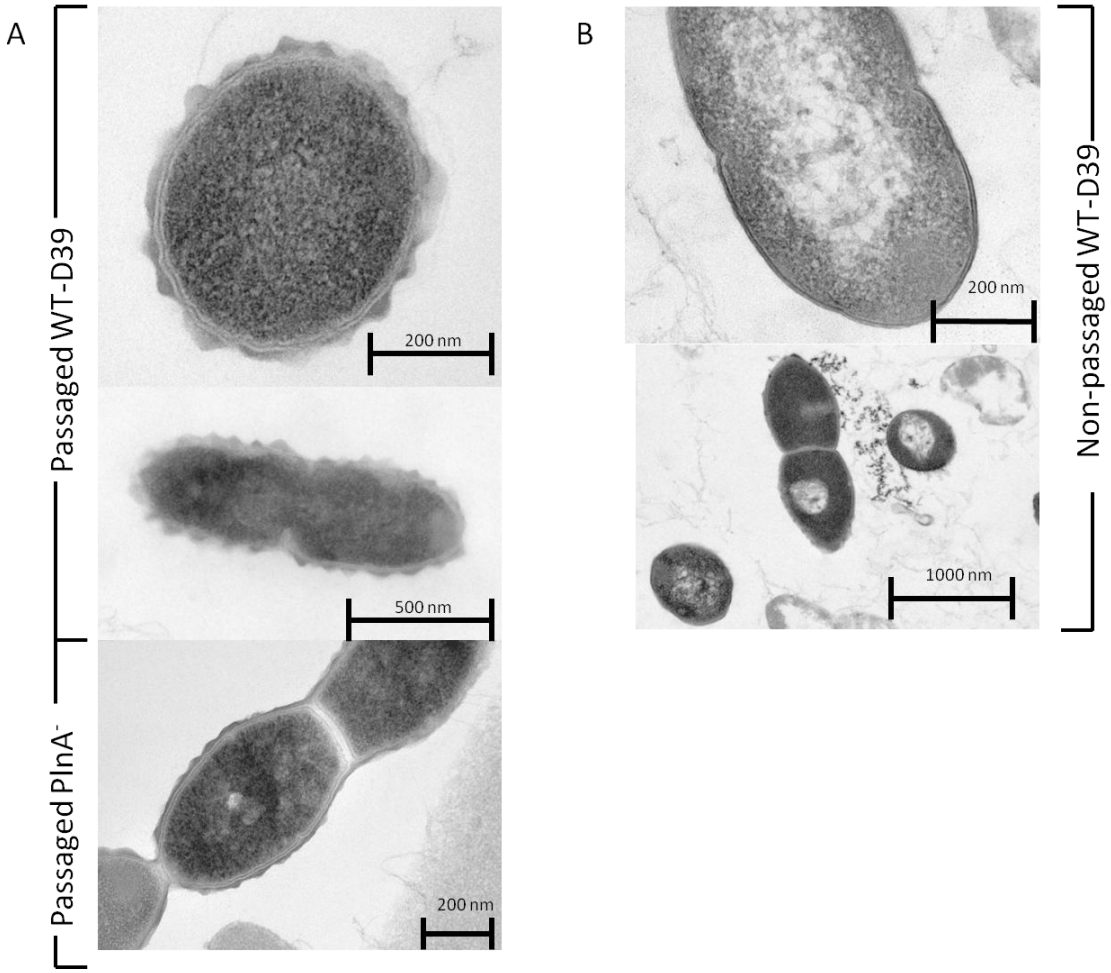

Figure 3.15 Electron microscopy of pneumococci. Scanning electron microscopy was performed on passaged WT-D39 (A, upper and middle panel), and passaged $PlnA^-$ (A, lower panel), and non-passaged WT-D39 (B, upper and middle panel). Larger capsules can be seen on the encapsulated strains. Images are at different magnification, with the scale indicated in each image.

3.11 Conclusions

The data in this thesis is the first description of a 'long term' asymptomatic nasopharyngeal carriage model using serotype-2-D39 pneumococci, which both reflects the duration of natural carriage in the human host, and incorporates the use of outbred mice. D39 is not a commonly used serotype in colonisation models, and was absent from one of the first descriptions of pneumococcal nasopharyngeal carriage in mice which showed carriage for serotypes; 3, 4, 6A, 6B, 14, 19 and 23F (Wu *et al*., 1997b). Seortype-2 was perhaps excluded due to its high level of virulence in the mouse model, which, when delivered via the intranasal route usually causes fatal pneumonia within 48 hours in MF1 mice (Alexander *et al*., 1998; Gingles *et al*., 2001). Despite this, in these data, WT-D39 was able to be carried asymptomatically in MF1 mice for >28 days, without leading to invasive disease within this period. One group which has continued to study primary pneumococcal colonisation in mice (and has shown long term carriage), consistently uses a strain derived from a patient with otitis media, serotype 23F, which is known to colonise, but has reduced virulence in mice (McCool and Weiser, 2004). However, as pneumococcal carriage is the route of entry for all subsequent infection in humans, and even highly virulent strains such as D39 must first normally colonise the nasopharynx as part of this process, it is these strains (rather than those associated solely with carriage) which are therefore of more clinical importance (Bogaert *et al*., 2004).

In this model, the amount of D39 pneumococci needed to establish a stable colonisation in MF1 mice was shown to be ~5 x 10^5 CFU in 10 µl, which is half the number of viable bacteria used in the acute pneumonia model, 1x10^6 CFU, albeit in a higher volume of 50 µl. However, even doses above 10^7 CFU of some serotypes in the smaller volume can be tolerated intranasally and do not normally lead to invasive disease (Ogunniyi *et al*., 2007; Wu *et al*., 1997b). It is therefore the volume and not only the CFU used in carriage that is crucial in determining the outcome of infection, as it was considered that a 10-20 µl dose stays within the nasopharynx and only after the intranasal volume passes 30 µl, does any significant amount of bacteria reach the lungs, and allow an invasive pneumonia to develop (Aras Kadioglu, unpublished observations). Interestingly, contamination of the lungs could be identified soon after inoculating the mice with both WT and attenuated pneumococci, although the levels of

bacteria were lower than observed when using the invasive challenge volumes, which were typically >3 log CFU/mg of lung tissue, immediately following inoculation (see Fig 3.3 and Fig 5.5). The small numbers of bacteria at early time points quickly declined and were below the levels of detection after day 3, which may reflect them being overwhelmed by innate immune mechanisms in the lungs. The appearance of pneumococci in the lungs (although using higher volumes than used in this thesis) has been speculated to result from aspiration of bacteria under anaesthesia (Briles *et al*., 2003). Other studies have employed a method without anaesthesia in mice, which also results in low numbers of pneumococci in the lungs, but may still lead to sepsis in certain serotypes (Wu *et al*., 1997b). Ultimately, the advantages of using anaesthesia outweigh the disadvantages, as the risk of the discharge of the intranasal dose through the nostrils during infection is reduced. The appearance of true long term carriage of the nasopharynx without some early contamination of the lungs may be unavoidable in mice.

In existing carriage models the dose required to maintain long term carriage in mice is dependent on both the mouse strain, and serotype (Wu *et al*., 1997b). However, many of the strains routinely used in colonisation models are almost completely avirulent in mice, such as serotype 23F, and thus the mice will tolerate high inoculum without risk of developing invasive pneumococcal disease (McCool and Weiser, 2004). Interestingly, in this model with virulent WT-D39, when a higher starting inoculum was used, similarly, it did not lead to acute infection, but the duration of carriage was reduced, indicating a possible threshold in the response of the host immune system. It has been shown previously that carriage doses of pneumococci do not cause a strong inflammatory response in the nasopharynx, and although an influx of neutrophils has been identified soon after colonisation, in other studies this was not responsible for clearance of the carrier state (Wu *et al*., 1997b; Nelson *et al*., 2007). However, if a higher dose of virulent pneumococci are used then this response may be more robust, or involve other host factors that may lead to early clearance, as may be the case in this model (Fig 3.4). When a dose of 10^8 pneumococci was used in a nasopharyngeal colonisation model, the gene expression of type 1 interferons was upregulated early during the carriage, indicating an inflammatory response (Joyce *et al*., 2009). It is likely that higher amounts of bacterial factors such as PLY, which is known to stimulate interferon gamma (IFN-γ), may do so in a dose dependent fashion, as is the case with

other cytokine secretion in response to the toxin (McNeela *et al*., 2010). Interestingly, the number of pneumococci in the nasopharynx throughout long term carriage was not significantly different to the levels in the nasopharynx when using a lethal invasive challenge at 24 hours post infection (Fig 3.12a and Fig 5.5) and may represent the maximum level which can be maintained in the upper respiratory tract. The tolerance or control of any immune responses to pneumococci during carriage (even when faced with high levels of bacteria) may be testament to the immunosuppressive capabilities of *S. pneumoniae* (such as the evasion of opsonophagocytosis), which, although contributing to invasive disease, from an evolutionary aspect may be primarily adapted to persisting in the nasopharynx, in order to maintain a reservoir of bacteria for person to person transmission, (Weiser, 2010). The fact that a potentially invasive strain such as D39 was carried asymptomatically in the nasopharynx for 28 days supports this hypothesis, and is another illustration that in the adult immune-competent host *S. pneumoniae* act as part of the commensal human flora, and the risk of developing invasive disease from carriage is low.

The choice of mouse strain was crucial to the relevance of this work. Mice are not natural host species of pneumococcal infection and thus there exists a broad spectrum of susceptibility between different strains (Gingles *et al*., 2001). The resistant BALB/c (Magee and Yother, 2001) and C57BL/6 (Roche and Weiser, 2010; Malley *et al*., 2005b), and the susceptible CBA (Briles *et al*., 2005) which are commonly used in carriage models, represent extremes of outcomes during invasive disease and thus it is likely to assume there may also be differences between these strains during long term carriage. Indeed it has been demonstrated that there is as much as 100 fold difference in the density of nasopharyngeal colonisation between different inbred mouse strains with the same serotype of pneumococci (McCool and Weiser, 2004). Differences in mouse strain susceptibility to infection with another pathogen, *Yersinia entercolitica*, have been shown to be dependent on the production of different amounts of IFN-γ produced in these mouse strains (Autenrieth *et al*., 1994). In pneumococcal susceptibility to invasive disease, other groups have described an enhanced neutrophil response in the resistant mouse (Gingles *et al*., 2001), as well as increased amounts of the anti inflammatory IL-10 (Kerr *et al*., 2002) which is interesting in the context of carriage where inflammation is limited. More recently genetic differences in two mouse strains, BALB/c and CBA/Ca, have been mapped to a region in chromosome 7

and there are studies ongoing in our laboratory to better define the molecular basis for those differences (Denny *et al*., 2003). The MF1 mouse is less susceptible than the CBA/Ca mice but more susceptible than either the C57Bl/6 or BALB/c mice (Gingles *et al*., 2001), and can carry D39 pneumococci asymptomatically in their upper respiratory tract and yet remain susceptible to bacteraemia and septicaemia through intranasal infection by the same strain of pneumococci (Trappetti *et al*., 2009) (Kadioglu *et al*., 2002). This model therefore better mimic's carriage by a potentially invasive pneumococcal strain, such as D39, into a genetically diverse (outbred) susceptible host than has previously been recorded. This combination of factors is repeatable, as using the serotype-3 strain-A66, which has a slower onset of infection during invasive disease than D39, but is equally virulent, also exhibited similar levels of nasopharyngeal carriage.

Using this model we have shown for the first time that the toxin PLY is essential for the maintenance of long term pneumococcal carriage, as the nasopharynx of mice infected with the PLY deficient pneumococci were cleared 14 days after inoculation. PLY has been previously shown to be important in the pathogenesis of lung and blood infection, and was also required for short term carriage in the nasopharynx (Kadioglu *et al*., 2000; Ogunniyi *et al*., 2007). In contrast, longer term studies of nasopharyngeal carriage have shown that colonisation with PLY deficient strains were in fact extended compared to the WT, and existed at a higher density (van Rossum *et al*., 2005; Rubins *et al*., 1998). It has been suggested that the mild inflammatory response and neutrophil infiltration observed during PLY sufficient (but not PLY deficient) colonisation might promote its clearance from the nasopharynx by later adaptive immune responses, and thus result in bacteria being cleared earlier in the WT (van Rossum *et al*., 2005). A hypothesis behind pneumococci possessing a factor which initiates its own clearance could be that immune mechanisms that are attracted may facilitate transmission of the bacteria further down the respiratory tract, but as carriage is the primary route by which pneumococci are passed within a population it is more likely that PLY serves to lengthen rather than shorten nasopharyngeal carriage, as transmission will ultimately lead to death of the individual and the bacterium alike. There are many ways in which PLY can also suppress the host immune system; PLY released upon lysis can bind to complement via the classical pathway, and thus reduce opsonophagocytosis, by diverting crucial complement components away from intact pneumococci (Paton *et al*.,

1984), and PLY may promote the extension of carriage by slowing the rate of muco-ciliary beating and also by increasing attachment to host epithelial cells by inducing damage to the epithelium and the separation of tight junctions (Rayner *et al*., 1995). Binding of pneumococci to undamaged epithelium is modest, and thus the exposure of normally hidden residues by PLY may facilitate stronger (and therefore longer lasting) attachment to the nasopharyngeal epithelium (Feldman *et al*., 1992). Differences between this model and Van Rossum *et al* may be explained by the differences in mouse strain and serotype used, as they used the resistant C57Bl/6 mouse and an avirulent pneumococcal strain originally derived from otitis media (McCool *et al*., 2002). It may also be the case that because they used a nasal lavage to determine CFU, as opposed to the whole homogenisation process employed in this thesis that, PLY+ colonies were more tightly embedded in the tissue, and thus were not as readily detected as PLY$^-$ colonies. We have shown for the first time that domain-4 of PLY may be responsible for the attenuation of carriage observed using the full length toxin deletant. The Δ4 mutant, which does not cause haemolysis of sheep red blood cells (data not shown) but retains 3 quarters of the PLY molecule, was cleared from the nasopharynx after day 7, at the same time as PlnA$^-$. Domain-4 contains sites which are important both for the binding, haemolysis and complement activating properties of PLY (Rossjohn *et al*., 1998). The similarity in structure of domain-4 to the Fc portion of IgG may activate complement and it is also domain-4 that interacts with the host membranes during insertion of the PLY molecule to create pores (Rossjohn *et al*., 1998). The PLY molecule in sublytic concentrations is able to stimulate a plethora of cytokines *in vivo* which include IFN-γ and IL-17A, and it is likely that some of these factors have a immunosuppressive role in the nasopharynx (McNeela *et al*., 2010). Whilst the pore forming activity of PLY did not affect IFN-γ secretion, a mutant form lacking cytolytic activity was not able to cause secretion of IL-1β, a cytokine that aids the production of IL-17A (McNeela *et al*., 2010). From these studies it is impossible to interpret which aspect of PLY's specific activities is the most important for maintaining long term carriage, however with the recent finding that PLY may be localised to the cell wall and is accessible to the cell surface in the absence of cell lysis, there may be some as yet undefined function of PLY which contributes to carriage (Price and Camilli, 2009). More work with specific mutants that retain either haemolysis, or the complement activation properties would therefore, be informative.

Of all the isogenic mutants tested in carriage, the neuraminidase-A mutant was cleared from the nasopharynx in the shortest time frame, at some point 3>/<7 days after inoculation. The sharp drop in nasopharyngeal CFU's observed in both neuraminidase mutants at 24 hours post infection was also the most pronounced of any pneumococcal strain tested. As the *in vitro* growth of this mutant was equivalent to the parental D39 and its other isogenic mutants (data not shown) we can conclude that neuraminidase-A is vitally important in the initial attachment to nasal epithelium. NanA is one of three enzymes which cooperate to sequentially to remove sialic acid, galactose and N-acetylglucosamine respectively from host glycoproteins, which ultimately exposes mannose residues, providing access to previously inaccessible adherence factors and also yielding carbon nutrients in the process (King *et al*., 2006). Because these exoglycosidase enzymes must work consecutively (Burnaugh *et al*., 2008), by knocking out the first enzyme in the sequence, NanA, pneumococcal growth and adherence is severely decreased and survival is difficult in the nasopharynx. It has been suggested that two phases of pneumococcal colonisation may be needed, one to establish efficient initial colonisation (transparent colonies), and one that promotes invasion of nasopharyngeal tissue and leads to systemic disease (opaque colonies) (Briles *et al*., 2005). Transparent colonies of pneumococci also express higher levels of NanA which provides further evidence of the importance of NanA in the upper respiratory tract (King *et al*., 2004). The ability to switch between these two subtypes (opaque and transparent) is logical for the pneumococcus as it would allow a subpopulation which is loosely attached to the nasopharynx to serve as a permanent reservoir for the transmission from person to person, whilst also maintaining long term stable carriage within an individual. This hypothesis is supported by the simultaneous detection of two colony types during carriage by using a whole nasal homogenate (such as the method employed in this thesis) compared to a nasal wash or lavage, which only detects loosely attached pneumococci, although this has not been demonstrated for D39 (Briles *et al*., 2005). Like PLY, neuraminidase-A has also been shown to reduce the effect of complement dependent phagocytosis, by interfering with the deposition of the complement component C3 on the surface of pneumococci (Dalia *et al*., 2010). The neutrophil infiltration of nasal spaces observed at 24 hours in WT colonised mice is not responsible for the clearance of carriage (van Rossum *et al*., 2005), but in conjunction with increased complement deposition, due to the absence of NanA, may be more effective, and thus, may also be a contributing factor to the early disappearance of

NanA$^-$ in this model. In addition to binding host sialic acid, neuraminidases remove sialic acid from the surface of other nasopharyngeal flora. *Neisseria meningitidis* and *H. influenza* are able to coat their surface in sialic acid in an attempt to avoid complement activation, and thus the co-incubation with pneumococci could disadvantage these organisms (Shakhnovich *et al*., 2002). In our model there are a variety of non-pneumococcal species that can be isolated from the nasopharynx in conjunction with pneumococci (data not shown); however, contaminants are not normally visible as they do not grow on gentamicin supplemented plates (which were used for carriage experiments). In the future it would be interesting to assess the importance of neuraminidases during competition for the niche of the nasopharynx.

For the first time we have shown that a NanB$^-$ mutant persisted longer in the nasopharynx than NanA$^-$ during carriage, (although both mutants were cleared from the nasopharynx before the WT). This data concurs with evidence from previous invasive challenges with these mutants, where the main feature of the NanA mutant was the early reduction of nasopharyngeal CFU, whereas the NanB mutant was attenuated at both tracheal and lung infection (Manco *et al*., 2006). It was recently indentified that in addition to having different optimum pH (Nan A = pH 6.5, Nan B = pH 4.5) (Berry *et al*., 1996), neuraminidases cleave different residues of sialic acid with the products of NanA cleavage acting as a substrate for NanB, although recently it has been identified that NanB may partially substitute for the absence of NanA under certain conditions (Gut *et al*., 2008). This may explain why NanA$^-$ colonisation was shorter, and why NanB may not be important until later during carriage and its progression down the respiratory tract.

Although colonisation of a PspA$^-$ mutant was stable until 3 days after inoculation, it was cleared from the nasopharynx before 14 days, corroborating previous findings (Ogunniyi *et al*., 2007). Many of the actions of PspA may promote the survival of pneumococci against the actions if the host immune system, either by preventing complement activation via the classical pathway (Ren *et al*., 2004), or by blocking direct killing by apolactoferrin (Shaper *et al*., 2004). As well as preventing C1q binding to the surface of bacteria (and thus blocking the initiation the classical pathway of complement), PspA may also prevent amplification via the alternative pathway, through directly preventing C3 deposition on the surface of pneumococci (Li *et al*., 2007). A consequence of the binding of C1q, C3 and C4 (also increased in the absence

of PspA) is immune adherence of pneumococci to erythrocytes, and ultimately transfer of this complex to macrophages for phagocytosis, which is also enhanced in pneumococci which do not possess functional PspA (Li *et al*., 2007; Nelson, 1953). Binding of C3 to the pneumococcal surface is a determinant of whether nasopharyngeal carriage can subsequently cause experimental otitis media (EOM) in mice, as strains which bound the most complement were responsible for less EOM (Sabharwal *et al*., 2009).

Colonies of the non-encapsulated 110.58 persisted in the nasopharynx for two weeks (the limit of observation), and were at their highest density at 14 days after inoculation, in contrast to the both encapsulated WT encapsulated strains used. This is the first description of long term carriage in mice with non-encapsulated pneumococci. Previously, it has been shown that expressing reduced amounts of capsule is not disadvantageous during colonisation *in vivo* (Magee and Yother, 2001), and that having less capsule or indeed no capsule is often highly advantageous for *in vitro* attachment to epithelial cells (Adamou *et al*., 1998; Hammerschmidt *et al*., 2005). However, a complete absence of the polysaccharide capsule has repeatedly been proven to induce rapid clearance of these bacteria during *in vivo* mouse carriage (Magee and Yother, 2001; Roche *et al*., 2007; Nelson *et al*., 2007). This is most likely due to the effects of host complement components, as laboratory generated non-encapsulated mutants are highly susceptible to deposition and activation of complement on their surface, and subsequent complement dependent phagocytosis, compared to parental encapsulated strains (Hyams *et al*., 2010a). Naturally occurring non-encapsulated strains such as 110.58 represent almost 1% of all strains detected in surveillance, and are predominately, but not exclusively recovered during pneumococcal carriage (Hathaway *et al*., 2004). In place of capsular genes, 110.58 (like other non-encapsulated strains identified) has a sequence which shares homology to an *aliB* like open reading frame (*aliB* like ORF I and ORF II), that may have been acquired by horizontal transfer between *S. pneumoniae* and *S. mitis* or *Streptococcus gordonii* (Hathaway *et al*., 2004). *aliB* is a gene which forms parts of *ami-aliA/aliB* oligopeptide permease (which is also expressed natively elsewhere in the *S. pneumoniae* genome), and is important both for the uptake of amino acids, and sensing the nutritional environment as a stimulus to change gene expression (Claverys *et al*., 2000). This may be of particular importance in carriage where nutrition, the ability to respond to the nutritional environment, or a

failure to compete nutritionally with commensal colonising species may affect survival in the nasopharynx (Kerr *et al*., 2004). Mutants constructed in true *aliB* have performed less well in a model of nasopharyngeal carriage, however there was no difference between an *aliB* deficient strain and WT-D39 in the kinetics of an invasive pneumococcal challenge (Kerr *et al*., 2004). It is thought that the addition of the *aliB* like ORF's to the true *aliB* already expressed by the pneumococcus might provide an extra ability to scavenge substrates from the nutritionally poor surfaces of the nasopharynx (Lucy Hathaway, unpublished observations). However, quite how the 110.58 strain manages to survive in the nasopharynx at the expense of losing capsule is as yet unknown, as the capsule remains indispensible for invasive pneumococcal disease. It is interesting to note that capsular thickness even within the same strain effects the rate at which pneumococci uptake genetic material, the increased competence of transparent strains which inhabit the nasopharynx may explain how non-encapsulated strains such as 110.58 develop (Weiser and Kapoor, 1999). It has been shown that in the nasopharynx, the expression of almost all pneumococcal virulence genes are higher than in the lungs, blood or *in vitro* culture (Mahdi *et al*., 2008). Furthermore, transparent colonies found preferentially in carriage have different levels of gene expression *in vivo* to their opaque variants, as well as having different phenotypic properties such as the ability to bind complement (which is increased in the transparent phase) (Hyams *et al*., 2010b). A mixed population of phase variants could not be distinguished by this study, however the differences in gene expression between transparent and opaque colonies were less acute than the differences between pneumococci occupying different niches (Mahdi *et al*., 2008). Nonetheless it was necessary to identify whether there was any differences in the ability to cause invasive pneumococcal disease between colonies isolated on day 7 of carriage with D39, compared to standard passaged WT-D39. In this study there was no statistical difference in either CFU or end point survival between the two strains of D39. This result is perhaps not surprising, as there is increasing evidence that bacterial factors required for carriage have a similar ability to aid invasive disease (Mahdi *et al*., 2008; Hava and Camilli, 2002; Orihuela *et al*., 2004a). Therefore colonies taken directly from the nasopharynx have the ability to cause invasive disease as readily as establishing carriage, which may explain why invasive disease commonly develops soon after individuals acquire a new strain in the nasopharynx (Gray *et al*., 1980).

The final results in this chapter aimed to explain some of the differences in carriage by using an *in vitro* model of adherence and invasion of epithelial cell lines. It has been shown previously that pneumococci can adhere to and invade host endothelial cells in the lungs and endothelium blood by exploiting the platelet activating factor receptor (rPAF), which may be an important part of the pathogenesis of bacteria reaching the blood during invasive disease (Cundell *et al*., 1995a). Pneumococci have also been shown to translocate through epithelial cells in the nasopharynx via binding of CbpA/PspC to the polymeric immunoglobulin receptor (pIgR), a process they may exploit to enter cells and avoid detection by aspects of host immunity (Zhang *et al*., 2000). The level of *in vitro* adherence to nasopharyngeal and epithelial cells lines in experiments here were generally low (Fig 3.14), but do not differ from what has been observed previously (Hammerschmidt *et al*., 2005). Reduced adherence *in vitro* is a feature of pneumococci which have large polysaccharide capsules as, although at least 6% of capsule was previously thought to be indispensible to carriage *in vivo* (Magee and Yother, 2001), non-encapsulated pneumococci adhere better *in vitro* (Talbot *et al*., 1996). Pneumococci which have a transparent colony phenotype (which have thinner capsules and are associated with nasopharyngeal carriage) (Kim and Weiser, 1998), also consistently adhere better than colonies with an opaque phenotype (associated with invasive disease) within the same strain (Cundell *et al*., 1995b). Studies on gene expression during pneumococcal pathogenesis have shown the capsule genes are upregulated *in vivo* (Ogunniyi *et al*., 2002), and bacteria recovered from the blood (such as the case during animal passage of pneumococci), often have thicker capsules, and thus it was expected that there could be a difference between animal passaged and non-passaged strains in this assay (Mahdi *et al*., 2008; Ogunniyi *et al*., 2002). The lack of difference between passaged and non-passaged pneumococci in the adherence or invasion assay may be explained by the speed at which pneumococci can change gene expression of capsule in response to environmental conditions (Hammerschmidt *et al*., 2005), however the thickness of capsule did vary between these strains, as measured by electron microscopy (Fig 3.15, A, B). The ability of pneumococci to invade epithelial cells has also been demonstrated in some serotypes but not others (Talbot *et al*., 1996), and this may explain why there was no invasion observed in serotype-2 pneumococci, but serotype-3 and 23F pneumococci were able to invade epithelial cells (Fig 3.14, C, D). The genetic background of the strain has also been implicated to have a role in adherence and the ability to cause infection in mouse models irrespective of capsular

type (Kadioglu *et al*., 2002). A number of factors other than capsule have been implicated to have a clear role in adherence, such as the expression of pneumococcal surface adhesion A (PsaA), which may bind to glycoconjugate receptors on epithelial cells (Romero-Steiner *et al*., 2003), and factor H, which helps may help the interaction of PspC and the polymeric Immunoglubulin receptor (PIgR) (Quin *et al*., 2007). Neuraminidases either from pneumococcus or viruses can have an indirect effect on adherence through the removal of sialic acid residues, in order to yield more adhesive moieties on the epithelial surface (McCullers and Bartmess, 2003). Slight differences in adherence were also detected in PLY deficient pneumococci, although the differences between sufficient and deficient strains were less than between different serotypes (Rubins *et al*., 1998). As the *in vitro* adherence of D39 is generally poor compared to other strains it is unlikely that the expression of PLY, neuraminidases or capsule would have had a noticeable effect in this strain. Pneumococcal adherence *in vivo* is a two stage process, with the first stage; the primary adherence acting as a stimulus for host activation and upregulation of the factors which enhance subsequent adherence. The actions of cytokines TNF-α, and IL- 1α have been shown to enhance subsequent adhesion to epithelium in a dose dependent response (Cundell *et al*., 1995a). TNF-α and IL-1α can be released by dendritic cells in response to stimulation by sublytic concentrations of PLY, the lack of immune response cells in the *in vitro* system may explain the differences in adherence/carriage between the animal experiments and *in vitro* data (McNeela *et al*., 2010).

3.12 Summary

All of the pneumococcal mutants tested in the mouse experiments were similarly attenuated at causing both invasive disease and long term carriage (with the exception of the mutant lacking the polysaccharide capsule) and thus all have an essential role to play in the nasopharynx, which cannot be substituted by other factors. The first virulence factor to have an effect on carriage is likely to be neuraminidase A, and later neuraminidase B, helping the sequential attachment and release of nutrients from the nasopharynx. PLY is needed afterwards to penetrate deeper into the tissue to provide a lasting colonisation, and PspA may be required to evade host immunity (Fig 3.16).

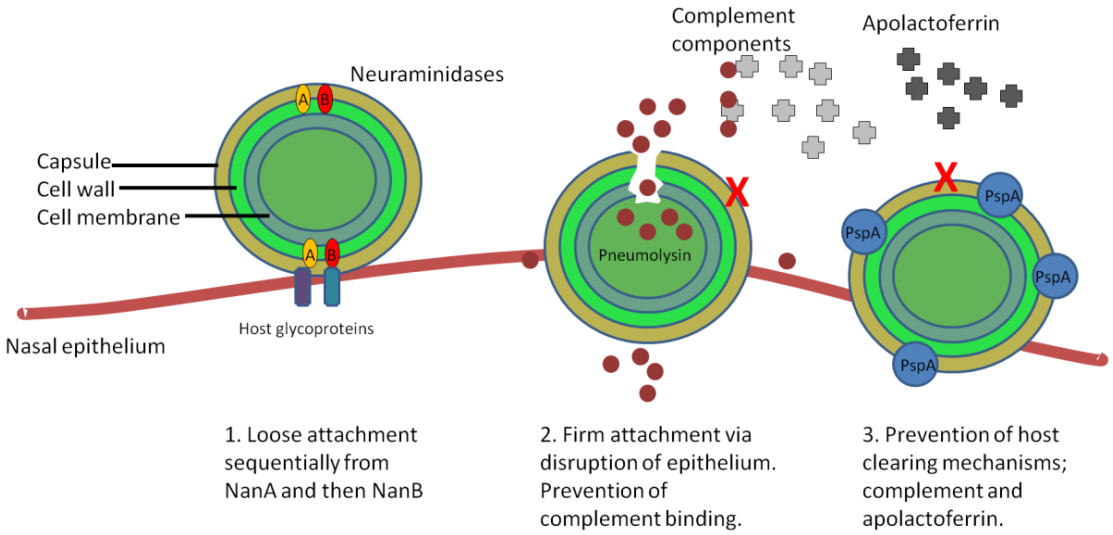

Figure 3.16 Schematic representation of the sequential impact of different virulence factors tested during carriage. First neuraminidase-A and later NanB promote the attachment and survival of pneumococci to the surfaces of the nasopharynx. PLY promotes invasion of the tissue, and thus permits a firmer adhesion to the nasopharynx. The action of PLY can also repel host complement binding to the pneumococcal surface. PspA acts later in carriage to prevent clearance by innate host immune mechanism, such as complement components and apolactoferrin.

The next chapter will show how some of these bacterial virulence factors become targets for the host immune response to carriage.

Chapter 4: Host immunity during nasopharyngeal carriage of *S. pneumoniae*

4.1 Preface

There has been a large amount of research undertaken into the roles of the innate and adaptive immune responses during invasive pneumococcal disease, however, by comparison, knowledge of the host immune response to nasopharyngeal carriage only, is limited and has focussed largely on the generation of anti-pneumococcal antibody (Goldblatt *et al*., 2005a). Adults produce strong IgG anti-capsular antibody responses to natural colonisation, which can be measured in serum by ELISA (Goldblatt *et al*., 2005b), and both adults and younger children (who have difficulties in developing anti-capsular antibodies) also generate serum IgG antibodies to conserved pneumococcal proteins such as PLY, PsaA and to a lesser extent PspA after episodes of natural nasopharyngeal colonisation (Rapola *et al*., 2000; Goldblatt *et al*., 2005b). In mice, anti-protein antibodies raised against PspA and PsaA, but not capsular antibodies can be routinely detected during carriage (McCool and Weiser, 2004; Trzcinski *et al*., 2005; Malley *et al*., 2005a). However, whilst levels of anti-protein antibodies were found to correlate to the density of colonisation in individual mice, it was shown that mice incapable of producing normal antibody responses did not necessarily clear colonisation at a slower rate (Trzcinski *et al*., 2005; McCool and Weiser, 2004). In contrast, CD4+ T-cells, along with the cytokine IL-17A were found to be essential for clearing carriage and in protecting mice from subsequent colonisation (van Rossum *et al*., 2005; Malley *et al*., 2005b). Despite these findings cellular immunity remains poorly described in murine nasopharyngeal carriage.

To this end it was sought to describe the humoral and cellular immune response to nasopharyngeal carriage during both WT and PLY negative pneumococcal carriage.

Results

4.2 Humoral immune response to long term WT nasopharyngeal carriage

In order to assess the systemic immune response to nasopharyngeal carriage, levels of serum antibody were assessed from mice during carriage with WT-D39. For capsule specific antibody, only IgM (and not IgG or IgA, data not shown) could be found at any time point above background level (Fig 4.1). Anti-capsular IgM was detected as early as 3 days post infection ($p=0.0088$), however levels of IgM appeared to reach the maximum titre at day 7 (0.9633 Log titre ± 0.1136 (S.E.M.), and did not significantly increase after day 7, remaining at around Log 1 until day 28 of carriage (the limit of observation) (Fig 4.1). Despite bacterial numbers during the carriage of WT-D39 being relatively stable, there was a strong inverse correlation between CFU and anti-capsular IgM titre, Pearson $r=0.4437$ ($p=0.0141$), so that where the titre of antibody was high the CFU in the nasopharynx was low (Fig 4.1, B). Anti-PspA antibodies but not PspC (data not shown), of subclass IgG were detected in the later stages of carriage (Fig 4.1), and in contrast to the anti-capsular antibodies observed, no IgM or IgA specific PspA antibodies could be found in serum during carriage (data not shown). Anti-PspA antibodies were first detected on day 14 post infection, and the first significant change above background levels was not observed until day 21 of the experiment ($p=0.0092$) (Fig 4.1, C). The highest titre of anti-PspA was detected at 28 days post infection ($p=0.0088$) (Fig 4.1, C). Although correlation was not significant in this instance ($p=0.0545$) there was a trend towards those mice with higher titres of anti-PspA antibodies having lower nasopharyngeal CFU at the corresponding time points.

The generation of anti-PspA antibodies in the serotype 3-(A66) carriage was not significantly different to the serotype-2 WT-D39 at any time point (Fig 4.2). However, in this instance a significant inverse correlation did exist between the nasopharyngeal CFU of A66 and anti-PspA specific IgG titre, Pearson $r=0.4983$ ($p=0.0495$) (Fig 4.2, B).

4.3 Humoral immune response to PLY negative colonisation

Antibody levels were assessed during the cleared colonisation of $PlnA^-$ in the same way as for WT-D39. Once again, only IgM anti-capsular and IgG anti-PspA antibodies could be detected at any time during the colonisation (Fig 4.3). Levels of anti-capsular

IgM were generally higher than was observed in the parental strain although only significantly at day 14 (p=0.0223), which also coincided with the clearance of bacteria from the nasopharynx in the mutant (Fig 4.3, A). Additionally, there was an even stronger inverse correlation between CFU and anti-capsular antibody titre than existed in the WT carriage, Pearson r=0.5121 (p=0.0017) (Fig 4.3, B). IgG anti-PspA antibodies were also detected earlier in PlnA$^-$ carriage than during the WT-D39 carriage, at day 7 (p=0.0232), but levels did not differ significantly at any other time points tested (Fig 4.3, C). In contrast to the WT (Fig 4.1, B), significant inverse correlation existed between nasopharyngeal CFU and systemic anti-PspA antibody titre (Fig 4.3, D), Pearson r=0.4309 (p=0.0098).

4.4 Effects of capsule on immunity to pneumococcal carriage

Due to the unexpected finding that the non-encapsulated strain 110.58 could be carried 'long term' in the nasopharynx, the humoral immune response during this carriage was also examined. During the first 14 days, it was not possible detect specific anti-PspA antibodies significantly above background levels by ELISA (data not shown), and therefore the ability of total IgG and IgM antibody from immune serum of 110.58 colonised mice to bind to the whole pneumococci was determined by flow cytometry. There was no significant difference in the binding properties of IgG antibody from day 1 of carriage with either WT-D39 or 110.58 (data not shown). However, the binding of both serum IgG and IgM antibodies from day 14 of the respective carriage experiments to D39 pneumococci was significantly stronger in the WT-carriage serum (p=0.0286). In contrast there was no significant change in either IgG or IgM antibody binding in the 110.58 mutant between day 1 and 14 of carriage (Fig 4.4). This lack of humoral response could explain why 110.58 persisted and was at its highest level at 14 days post infection.

4.5 Effects of PspA on immunity to pneumococcal carriage

Earlier I showed that it was possible to detect robust serum anti-PspA responses in mice that were subjected to both WT and PLY deficient carriage, which correlated to the density of bacteria in the nasopharynx. Next it was sought to identify if a mutant that lacked PspA (which does not cause long term carriage) was able to generate an equivalent amount of serum IgM anti-capsular antibody as those strains. Whilst it was not possible to find anti-PspA generated during 14 days of this colonisation (data not

shown), anti-capsular antibody in this strain was found at all days tested (Fig 4.5) and was not significantly different to that observed in either the parental WT-D39, or PLY negative PlnA$^-$ colonisation. Levels of anti-type-2 capsule antibodies in the PspA mutant correlated significantly to the decline and clearance of pneumococci in the nasopharynx (Fig 4.5, B), which taken with the results from the non-encapsulated strain indicate a protective role for anti-capsular antibody during primary carriage.

4.6 Is the antibody generated during carriage functionally active?

In order to establish whether the antibody generated during carriage was able to functionally bind to pneumococci *ex vivo*, serum taken from mice during colonisation or carriage with both WT and PlnA$^-$ pneumococci were incubated with D39 and the total bound IgM and IgG quantified using flow cytometry. It is firstly interesting to note that at time zero (naive mice), the percentage of pneumococci which had bound IgM to the surface was significantly higher than the isotype control, indicating that there is pre-existing IgM natural antibody which is able to bind to the surface of *S. pneumoniae* (Fig 4.6). In contrast, there appeared to be no pre-existing IgG antibody. There were no significant differences in antibody development between PLY negative and WT colonisation, for either IgM or IgG, which in general followed the pattern shown for specific antibodies by the ELISA method, with a rapid but modest increase in IgM antibody and a slower emergence of IgG (Fig 4.6, 4.7, 4.8). However the level of IgM in the mutant was higher at both day 7 and 14 ($p=0.0079$) compared to the WT-D39 (Fig 4.6), which concurs with the result for specific anti-capsular IgM determined by ELISA.

4.7 Local immunity to nasopharyngeal carriage

To measure the local immunity generated to *S. pneumoniae*, cervical lymph nodes (CLN) which drain the region of the nasopharynx were dissected and analysed from mice during carriage. The total numbers of IgA secreting B-lymphocytes in the CLN were quantified from mice during carriage with both WT-D39 and its isogenic mutant PlnA$^-$ (Fig 4.9). IgA positive spots were first detected above background levels in both strains at day 3, and peaked significantly at day 7 ($p=0.0097$) (Fig 4.9, A). IgA was also above background at day 14 ($p=0.0449$) although in reduced amounts that were significantly lower than the peak observed after 7 days ($p=0.0317$). The amount of IgA secreting B-cells was generally low after day 7 and were indistinguishable from the

background level by day 28 (Fig 4.9, A). The same samples were simultaneously assessed for capsule specific IgA, and the results indicated that the majority of total IgA was raised against the capsular polysaccharide of D39, as the peak of serotype-2 capsule specific IgA (not significant) coincided with the total IgA peak (4.9, B).

4.8 Cellular immunity during pneumococcal carriage

The cellular composition of the cervical lymph nodes was also assessed in both WT and attenuated PlnA$^-$ colonisation by flow cytometry. As CD4+ T-cells had been shown to be essential to the clearance of long term carriage, the levels of lymphocytes were predominantly measured (van Rossum *et al*., 2005). The relative amounts of CD4+ T-cells (which accounted for approximately 40% of lymphocytes), CD8+ T-cells (20% of lymphocytes) and CD19+ B-lymphocytes (40% of lymphocytes) did not deviate significantly from pre-inoculated levels throughout the duration of carriage, in either the WT, or PLY deficient mutant (Fig 4.10). The levels of naive CD4+ or CD8+ lymphocytes (CD44^{lo}, CD62L^{hi}) *vs* memory CD4+ or CD8+ lymphocytes (CD44^{hi}. CD62L^{lo}) were also stable throughout carriage (data not shown). In WT-D39 carriage however, the percentage of the cells in the lymph node which fell into the lymphocyte gate fell at 24 hours after infection, from a mean of 81.07% ± 0.7480, to just 68.32% ± 3.640 ($p=0.0142$) (Fig 4.10, D). By day 3, levels had return to pre-colonisation levels, after which time the non-lymphocyte population was maintained at time zero equivalent levels for the duration of carriage (data not shown). It is interesting to note that this temporal granulocyte infiltration to the draining lymph nodes is only evident in the WT-D39 carriage and not in PlnA$^-$ cleared colonisation (Fig 4.10, D). This evidence was supported by differential counting of CLN cytospin preparations made during carriage which were stained with Giemsa (data not shown).

4.9 Confocal microscopy of mouse tissues during carriage with WT-D39

Following on from the flow cytometry of lymph node samples, confocal microscopy was performed on cervical lymph nodes removed throughout long term carriage with WT-D39 pneumococci. On all days analysed the presence of distinct B-cell areas (indicated by expression of B220), and T-cell areas (indicated by the expression of CD3) could be observed (Fig 4.11). T-cell areas are typically central in the node and can be distinguished by the high expression of MHCII from dendritic cells in these areas, whereas B-cell areas are generally peripheral and low in MHCII expression (Fig

4.11). Throughout carriage these areas remained separate and there was no obvious movement of T-cells into B-cell areas, which is indicative of the formation of a germinal centre. Concurrent with the flow analysis, the relative numbers of T and B lymphocytes did not appear to vary throughout carriage (Fig 4.11). To assess the population of granulocytes in the cervical lymph nodes, sections were stained with antigen 7/4, which is present on neutrophils and Gr-1 (present on both neutrophils and monocytes) (Fig 4.12). The number of neutrophils were low in all days tested and did not appear to increase in number significantly at any day tested, which supported flow analysis of these samples.

It was recently identified that macrophages within the luminal spaces of the nasopharynx may also contribute to the clearance of primary pneumococcal carriage (Zhang *et al*., 2009). Macrophage populations in the cervical lymph nodes were stained with markers for sialoadhesin (CD169), CD11c and CD68 (Fig 4.13). CD169 which along with CD68, is normally phenotypically indicative of subcapsular sinus macrophages (SCS) is localised only at the periphery of the node, and can be seen partially surrounding B-cell rich areas (Fig 4.13). Although there is CD68 staining distributed throughout the node, interestingly, some of the CD68 (a macrophage marker) is associated with both CD11c and CD169 which may indicate a proportion of these are similar to the macrophage population which is described below in nasal mucosal tissue taken from the same mice (Fig 4.16). Dendritic cells, which highly express MHCII and CD11c, were evenly distributed throughout the tissue at all time points measured, with the highest intensity of staining seen on day 7 and 14 of carriage (Fig 4.14). In contrast, the proportion of CD68+ CD11c+ and CD68+ expressing macrophages was lowest in naive mice and increased to maximal levels on day 3 of carriage, but was strongly present for the remaining days of carriage (Fig 4.14).

A further observation of the confocal microscopy was an apparent accumulation of mannose receptor (CD206) at the sites where CD169 expressing macrophages were present (Fig 4.15, top panel). Mannose receptor was not present during the early stages of carriage (data not shown) but is found strongly expressed at day 7 and day 14. Furthermore, it appears that neutrophils/monocytes which are evenly distributed throughout the tissue at earlier time points (Fig 4.12), accumulate in the same areas as mannose receptor binding at day 7 and 14 (Fig 4.15 lower panel).

4.10 Contribution of nasal mucosa to carriage

Nasal mucosal tissue dissected from the same mice as for CLN confocal, during carriage with WT-D39, was sectioned and stained for the presence of macrophages, dendritic cells, and T and B lymphocytes. Interestingly, there was no evidence of T or B-cells present in the tissue throughout the duration of carriage, and few dendritic cells, which correlated to flow cytometry of this tissue (data not shown). The most striking observation was the presence of a macrophage population defined by the expression of CD11c, CD169, CD68 and MHCII, which appears to be resident in naive mucosa, and is upregulated during carriage (Fig 4.16). This population was confirmed as macrophages by the expression of the common macrophage marker F4/80, but lacked SIGNR1 (a type II transmembrane protein which normally defines the macrophage population in the spleen and lymph nodes) (data not shown). This population of macrophages which has not been previously described in mucosal tissue, is, by virtue of its surface marker expression is most phenotypically similar to alveolar macrophages. As a measure of an immune response to pneumococcal carriage, the nasal mucosa was stained with MECA 32, a marker of endothelium which is upregulated during inflammation and cellular infiltration (Engelhardt *et al*., 1994; Leppink *et al*., 1989). MECA 32 expression appears to define the edges of high endothelial venules (HEV's), the numbers of which are increased at each time point measured for the duration of carriage, indicating neo-angiogenesis (Fig 4.17). MECA32 staining is localised with the expression of peanut agglutinin (PNAg), a marker which normally indicative of germinal centre formation (Fig 4.17).

4.11 *In vitro* stimulation of chemotaxis of CD4+ T-cells

Because of the consistent finding that CD4+ T-cells in mice are necessary for the protection against primary pneumococcal carriage (van Rossum *et al*., 2005), and against subsequent pneumococcal challenges (Malley *et al*., 2005b), the pneumococcal strains used in animal infections were assessed for their ability to recruit CD4+ T-cells. Both WT-D39 (63.18% ± 7.12, p=0.0003) and A66 (49.5% ± 5.98, p=0.0016) which had been animal passaged, caused considerably more migration of human CD4+ T-cells towards whole bacteria than the negative control (which contained no bacteria) in a Transwell transmigration assay (Fig 4.18). However, non-passaged WT-D39 which were otherwise identical, induced significantly less migration of CD4+ T-cells than the

passaged form (p=0.0004). To determine whether the migration process was unique to virulent strains only, the PLY deficient mutant (which was passaged), was tested in the same model. PlnA$^-$ (p=0.0003) caused significantly less migration than the passaged WT-D39, at levels that were equivalent to the negative control (Fig 4.18).

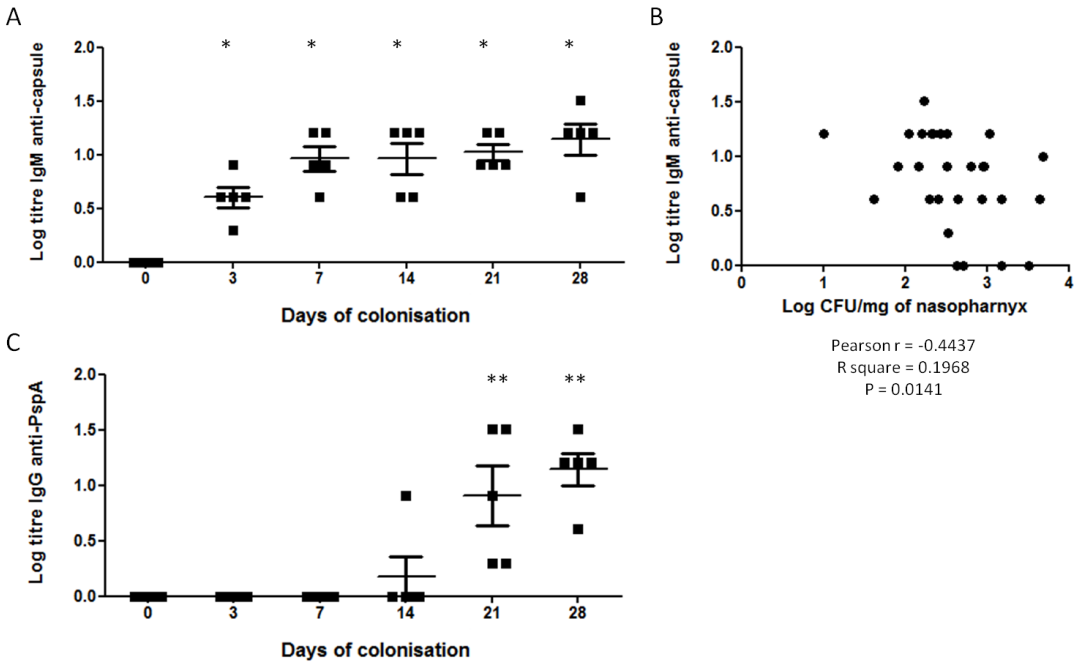

Figure 4.1 Serum antibody levels during carriage of WT-D39. Concentrations of (A) IgM anti-capsular and (C) IgG anti-PspA antibodies were assessed by ELISA. Anti-capsular antibody as a function of nasopharyngeal CFU is represented in (B). Each square represents the analysis of one mouse, n= 5 for all time points and Log titre data represents the average of duplicate wells analysed on two separate days. Log of the endpoint titres ± S.E.M. were calculated as the last dilution to reach 0.1 absorbance at 405 *nm*. Statistical significance of changes in antibody compared to day 0 were assessed using the Mann-Whitney test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001). Pearson correlation (which assumes Gaussian distribution) and statistical significance was calculated using Graphpad prism software.

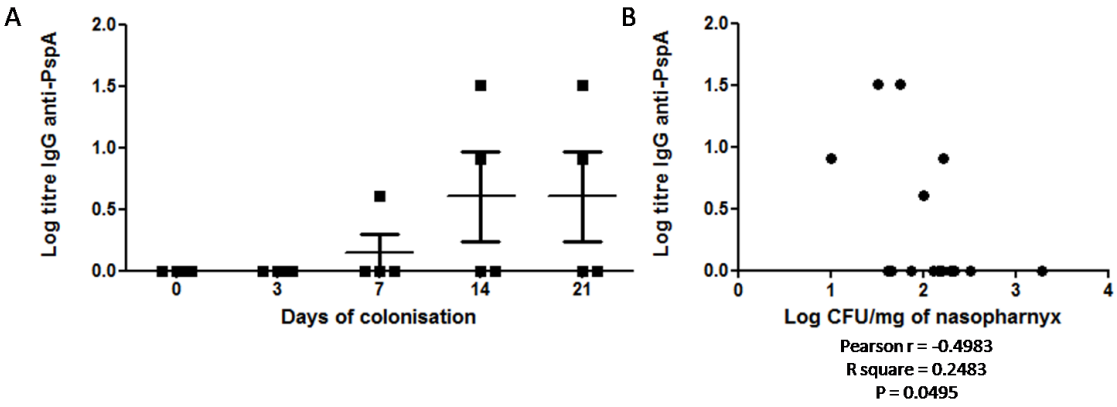

Figure 4.2 Serum antibody levels during colonisation with WT-A66. Concentrations of (A) IgG anti-PspA antibodies were assessed by ELISA. Anti-PspA titre as a function of nasopharyngeal CFU is also shown (B). Each square represents the analysis of one mouse, n= 5 for all time points and Log titre data represents the average of duplicate wells analysed on two separate days. Log of the endpoint titres ± S.E.M. were calculated as the last dilution to reach 0.1 absorbance at 405 *nm*. Statistical significance of changes in antibody compared to day 0 were assessed using the Mann-Whitney test. Pearson correlation (which assumes Gaussian distribution) and statistical significance was calculated using Graphpad prism software.

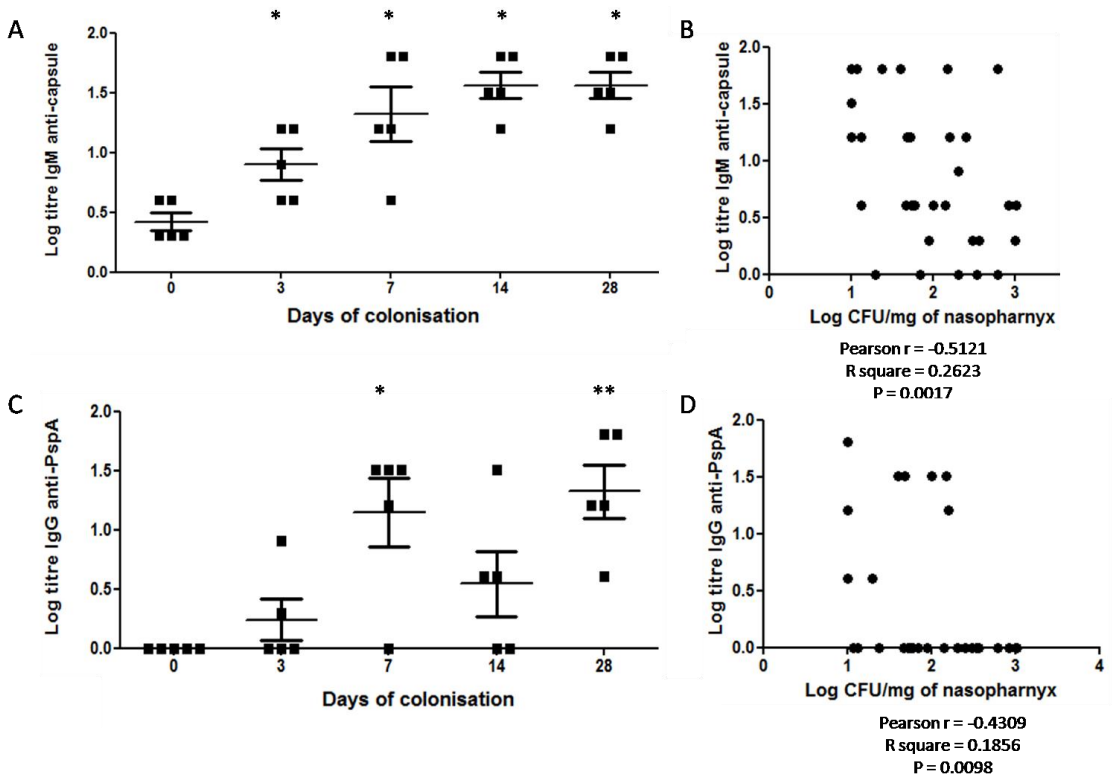

Figure 4.3 Serum antibody levels during colonisation with PlnA$^-$. Concentrations of (A) IgM anti-capsular and (C) IgG anti-PspA antibodies were assessed by ELISA. Anti-capsular antibody as a function of nasopharyngeal CFU is represented in (B), and anti-PspA titre as a function of nasopharyngeal CFU is also shown (D). Each square represents the analysis of one mouse, n= 5 for all time points and Log titre data represents the average of duplicate wells analysed on two separate days. Log of the endpoint titres ± S.E.M. were calculated as the last dilution to reach 0.1 absorbance at 405 *nm*. Statistical significance of changes in antibody compared to day 0 were assessed using the Mann-Whitney test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001). Pearson correlation (which assumes Gaussian distribution) and statistical significance was calculated using Graphpad prism software.

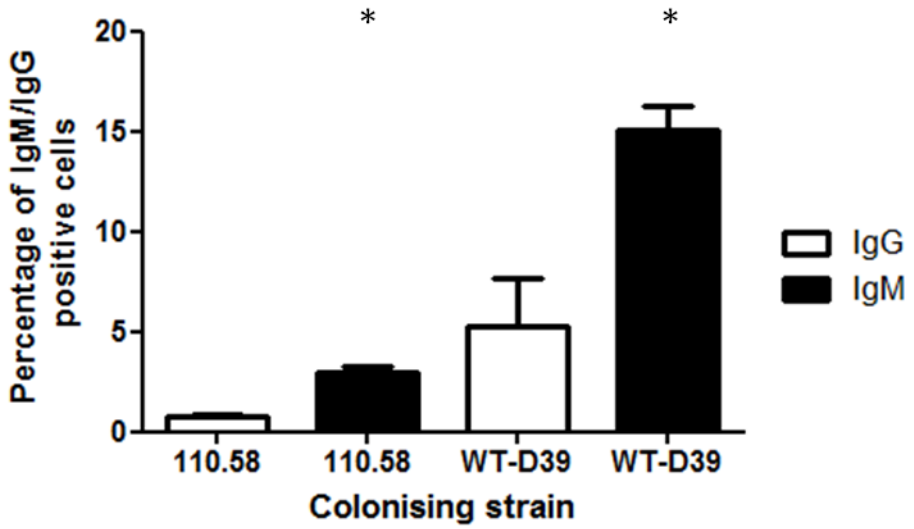

Figure 4.4 Comparison of humoral immune to WTD39 and 110.58 carriage. The binding of serum antibodies, IgG (open bars) and IgM (filled bars), to the surface of D39 pneumococci was compared in day 14 serum from the carriage of non-encapsulated 110.58 and WT-D39. 100 µl of pneumococci at 0.1 OD 600 *nm*, was incubated overnight with 10 µl of mouse immune serum, and stained the next day with fluorescently conjugated anti-IgG, or anti-IgM. 100,000 gated events of intact pneumococci were acquired on n=5 samples for each strain, with the percentage of cells expressing fluorescence above the level of isotype control recorded. Statistical significance of the comparison of between strains were assessed using the Mann-Whitney test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001).

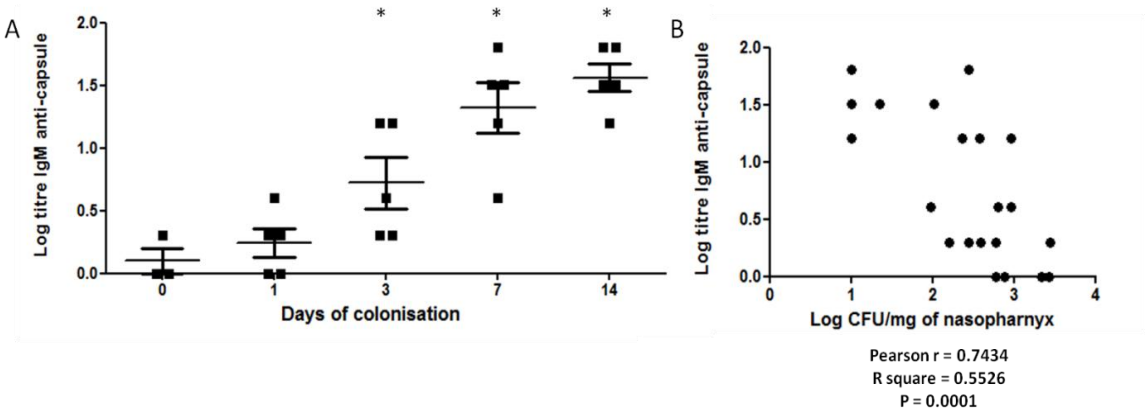

Figure 4.5 Serum antibody levels during colonisation with a mutant lacking PspA. Concentrations of (A) IgM anti-capsular were assessed by ELISA. Anti-capsular antibody as a function of nasopharyngeal CFU is represented also (B). Each square represents the analysis of one mouse, n=5 for all time points and Log titre data represents the average of duplicate wells analysed on two separate days. Log of the endpoint titres ± S.E.M. were calculated as the last dilution to reach 0.1 absorbance at 405 *nm*. Statistical significance of changes in antibody compared to day 0 were assessed using the Mann-Whitney test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001). Pearson correlation (which assumes Gaussian distribution) and statistical significance was calculated using Graphpad prism software.

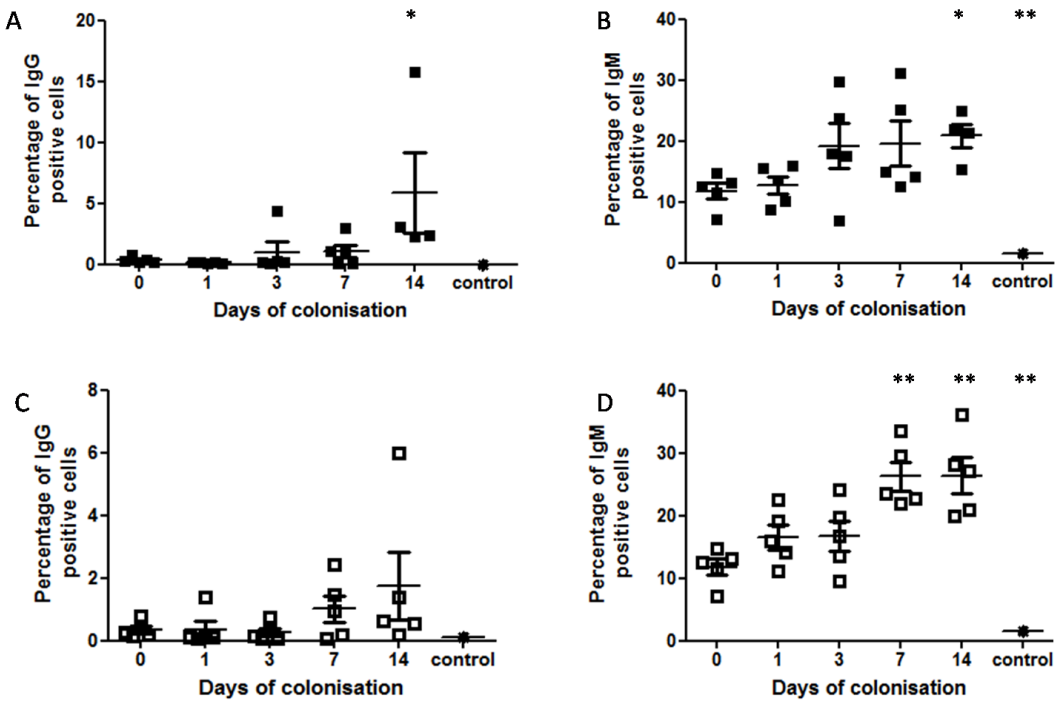

Figure 4.6 Total serum antibody during carriage. MF1 mice were colonised with WT-D39 (closed squares), or PlnA$^-$ (open squares), and serum analysed for the ability of IgG (A, C) and IgM (B, D) to bind to the surface WT-D39, by flow cytometry. Briefly, 100 µl of pneumococci at 0.1 OD 600 *nm*, was incubated overnight with 10 µl of mouse immune serum, and stained the next day with fluorescently conjugated anti-IgG, or anti-IgM. 100,000 gated events of intact pneumococci were acquired per sample, with the percentage of cells expressing fluorescence above the level of isotype control recorded. Each square represents one mouse and n=5 for each time point and each strain. Statistical significance of changes in antibody binding compared to day 0 were assessed using the Mann-Whitney test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001).

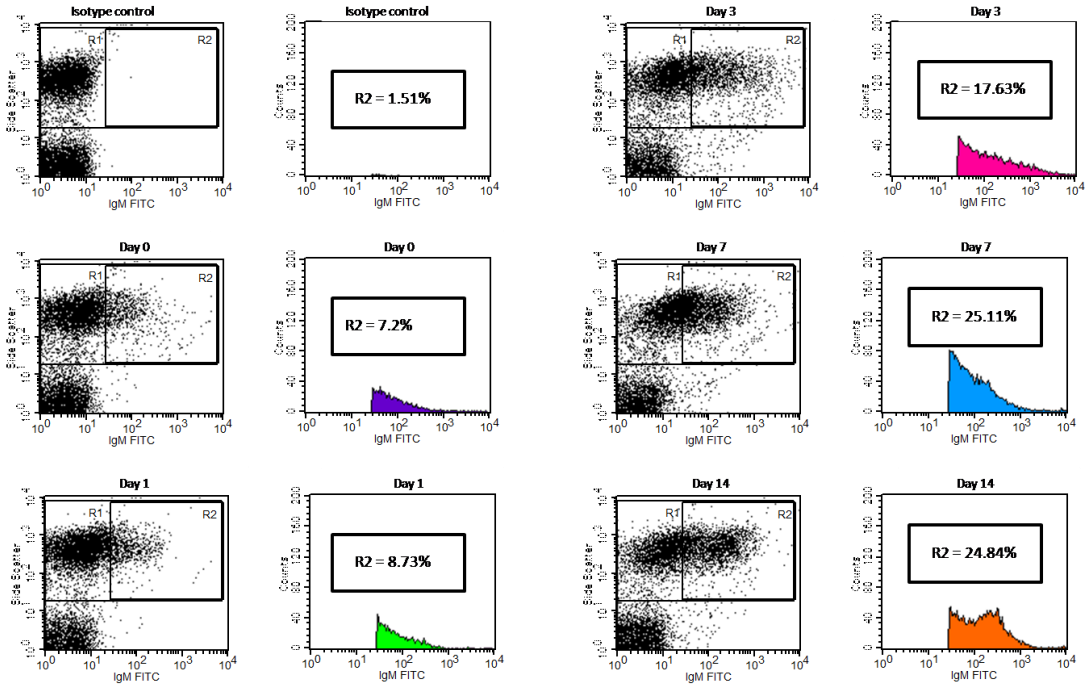

Figure 4.7 Total serum antibody during carriage. Representative data of total IgM bound to the surface of WT-D39 pneumococci from serum taken from mice during different days of WT carriage. 100,000 gated events of intact pneumococci were acquired per sample, with the percentage of cells expressing fluorescence above the level of isotype control recorded.

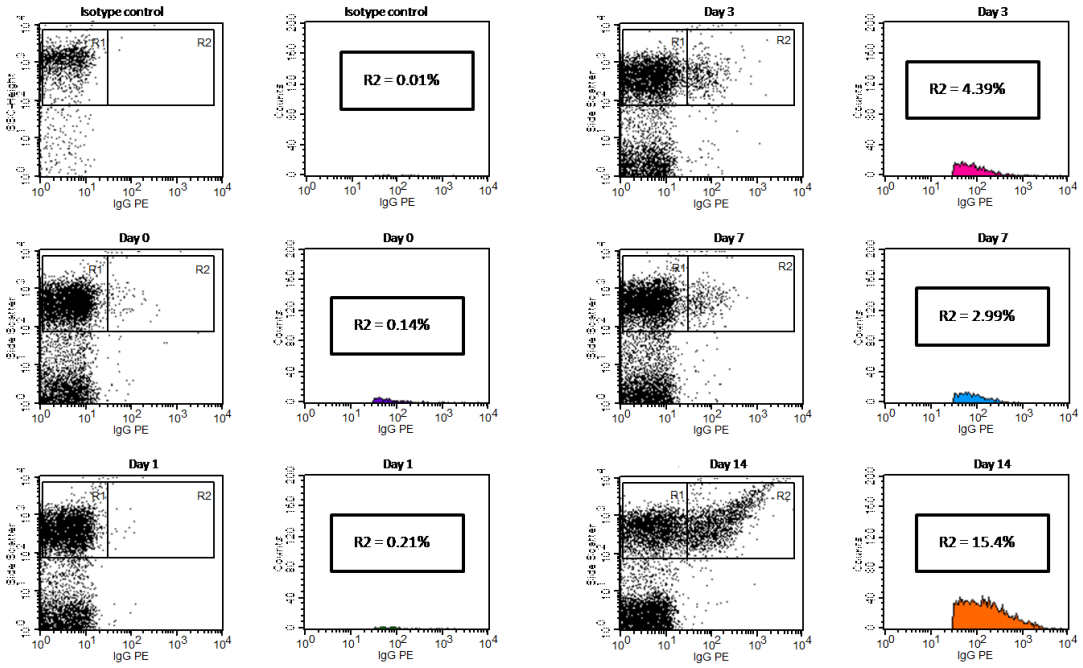

Figure 4.8 Total serum antibody during carriage. Representative data of total IgG bound to the surface of WT-D39 pneumococci from serum taken from mice during different days of WT carriage. 100,000 gated events of intact pneumococci were acquired per sample, with the percentage of cells expressing fluorescence above the level of isotype control recorded.

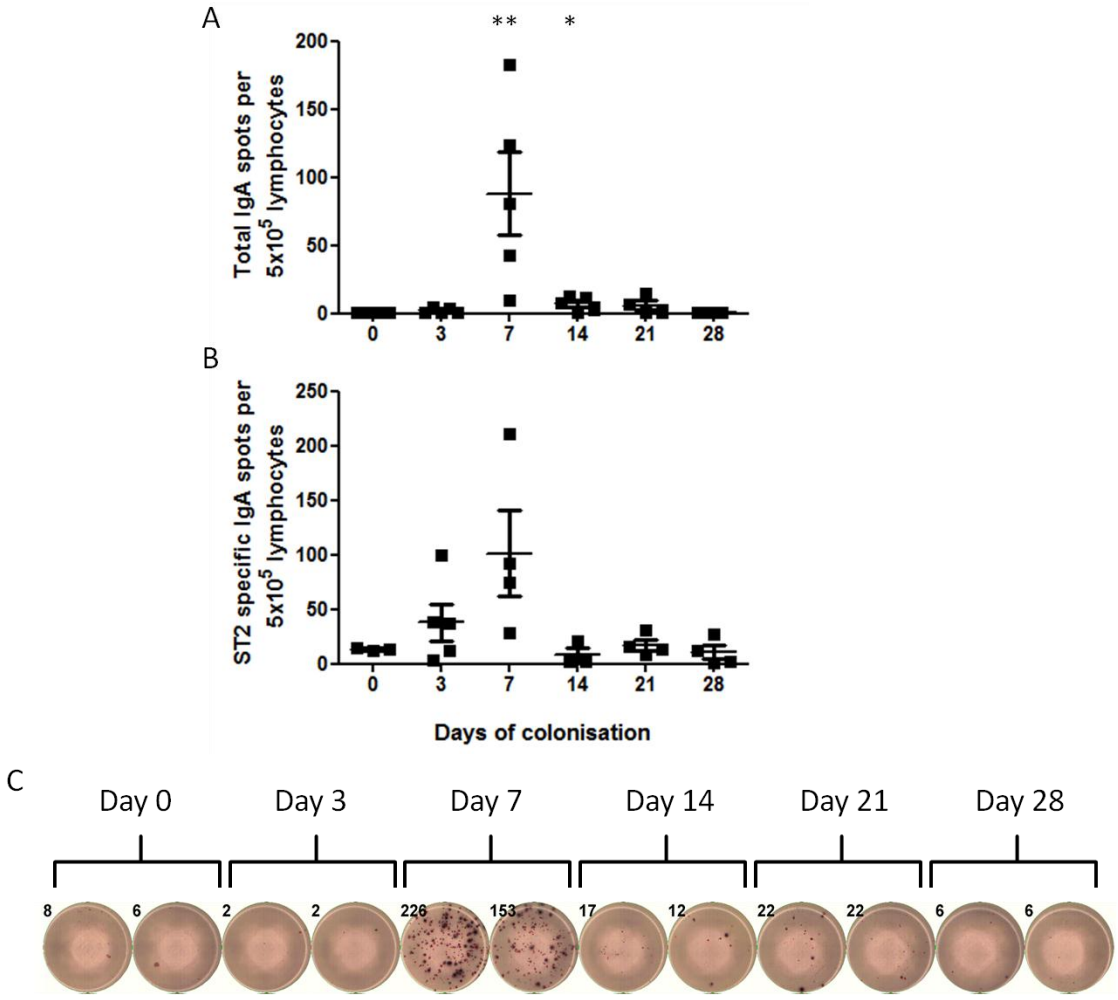

Figure 4.9 Local immunity to nasopharyngeal carriage. *Ex-vivo* ELISPOT's were performed on mixed cell suspensions from CLN samples taken at different times during carriage with WT-D39 and PlnA$^-$ (data pooled). Total IgA (A) and serotype-2 capsular specific IgA (B) were determined in 5 x10^5 cells, in duplicate wells. Values represent the mean spots per well in duplicate wells ± SD, of n=5 mice. The background value of IgA secondary antibody alone, and serum alone were subtracted from the number of positive spots in each well. Representative data is shown (C). Statistical significance of spots on each day was compared to naive mice (Day zero) and were calculated using the Mann-Whitney test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001).

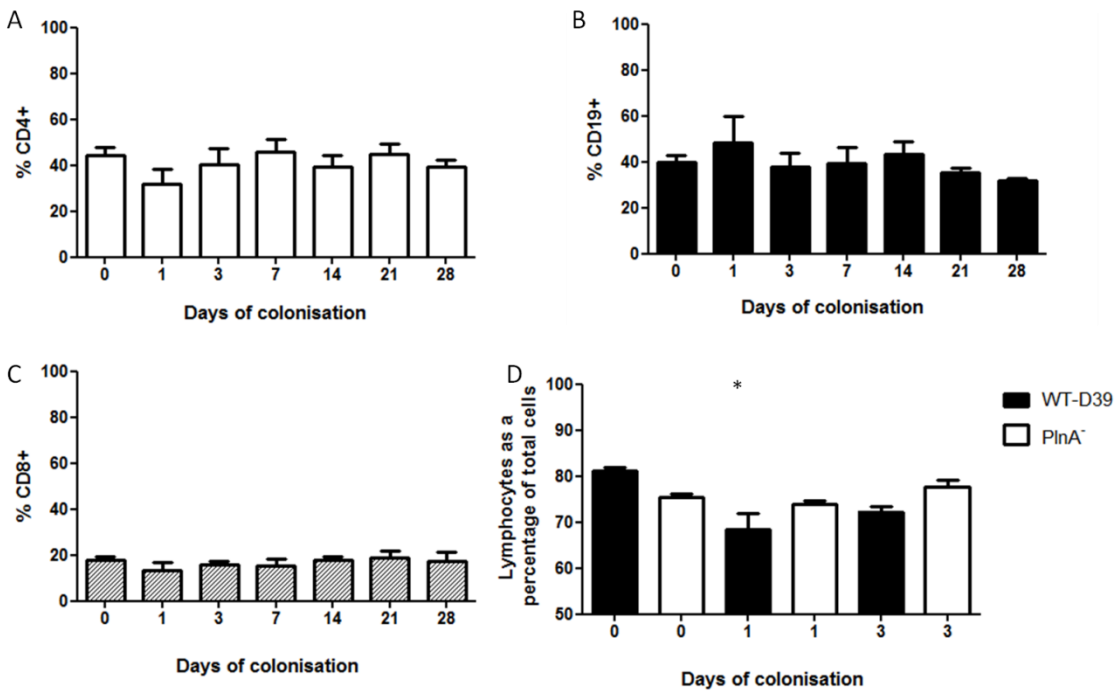

Figure 4.10 Cellular composition of CLN during WT-D39 and PlnA⁻. Whole cervical lymph nodes suspensions were stained individually with anti-CD4+ (A), anti-CD8+ (B) and anti-CD19+ (C). 10,000 'live' events inside a lymphocyte gate were acquired per sample and the values expressed are the mean percentages of the cells in the gate ± SD for 8 mice per time point (4 WT and 4 PlnA⁻). The percentage of lymphocyte gated cells out of total cells analysed is shown for day 0, 1, and 3, of carriage only, in both WT-D39 and PlnA⁻ carriage. Statistical differences between day 0 and subsequent days of carriage within strains was calculated using a students t test, *(p=<0.05).

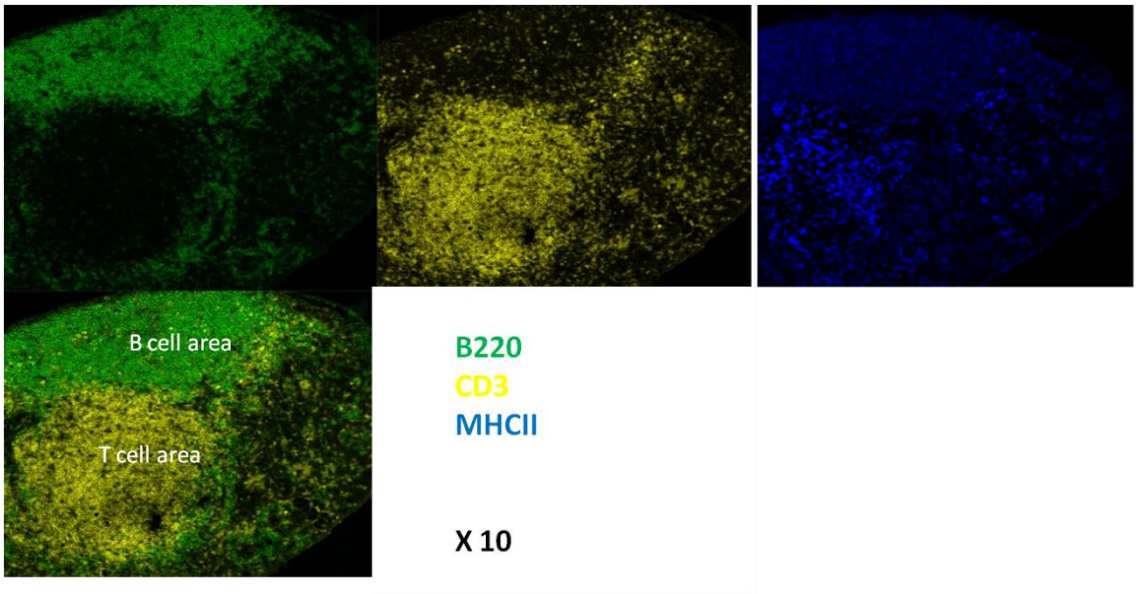

Figure 4.11 Confocal microscopy of B and T lymphocytes in cervical lymph nodes during carriage. 2 CLN samples from different MF1 mice on day 0, 1, 3, 7 and 14 of carriage with WT-D39 were sectioned and stained with the surface markers B220 (B-cells) (Green) CD3 (T-cells) (yellow) and MHCII (blue). The lower image is a composite image of B220 and CD3 only. All fields shown are from day 3 of carriage but were representative of all days analysed (data not quantitative). Pictures were taken with objective lens x 10.

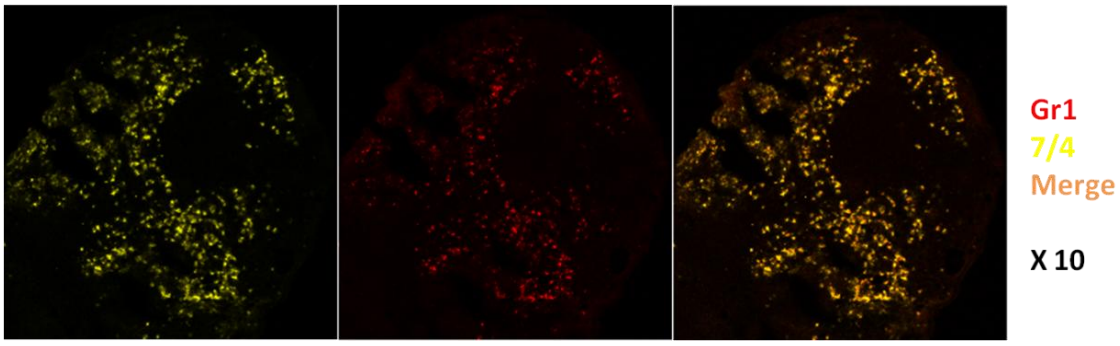

Figure 4.12 Confocal microscopy of granulocytes in cervical lymph nodes during carriage. 2 CLN samples from different MF1 mice on day 0, 1, 3, 7 and 14 of carriage with WT-D39 were sectioned and stained with the surface markers antigen 7/4 (yellow), GR-1 (red) and the image furthest to the right is a composite of GR-1 and antigen 7/4. All fields shown are from day 3 of carriage but were representative of all days analysed (data not quantitative). Pictures were taken with objective lens x 10.

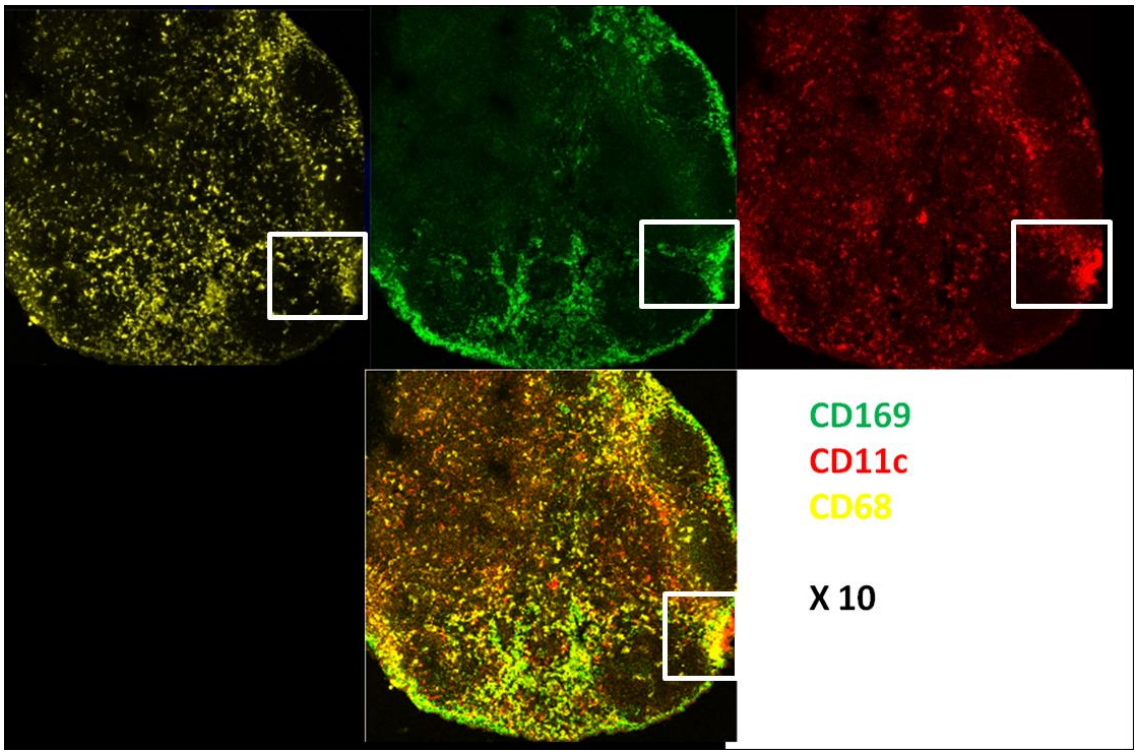

Figure 4.13 Confocal microscopy of macrophages in cervical lymph nodes during carriage. 2 CLN samples from different MF1 mice on day 0, 1, 3, 7 and 14 of carriage with WT-D39 were sectioned and stained with the surface markers CD68 (yellow), CD169 (green) and CD11c (red). The lower image is a composite of all three markers. Co-localisation of CD68, CD169 and CD11c, represents a population of macrophages similar in phenotype to alveolar macrophages and is indicated by the co-expression of these markers inside the white boxes. Analysis is not quantitative. Shown are lymph nodes from day 3 of carriage. Pictures were taken with objective lens x 10.

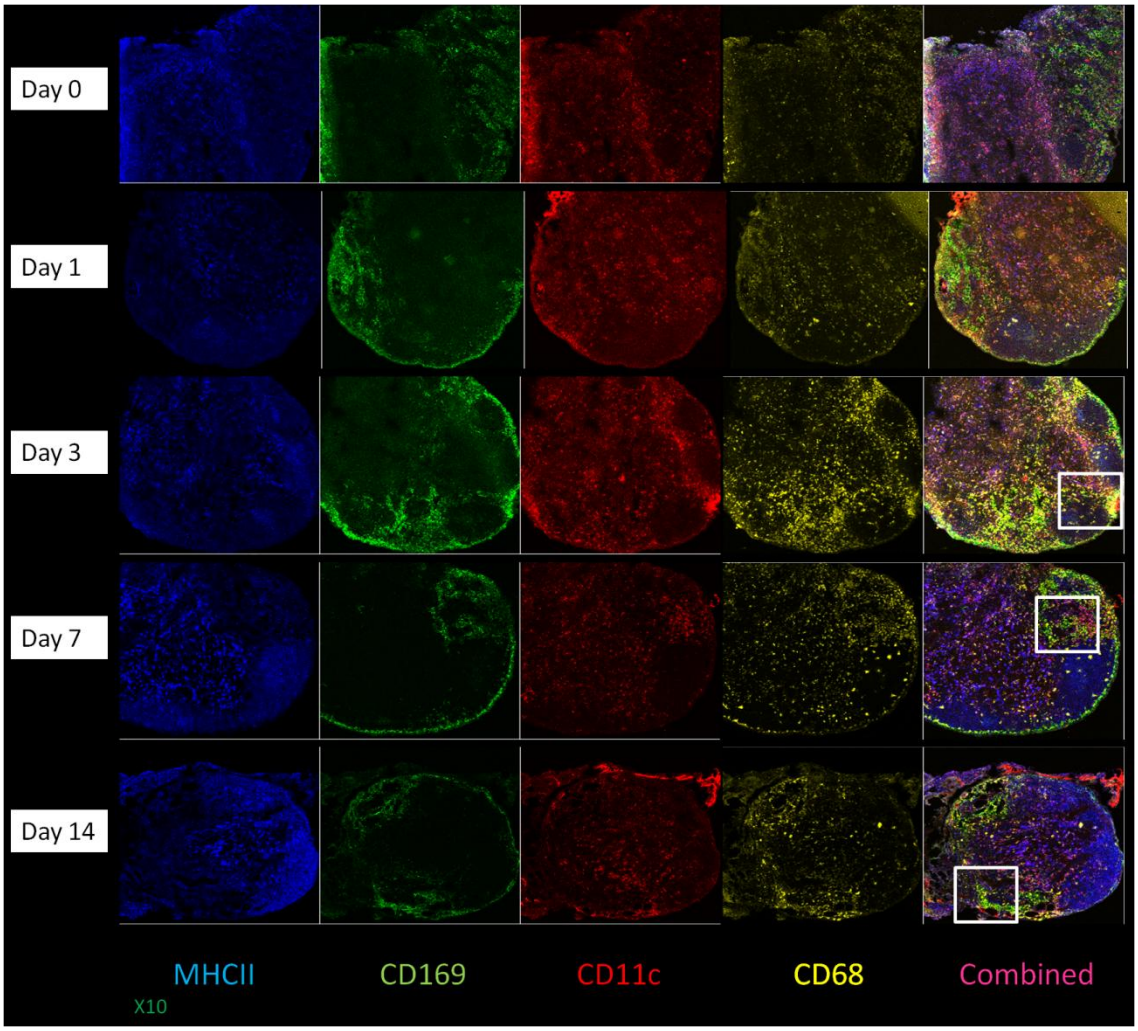

Figure 4.14 Confocal microscopy of macrophages and dendritic cells in cervical lymph nodes during carriage. 2 CLN samples from different MF1 mice on day 0, 1, 3, 7 and 14 of carriage with WT-D39 were sectioned and stained with the surface markers CD68 (yellow), CD169 (green) and CD11c (red) and MHCII (blue), and a composite image. CD11c+ CD68+ CD169+ macrophages are present in higher amounts during carriage than at day 0, as shown by the co-expression of those markers indicated in the white boxes (analysis not quantitative). Dendritic cells (CD11c+ $MHCII^{hi}$) also increase in numbers throughout carriage. Images shown are representative of all nodes for those days. Pictures were taken with objective lens x 10.

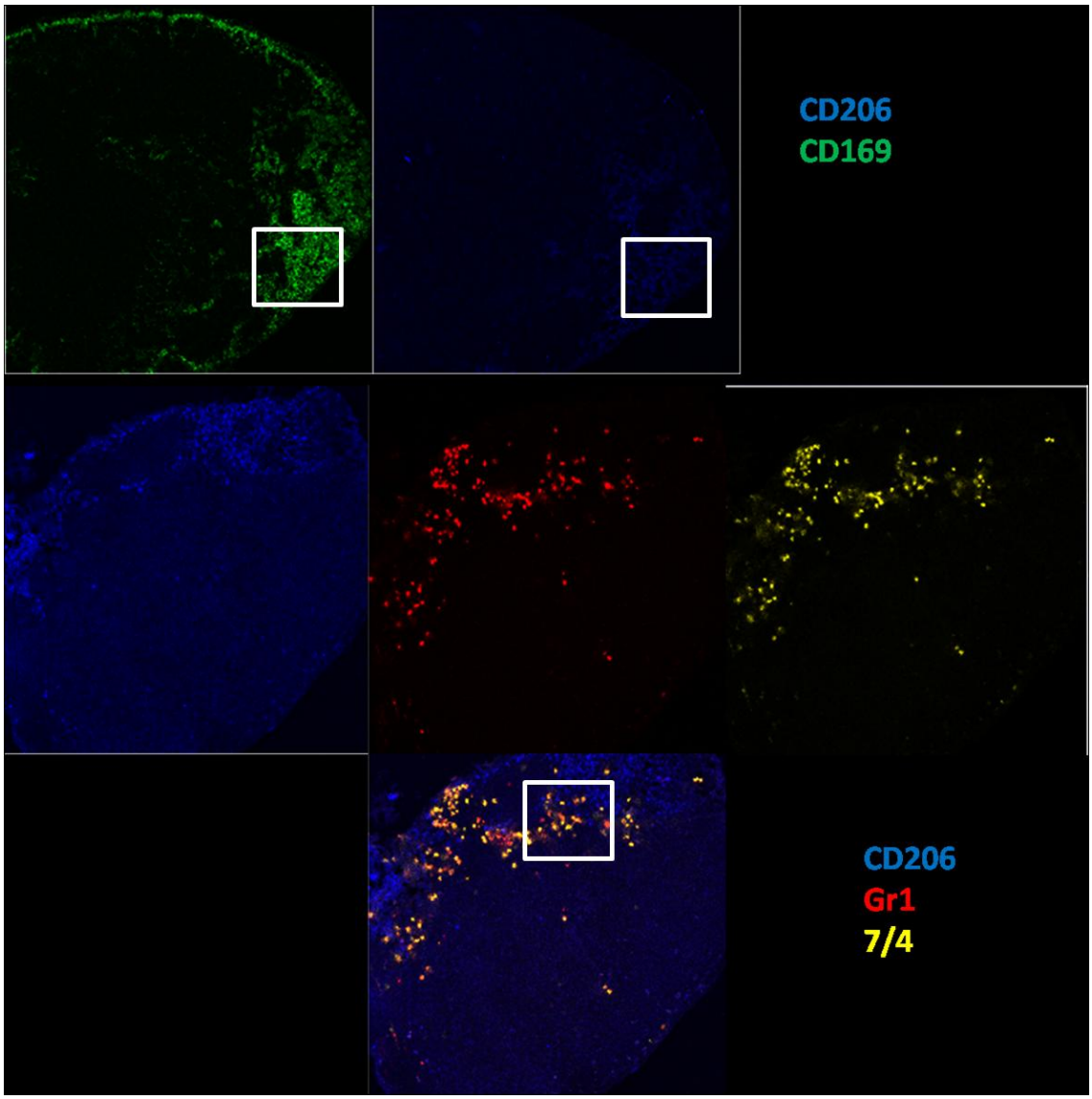

Figure 4.15 Confocal microscopy of mannose receptor binding in cervical lymph nodes during carriage. 2 CLN samples from different MF1 mice on day 0, 1, 3, 7 and 14 of carriage with WT-D39 were sectioned and stained with the surface markers CD206 (blue) and CD169 (green) (Top panel), and CD206 (blue), Gr-1 (red) and antigen 7/4 (yellow) and a composite image (lower panel). CD206 (mannose receptor) is seen to localise with CD169+ macrophages throughout carriage (white boxes, top panel), but with increasing amounts at the later stages of carriage (analysis not quantitative). At day 7 and 14 of the carriage, neutrophils/monocytes indicated by expression of Gr-1 and antigen 7/4 accumulate adjacent to these macrophage areas (white box, lower panel). All images shown are from day 7 of carriage. Pictures were taken with objective lens x 10.

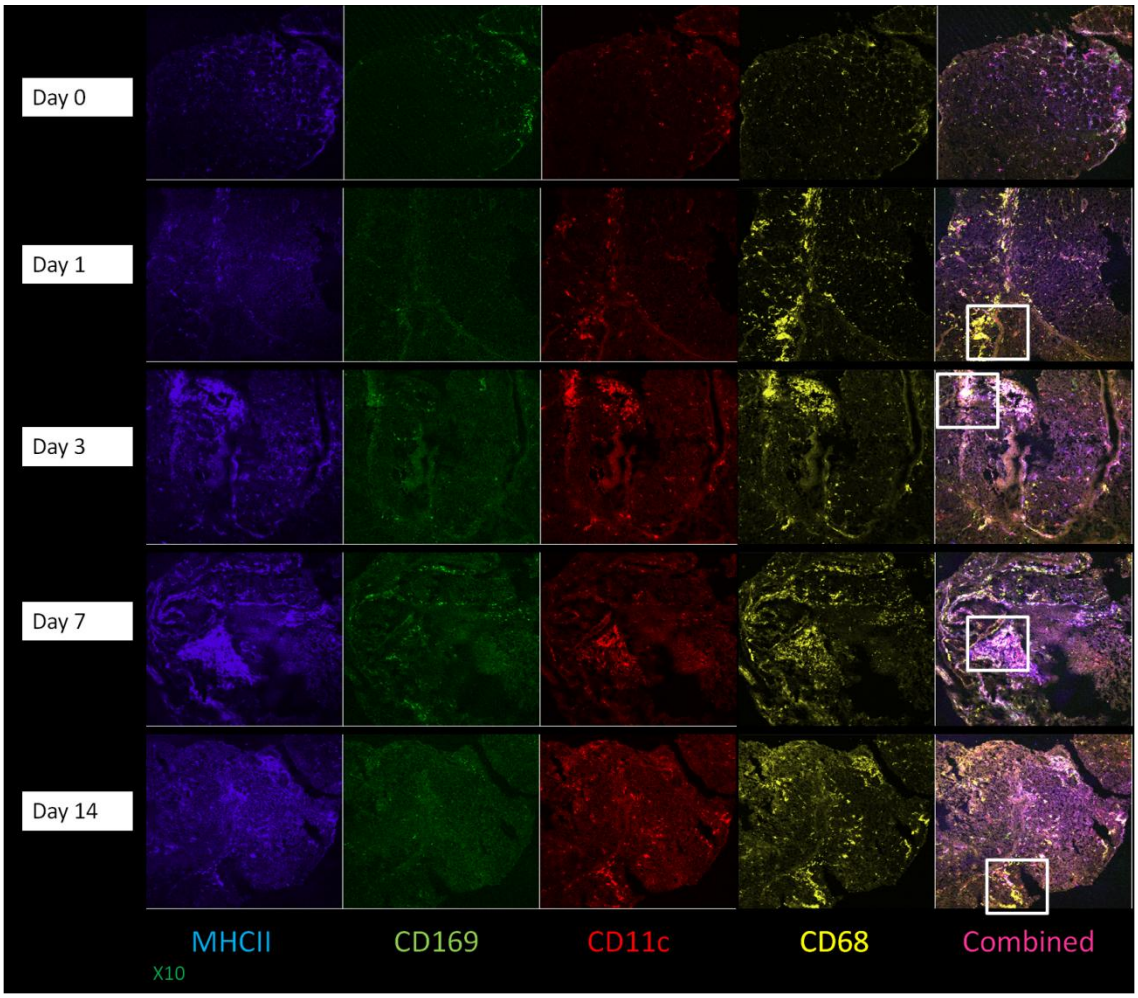

Fig 4.16 Confocal microscopy of nasal mucosa during carriage. 2 areas of nasal mucosa from different MF1 mice on day 0, 1, 3, 7 and 14 of carriage with WT-D39 were sectioned and stained for surface markers MHCII (blue), CD169 (green), CD11c (red) and CD68 (yellow), and the final image from each day is a composite of all markers. CD11c+ CD68+ CD169+ macrophages can been seen in increasing numbers over the course of carriage, indicated inside the white boxes (analysis not quantitative). The number of CD11c+ MHCII^{hi} dendritic cells also increase at the later stages of carriage. Pictures were taken with objective lens x 10.

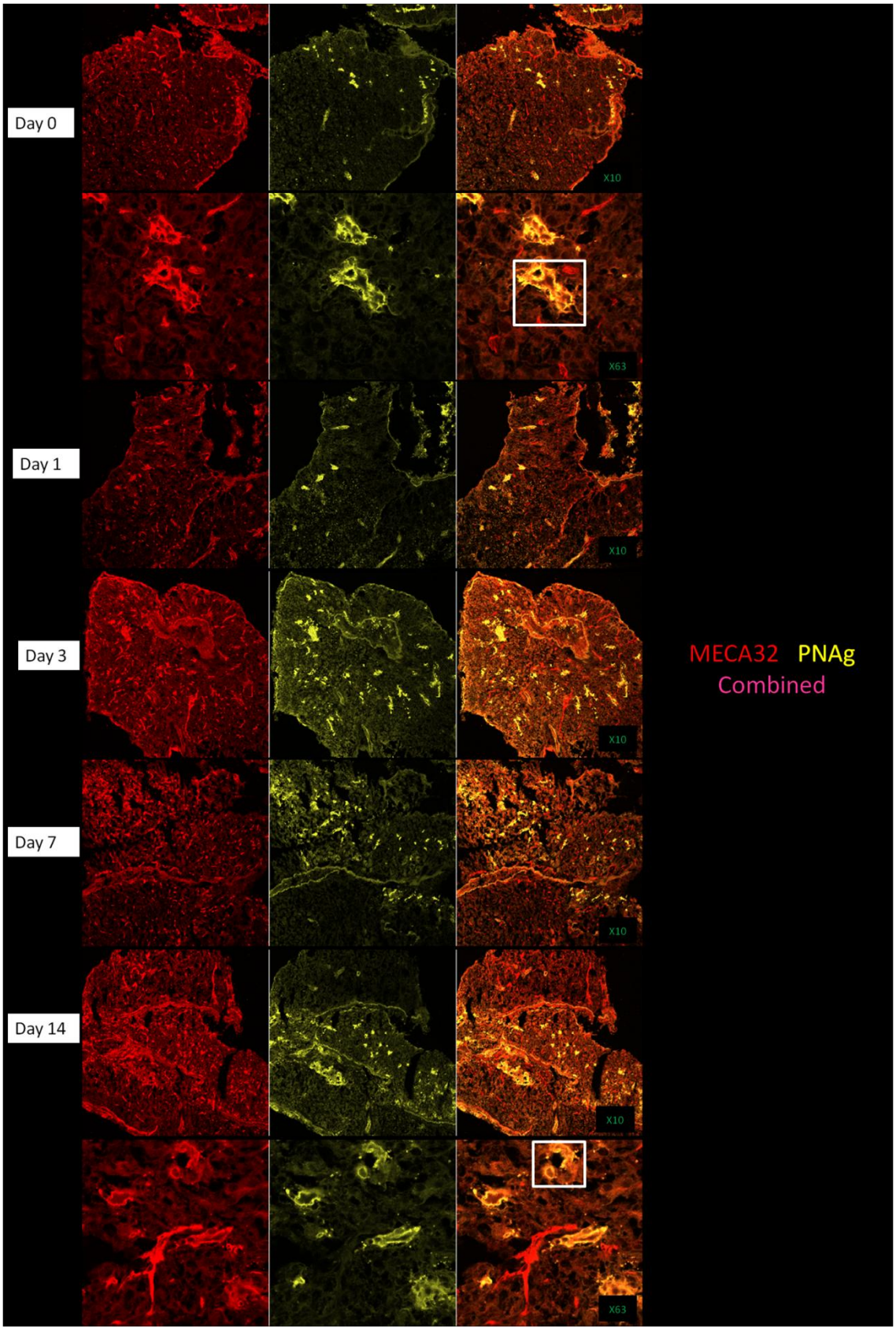
Day 0
X10
X63
Day 1
X10
Day 3
X10
Day 7
X10
Day 14
X10
X63
MECA32 PNAg
Combined

Fig 4.17 Confocal microscopy of nasal mucosa during carriage. 2 areas of nasal mucosa from different MF1 mice on day 0, 1, 3, 7 and 14 of carriage were sectioned and stained for surface markers MECA32 (red) and peanut agglutinin (PNAg) (yellow). Both MECA32 and PNAg appear to co-stain endothelial venules (indicated inside the white boxes) which increase in number throughout carriage (analysis not quantitative). Pictures were taken with objective lens x 10 and x 63 as indicated.

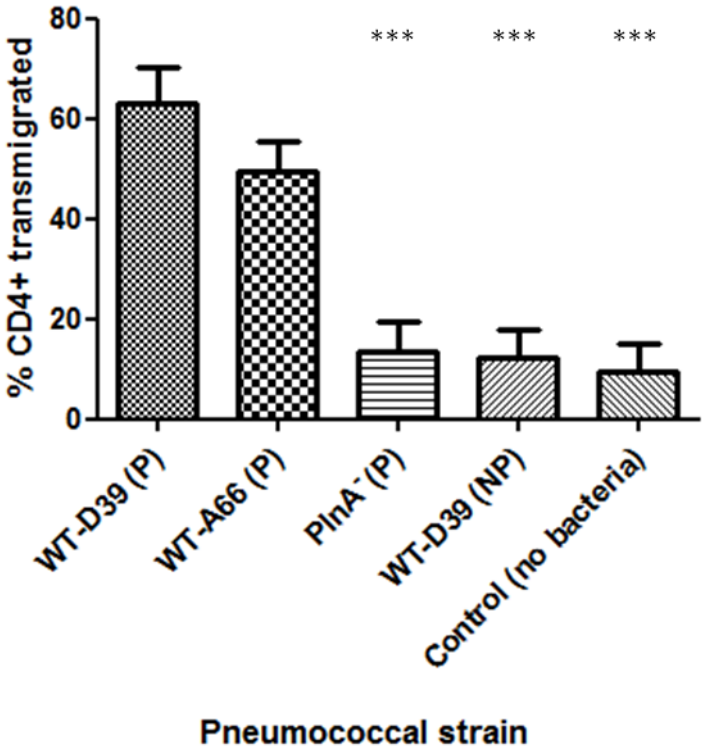

Figure 4.18 Transmigration of CD4+ T cells towards pneumococci. Strains of WT and isogenic pneumococcal mutants were tested for their ability to stimulate chemotaxis of purified human CD4+ T lymphocytes in a Transwell transmigration apparatus. Strains which had been animal passaged (P) or non passaged (NP) are indicated respectively. Shown is the mean % of cells which migrated towards the stimulus ± S.E.M. Cells were counted twice (upper and lower chamber of haemocytometer) twice, in duplicate wells in n=4 separate experiments. The statistical significant of strains compared to WT-D39 passaged was calculated using a students t test, *** ($p=<0.001$).

4.12 Conclusions

It has been previously shown that anti-polysaccharide antibody is the most effective protective antibody against pneumococci in a mouse septicaemia model (Schiffman, 1983), and also that anti-capsular antibody generated by vaccination alone can reduce the rate of acquisition of new colonising strains humans (Dagan *et al*., 2002). The data in this chapter represents the first description of serum capsular specific antibody being generated during murine nasopharyngeal carriage with live *S. pneumoniae*. Whilst anti-capsular IgG can be detected in human sera following episodes of natural carriage (Goldblatt *et al*., 2005b), recent mouse models have failed to identify any capsule specific antibodies (of any subclass) during live carriage of pneumococci (van Rossum *et al*., 2005; Roche *et al*., 2007; Malley *et al*., 2005b). Anti-capsular antibodies can be readily induced under the correct conditions in mice, as a robust IgG serum response to intranasally administered soluble capsular polysaccharide with cholera toxin can be observed (Malley *et al*., 2006) but it is known that the antibody response to soluble pneumococcal antigens and intact bacteria is different (Wu *et al*., 1999). Polysaccharides alone associate poorly with MHCII (Harding *et al*., 1991) and are thus considered thymus independent antigens (TI) and, in the absence of cognate T-cell help, they fail to generate germinal centres (Weissman *et al*., 1976), or induce the required affinity maturation of antibody subclasses (Klaus and Humphrey, 1974) and thus produce poor B-cell memory responses (Baker *et al*., 1971). The protective immune response to T-independent antigens such as the pneumococcal polysaccharide vaccine (Pneumovax-23) is particularly poor in young children, for reasons that are not well understood. This was the rationale behind the introduction of the polysaccharide protein conjugate vaccine which covalently links a protein carrier to polysaccharides, which can then be processed by internally by antigen presenting cells such as B-cells, presented in the context of MHCII to T-cells and provide the necessary costimulation for antibody isotype class switching and the production of germinal centres, a response which is comparatively more robust in infants (Dagan *et al*., 2002). In the presence of whole bacteria, there is a constant source of pneumococcal protein antigens which can be processed and presented through MHCII, however T-cell independent stimulation of polysaccharide antibody production can also occur by a number of different mechanisms (summarised in Fig 4.19). The extensive cross-linking of B-cells by the polysaccharide repeating structure or via the binding of complement receptor-2 (CR2)

to complement component C3d deposited on the surface of pneumococci (Griffioen *et al*., 1991) can enhance antibody secretion by plasma B-cells (Snapper and Mond, 1996). It is noteworthy that marginal B-cells of neonates are deficient in CR2 (Timens *et al*., 1989) which may explain the poor response to polysaccharide vaccine in this group. Additionally the influence of soluble factors such as IFN-γ and granulocyte macrophage colony stimulating factor (GM-CSF) released by natural killer cells and macrophages respectively, may provide the necessary co-stimulation required to produce an effective B-cell response in the absence of CD4+ lymphocytes (Snapper and Mond, 1996). Other antigenic components such as the CpG DNA motif and lipoproteins may also non-specifically act directly on B-cells to induce this effect (Snapper and Mond, 1996). Both the action of cytokines and bacterial antigens on B-cells result in the secretion predominantly of IgM subclass of antibody (Snapper and Mond, 1996), which concurs with the findings presented here, as no IgG specific to capsular polysaccharide could be detected throughout 28 days of carriage with WT-D39 (Fig 4.1).

Anti-capsular IgM increases rapidly throughout the first 7 days of nasopharyngeal carriage in mice, which agrees with other previous work using whole heat killed pneumococci administered I.P., which showed that IgM antibody raised against capsule appears much earlier than IgG antibody against proteins, which in contrast required CD4+ T-cells and CD40L costimulation (Khan *et al*., 2004). In the data presented here, serum IgM antibody correlated with lower bacterial CFU in the nasopharynx of individual mice carrying WT-D39 (although not complete clearance) and with the complete clearance of PLY negative bacteria, where bacterial numbers fell at each day that IgM levels increased (Fig 4.1, B & Fig 4.3, B). IgM has been implicated to have an important role during invasive disease as IgM specific memory B-cells and serum IgM levels are reduced in the elderly, a group which are particularly vulnerable to pneumococcal pneumonia (Shi *et al*., 2005), and a natural IgM deficiency in otherwise normal adults increases the risk of developing pneumococcal pneumonia (Yel *et al*., 2009). Natural IgM antibody, which could be detected in naive mice (Fig 4.6, B & D) has been shown to enhance activation of the complement system by the classical pathway, which is the dominant pathway of innate immunity to the pneumococcus (Brown *et al*., 2002), and immunisation with a monoclonal antibody that mimicked natural IgM antibody to type-2 polysaccharide was able to significantly delay the onset of fatal disease with D39 (Baxendale *et al*., 2008). However, the inability to clear fully

virulent pneumococci from the nasopharynx despite high levels of serum anti-capsular IgM, as is seen in the carriage of WT-D39, questions its protective capacity at mucosal surfaces. In contrast, IgG antibody levels in the saliva of children have been shown to correlate with serum IgG titres, and may more easily enter mucosal sites (Nurkka *et al*., 2001)

It is hypothesised that the development of antibodies to conserved pneumococcal antigens, rather than heterogeneous polysaccharide antigens could account for the simultaneous decline in carriage rates of multiple serotypes in children (Lipsitch *et al*., 2005). IgG anti-PspA antibodies could be detected in the later stages of WT-D39 and A66 carriage where correlation was found between a high PspA titre and lower bacterial numbers (Fig 4.2, B), and also in PlnA$^-$ carriage where the levels of PspA specific antibody correlated with the clearance of pneumococci from the nasopharynx (Fig 4.3, D). Antibodies against PspA have been shown to develop after intranasal infection with live pneumococci in both human experimental carriage (McCool *et al*., 2002), natural human carriage (Goldblatt *et al*., 2005a) and experimental carriage in mice, where the titre of anti-PspA antibody correlated with subsequent protection against re-colonisation (Trzcinski *et al*., 2005). PspA antibody was also the dominant IgG antibody detected during colonisation with a non-encapsulated serotype-4 pneumococcus (Roche and Weiser, 2010). Despite these findings, in many instances the clearance of colonisation itself has not been shown to be significantly different in mice that are deficient in producing antibody and WT mice (McCool and Weiser, 2004; Malley *et al*., 2005b). BALB/c mice which had both the longest and highest density of pneumococci of three mouse strains tested in a carriage model, also had the highest antibody titres of PspA (although, in contrast to our results, no anti-capsular antibody could be detected in this model) (McCool and Weiser, 2004). In a more recent study, C57Bl/6 mice were colonised with attenuated strains of pneumococci, lacking either polysaccharide capsule, PLY or PspA, and showed that antibody was crucial to subsequent protective responses, suggesting that antibody responses may be different to attenuated and WT bacteria (Roche *et al*., 2007). In the model presented here the level of anti-PspA IgG was higher in PlnA$^-$ colonised mice (Fig 4.3, C) compared to those carrying WT-D39 (Fig 4.1, C) or A66 (Fig 4.2, A) and similarly, serum IgM antibody from the colonisation with PlnA$^-$ was better able to bind to the surface pneumococci *in vitro* than serum IgM from WT-D39 carriage (Fig 4.6, D & B respectively). In addition

to the previously discussed mouse strain and bacterial strain variability in immune response to nasopharyngeal carriage, it is likely that the reduced ability of PlnA$^-$ bacteria to escape host immune mechanisms such as complement mediated phagocytosis, make them vulnerable targets to antigen presenting cells resident in the nasopharynx.

One of the reasons that intranasal immunisation is thought to be effective at preventing subsequent disease is because it can induce both mucosal and systemic responses, which, in line with what was observed here, is characterised by local secretory IgA antibodies and serum IgG and IgM responses (Lynch *et al*., 2003). IgA is the dominant pneumococcal antibody at mucosal sites such as the nasopharynx (Virolainen *et al*., 1995), and it has been shown that IgA was crucial in protecting mice from colonisation after intranasally administered conjugate vaccine (Lynch *et al*., 2003). In this model IgA could not be detected in the serum (data not shown) but pneumococcal capsule specific IgA and total IgA was released from B-cells from the cervical lymph nodes in both the WT-D39 and PlnA$^-$ carriage in equal amounts (Fig 4.9, A & B). The highest expression was observed on day 7 of carriage, however their absence from day 14, when both the WT-D39 and PlnA$^-$ CFU's are at the lowest level, suggests that IgA is not responsible for the decline in pneumococcal carriage. It is thought that secretory IgA may play a lesser role in reducing the duration of a carriage event as the pneumococcus possesses means to counteract IgA (IgA1 protease) but not other subclasses such as IgG and IgM (Kilian *et al*., 1996).

Recently, the importance of macrophages in pneumococcal infection was shown as two mouse strains, BALB/c and CBA/Ca which are respectively resistant and susceptible to pneumococcal infection, were shown to exhibit different cellular responses, with lower expression of TNF-α and increased necrosis of macrophages in the susceptible mouse (Ripoll *et al*., 2010). In the model presented here, the majority of the macrophages observed in the nasal mucosal tissue (and in the cervical lymph nodes) possess the surface markers CD169, CD11c and CD68, which meant that they most closely resembled alveolar macrophages (AM). Evidence from *in vivo* and *in vitro* models has shown that alveolar macrophages contribute to the clearance of bacteria in the lungs through phagocytosis of bacteria (Jonsson *et al*., 1985), a process which is enhanced by opsonisation by complement components and immunoglobulin (Gordon *et al*., 2000). It is noteworthy that the population of macrophages in the nasal mucosa and CLN of the

model presented here lacked the surface marker SIGNR1, a C-type lectin which has been implicated in uptake of capsular polysaccharide antigens by macrophages in the spleen (Kang *et al*., 2004). SIGNR1 was essential in protection against invasive pneumococcal disease (Lanoue *et al*., 2004) and suggests that an alternative pattern recognition receptor such as toll-like receptors or mannose receptor (which was detected during carriage in the CLN) may predominate in the upper respiratory tract of mice. Alveolar macrophages also play an immunosuppressive role during infection, and pneumococcal pneumonia in $AM^{-/-}$ mice produce higher amounts of TNF-α, IL-1β and KC but lower amounts of the anti-inflammatory IL-10 which can leads to a destructive increase in PMN cell infiltration to the lungs (Knapp *et al*., 2003). In mouse models using high bacterial loads, where PMN cells predominate regardless of macrophage expression (Kadioglu *et al*., 2000), neutrophil phagocytosis, apoptosis and their subsequent clearance by macrophages is vital for minimising inflammation that would otherwise be caused from necrotic PMN cells (Haslett, 1999). Recently, intracellular killing of pneumococci by macrophages *in vitro* was also shown to result in their apoptosis (Dockrell *et al*., 2001). In a low dose resolving mouse model of pulmonary pneumonia, early AM apoptosis was associated with clearance of bacteria from the lungs, in the absence of neutrophil infiltration, but when AM's were depleted, the clearance of pneumococci was decreased (Dockrell *et al*., 2003). When faced with small numbers of bacteria, macrophage apoptosis may also therefore limit non-specific lung injury or damage to mucosal epithelium in the absence of large numbers of neutrophils, which may promote the dissemination of pneumococci further down the respiratory tract or into blood directly (Dockrell *et al*., 2003). In addition, apoptotic cells may present pneumococcal proteins to dendritic cells either locally (Yrlid and Wick, 2000), or in draining lymph nodes (Bellingan *et al*., 1996) and thus generate an adaptive immune response, which would explain why an antibody response is generated in the WT-D39 carriage. Interestingly, nitric oxide accumulation which is stimulated by *S. pneumoniae* and the toxin PLY (Braun *et al*., 1999) acts as a stimulus for apoptosis, and PLY deficient mutants showed both decreased apoptosis and internalisation of pneumococci, compared to the parent strain (Marriott *et al*., 2004). It is therefore unclear how the clearance of the PLY negative mutants from the nasopharynx, in contrast to the persistence of the WT, is related to direct killing by macrophages (Fig 3.5 & 3.6). However, as these macrophages which are phenotypically similar to AM's can be found in both the nasal mucosa and CLN in

increasing numbers during carriage (qualitative observation), an alternative hypothesis is that that antigens taken from the nasal mucosa by these macrophages can circulate to the draining lymph nodes, as AM's have been shown to traffic from the lung to the lung draining lymph node, where they can present antigen (Kirby *et al*., 2009). In this model AM's were described to interact with B-cell areas of the lymph node which appears to be similar to what was have observed here (Fig 4.13). As AM's can substitute for dendritic cells in this process, this may explain why there were few dendritic cells in the nasal mucosa. In this model, the mannose receptor (CD206) appeared to be upregulated in the later stages of carriage. Mannose receptor (MR) is a type I membrane binding protein, that can function as a pattern recognition receptor on the macrophage surface (Linehan *et al*., 1999), where it can initiate non-opsonic phagocytosis of many micro-organisms (Ofek *et al*., 1995). MR can also be shed from macrophages to produce soluble mannose receptor (sMR) (Martinez-Pomares *et al*., 1998). Both forms are believed to contribute to antigen capture and delivery to the spleen or secondary lymphoid organs such as the cervical lymph nodes where one of the known ligands for its binding is sialoadhesin (CD169) which is present on subcapsular sinus macrophages (Martinez-Pomares *et al*., 1999). As SCS macrophages are closely associated with B-cell follicles, it may be that MR is capturing pneumococcal antigen which can then be delivered to B-cells. Because macrophages in the nasal mucosa did not appear to possess MR (data not shown) it could be speculated that these macrophages may be shedding their MR in response to antigen, which is then circulating to the CLN with or without antigen. The proposed mechanism of macrophage involvement in the context of this model is presented in Fig 4.20.

A consequence of antigen circulating from the nasal mucosa to the CLN in a model of persistent colonising bacteria such in the WT-D39 may be the induction of tolerogenic immune responses, which are even more common than a protective immune response in mucosal sites (Pabst *et al*., 2007). In the gut, 'immature' dendritic cells which encounter antigen on mucosal surfaces travel to the local lymph nodes, and present antigen to naive T-cells, wherein the absence of inflammatory conditions the production of T-regulatory cells is initiated (Guermonprez *et al*., 2002). Furthermore, it was recently identified that intra-tracheal infection with *S. pneumoniae* can also lead to an increase in T-regs in the CLN, which ultimately triggers down-regulation of antigen specific CD4+ T-cell cytokine and antibody responses (Preston *et al*., 2010). Although

the dendritic cell expression of MHCII in T-cell areas of the CLN increased during carriage in the qualitative analysis of immunohistochemistry (Fig 4.14), it was not possible to identify an increase in T-cells, or T-regs (via folate-4 receptor expression, data not shown) using quantitative flow cytometry. Coupled with the lack of T-cells found in the nasal mucosa, it could be speculated that the immune-tolerance in this model of nasopharyngeal carriage may be controlled predominately by the CD11c+, CD68+, CD169+ macrophage population which is abundant in both the nasal mucosa and CLN and is phenotypically similar to alveolar macrophages which release immunosuppressive IL-10 (Knapp *et al*., 2003). IL-10, which may be produced by macrophages or dendritic cells in response to PLY (McNeela *et al*., 2010), can also, in the absence of other stimulating cytokines, inhibit the production of antibodies and in particular T-independent antibodies by B-cells (Pecanha *et al*., 1993). This may be a further explanation of why the antibody response in the WT was slightly weaker than observed in the PLY deficient strain.

Macrophages have previously been cited as the main effector cells responsible for clearing primary carriage of pneumococci, in a mouse model of nasopharyngeal carriage (Zhang *et al*., 2009). Although an early increase in neutrophils was identified in this model (day 3), these numbers declined before the reduction of bacteria from the nasopharynx and clearance was instead correlated to a sustained number of monocytes/macrophages in the lumen of the upper respiratory tract, which peaked 7 days after colonisation and was maintained until clearance. The accumulation of macrophages in this case was dependent on the presence of TLR-2, CD4+ T-helper cells and IL-17A (Zhang *et al*., 2009), and forms part of a theory of how nasopharyngeal carriage can be cleared in the absence antibody. Respiratory epithelial cells which can be activated by pneumococcal binding to TLR-2, release TGF-β which in turn increases the permeability of the epithelium thus increase access of immune cells to the bacterial site (Beisswenger *et al*., 2009). Epithelial cells also under the direction of TLR-2 can release IL-6 which helps to recruit IL-17 producing T-cells (Beisswenger *et al*., 2009), which then in turn have a chemotactic effect on both monocytes and macrophages (Sergejeva *et al*., 2005). In the model by Zhang *et al* they state that the clearance of a secondary carriage event may be enhanced by anti-pneumococcal antibody, however, as there is a relative lack of polysaccharide antibody in their primary carriage model compared to the model presented in this thesis and they

used a mutant with a truncated PspA as the immunising strain, it is possible that higher antibody levels could exert an effect more quickly than was previously thought, particularly with the rapid rise in anti-capsular IgM and locally produced IgA shown in this thesis.

That whole pneumococci can initiate the chemotaxis of human CD4+ T-cells *in vitro* has been previously shown (Kadioglu *et al*., 2004). The finding that bacteria which have undergone a period of *in vivo* growth were better able to stimulate chemotaxis, not surprising as, it is known that the expression of many virulence factors, including the expression of the capsule are upregulated *in vivo* (Ogunniyi *et al*., 2002). High levels of migration were only seen in PLY sufficient strains and it has been shown that PLY gene expression may be up to 10 times higher *in vivo* (Ogunniyi *et al*., 2002). A similar requirement for PLY was shown in the migration of neutrophils towards pneumococci (Moreland and Bailey, 2006). Although PLY has been predominately viewed as a extracellular factor, released due to the actions of autolysin, purified PLY alone or PLY containing supernatants from WT bacteria were able to cause the same levels of migration as WT cells (Moreland and Bailey, 2006; Kadioglu *et al*., 2004). This indicates there is a more complex interaction between the intact pneumococci and immune cells. It was previously shown that cells which migrate towards pneumococci have the features of T-regulatory cells, with expression of CD25 and the release of IL-4 (Kadioglu *et al*., 2004). This fits with a model of immuno-suppression which may be initiated by the presence of PLY.

Ultimately, despite the immune responses generated in MF1 mice during nasopharyngeal carriage, they are unable to initiate the clearance of either WT-D39 or serotype-3, strain A66. Whilst this may be testament to the evasive properties of these sufficient bacteria it is also likely that an immune-tolerant/immunosuppressive response is both normal for such a commonly colonising bacteria, and desirable, as a full immune response may actually be detrimental to the outcome of carriage, just as the early infiltration of CD4+ T-cells into the lungs of mice during invasive pneumococcal disease leads to a more lethal outcome (LeMessurier *et al*., 2010). As there are not large differences in the humoral immune response to either WT or PLY deficient bacteria, and the increased granulocyte infiltration of CLN observed in the WT strains only is not responsible for bacteria clearance, it seems that at least for PlnA$^-$, its ability to survive in the nasopharynx is determined by its inability to penetrate the epithelium,

leaving it vulnerable to host clearance mechanisms that the WT pneumococci are better able to evade.

From these data it is clear that pneumococcal nasopharyngeal carriage is an immunising event that elicits both a local and systemic immune response, and the consequences of this response in the context of future challenges with the pneumococcus will be reviewed in the next chapter.

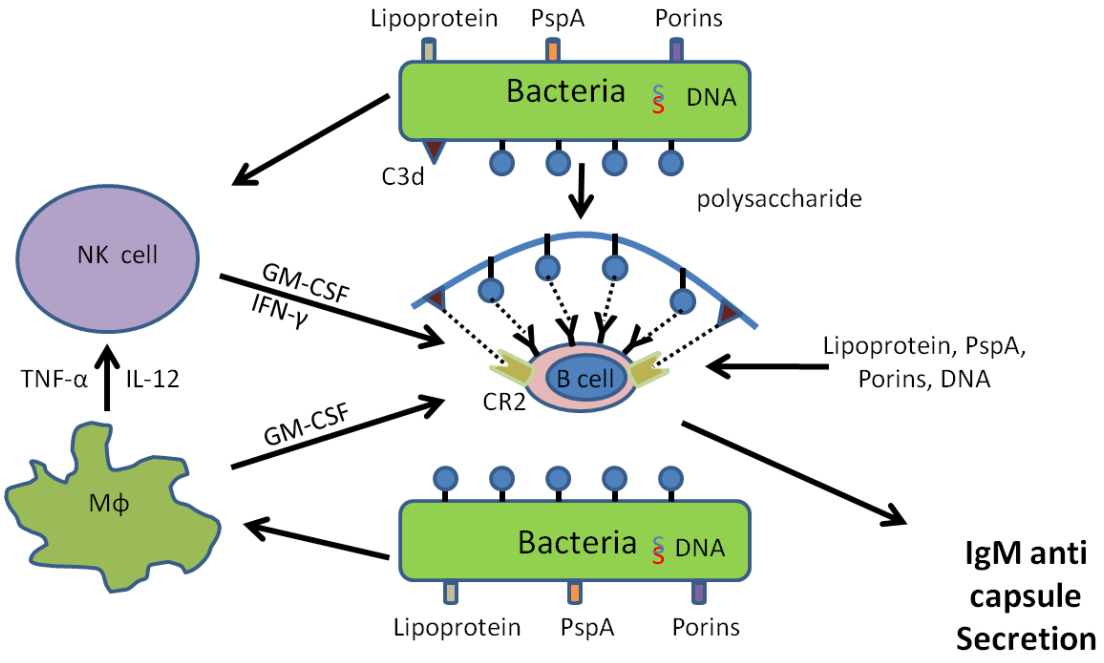

Figure 4.19 Model of T independent antigen stimulation of B cell responses. Adapted from (Snapper and Mond, 1996)**.** B-cells are activated in the absence of T-cells via the cross linking of antigen receptors by polysaccharide patterns, recognition of complement components via complement receptor-2, and recognition of other bacterial components through toll like receptors. Additionally, the action of IFN-γ and granulocyte macrophage colony stimulating factor (GM-CSF) released by natural killer cells and macrophages respectively, can provide the necessary signals to initiate antibody production of predominately IgM subclass.

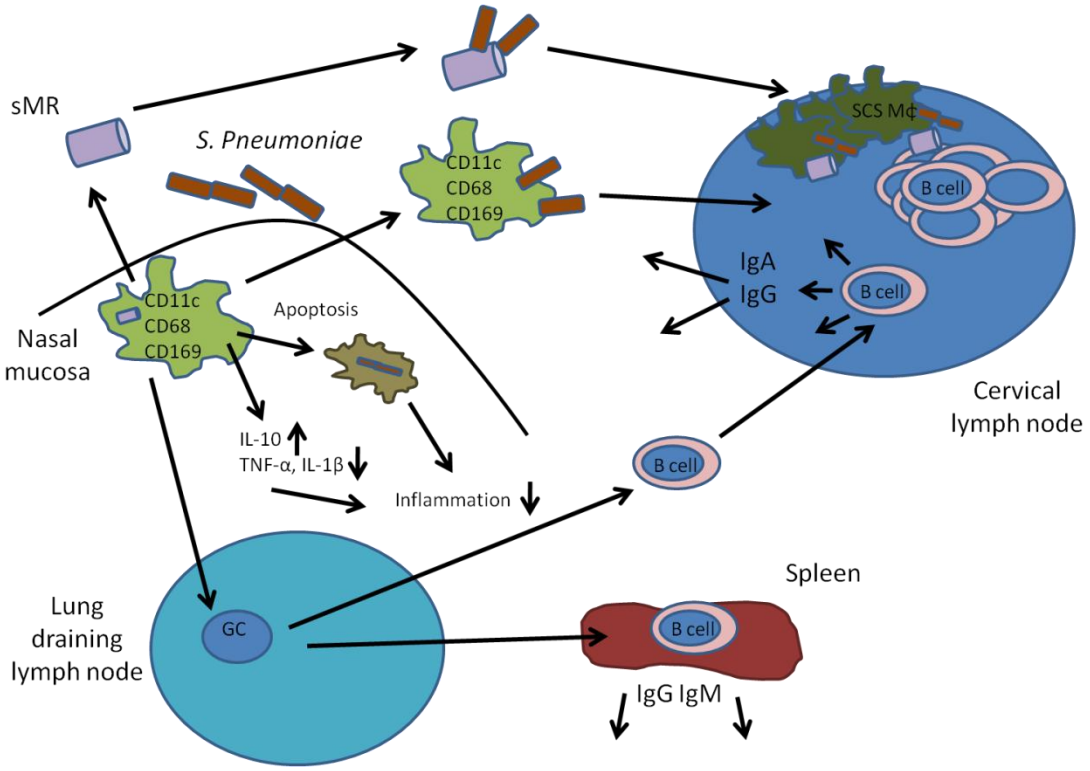

Figure 4.20 Role of macrophages in carriage. When encountering small numbers of pneumococci such as in carriage, resident macrophages may uptake bacteria which are in contact with the nasal mucosa. In an effort to not to make a damaging inflammatory response, macrophages which have phagocytosed pneumococci may initiate an anti-inflammatory cytokine cascade, with release of IL-10 and inhibition of TNF-α, and may also undergo apoptosis. Intact or apoptosed macrophages may traffic to the lung draining lymph nodes and the spleen carrying pneumococcal antigens to initiate a humoral immune response (those sites are implicated as there was significant systemic pneumococcal specific antibody but no evidence of germinal centres in the CLN). Pneumococcal specific B-cells may then return to the CLN to release antibody locally. Alternatively, macrophages carrying pneumococcal antigens bound to mannose receptor or soluble mannose receptor release by the cells may travel to the cervical lymph nodes and interact directly with B-cells to down-regulate the immune response.

Chapter 5: Future encounters with the pneumococcus; the immunising effect of nasopharyngeal carriage in mice

5.1 Preface

The main clinical aim of carriage studies is to inform future decisions concerning the treatment and prevention of pneumococcal disease through immunisation. Nasopharyngeal carriage is the gatekeeper for all subsequent pneumococcal disease, as well as its transmission to non-infected individuals, therefore a better understanding of the host-bacterial interactions could ultimately lead to vaccinations that reduce the incidence of carriage, which would then have a knock on effect on the incidence of invasive disease. The current conjugate polysaccharide vaccine, which is the preferred vaccination for children <2 years of age, is able to halve the carriage rates of vaccine serotypes, but at the cost of increasing carriage of non-vaccine serotypes (Mbelle *et al*., 1999). In non-immunised infants <2 years, where carriage rates are highest, individuals can reacquire prolonged carriage of all strains (even strains that have previously been detected) with the duration of the second carriage not significantly shorter than the first encounter of that strain (Hill *et al*., 2008). In contrast, carriage rates drop in adults without vaccination (Hansman *et al*., 1985), and the duration of individual carriage events are likely to be shorter by comparison (Goldblatt *et al*., 2005a). Whilst the relatively poor infant immune response, especially to polysaccharide antigens can be considered as a reason for these differences (Douglas *et al*., 1983), in the absence of vaccination the boosted memory immune response associated with natural exposure to pneumococcal antigens (both protein and polysaccharide) in infancy offers a simple explanation for this increased protection (Simell *et al*., 2002; Goldblatt *et al*., 2005b). Reductions in carriage rates can be observed in children after receiving the pneumococcal conjugate vaccine (Dagan *et al*., 2002), and in mice following intranasal doses of the conjugate vaccine and IL-12 (Lynch *et al*., 2003). Studies in mice have attempted to define correlates of protection against subsequent carriage and invasive pneumococcal disease and developed diverse immunisation strategies which include intranasal immunisation with purified pneumococcal proteins, whole-cell killed, and live non-encapsulated vaccines, cell wall polysaccharide vaccines, encapsulated live pneumococci and live-attenuated pneumococci, with varying degrees of success (Basset

et al., 2007; Malley *et al*., 2001; Malley *et al*., 2006; Malley *et al*., 2005b; Roche *et al*., 2007).

In this chapter I will define how primary carriage with a variety of attenuated strains of pneumococci alters both subsequent carriage events and invasive disease, and determine the correlates of the protection which I observed.

Results

5.2 Does nasopharyngeal carriage offer protection against future carriage events?

To determine whether a prior colonisation event could prevent or reduce subsequent nasopharyngeal carriage, mice which had cleared serotype-2 ($PlnA^-$) completely from their nasopharynx (see Fig 3.5) were subjected to a second WT-D39 (serotype-2) carriage dose, four weeks after the initial dose (two weeks after the initial clearance from the nasopharynx). The second colonisation was not significantly different to the primary WT-D39 carriage in naive mice during the first 7 days post infection (Fig 5.1). However, in contrast to the naive carriage, at day 14, pneumococci were completely cleared from nasopharynx in 100% of mice tested (p=0.0018) (Fig 5.1). The clearance of WT-D39 following $PlnA^-$ carriage was not statistically different when the 'immunising' strain was replaced with either of two D39 isogenic mutants lacking neuraminidase enzymes NanA and NanB (data not shown).

5.3 Is capsular serotype important for this protection against carriage?

In order to assess the effect of capsule specificity on future carriage events, mice which had cleared a serotype-2 colonisation with attenuated PLY negative bacteria ($PlnA^-$), were subjected to a secondary serotype-3 (A66) carriage, which is normally maintained in naive mice for at least 21 days (Fig 3.10). In contrast to the result of the homologous serotype re-colonisation, where colonies were cleared by 14 days of the second carriage event (Fig 5.1), colonies of A66 were maintained in the nasopharynx of all mice tested for two weeks post inoculation (the limit of observation) (Fig 5.2). Interestingly, there appeared to be the opposite effect as was seen in homologous serotype experiment (Fig 5.1), as, at all time points after day 1 the numbers of pneumococci in the nasopharynx were higher than previously observed in naive A66 carriage, day 3 (p=0.0159), day 7 (p=0.0195), day 14 (p=0.0159) (Fig 5.2). These results, suggest there may important role for the capsule in providing protection against a subsequent nasopharyngeal carriage event with the same serotype.

To test whether the effects of capsular based immunity generated through carriage are limited to particular serotypes, we assessed the ability of encapsulated serotype-2 ($PlnA^-$) to alter the carriage of a naturally occurring non-encapsulated pneumococcus, which normally persists in the nasopharynx for at least 14 days in naive mice (Fig

3.11). Four weeks after an initial carriage dose of PlnA$^-$ (which was cleared as previously described), the non-encapsulated mutant was introduced into the nasopharynx. Colonies of 110.58 were maintained in the nasopharynx until day 7 of the second carriage at the same density as was observed for 110.58 in naive mice (Fig 3.11), although carriage rates were lower in this case being <100% at day 3, and with one mouse exhibiting substantially lower colonisation at day 7 (Fig 5.3). However, in stark contrast to the naive carriage of 110.58, numbers of pneumococci present in all mice tested were below the level of detection at day 14 of the second colonisation ($p=0.0097$ compared to naive carriage of 110.58) (Fig 5.3), where previously naive mice had the highest burden of carriage on this day ($p=0.0151$) (Fig 3.11).

5.4 Are capsular antibodies required for clearance of subsequent carriage?

To further clarify the role of capsular antibodies during subsequent carriage, it was decided to test the effect of a non-encapsulated colonisation on subsequent encapsulated carriage. As the primary carriage of 110.58 was normally maintained strongly 14 days post inoculation (Fig 3.11) it was decided to artificially clear this mutant after 14 days, in order to replicate the timescale of the clearance of PlnA$^-$. 110.58 was cleared from the nasopharynx by the addition of rifampin as described elsewhere (Malley *et al*., 2005b), which abolished the carriage of pneumococci (Fig 5.4, A) but allowed additional nasal-flora to return to normal levels after 14 days (data not shown). Whilst control mice (which also received identical rifampin treatment) had colonies of WT-D39 present at day 14 of the second colonisation which were not statistically different to primary carriage levels, the mice pre-colonised with 110.58 had no detectable CFU of WT-D39 at day 14 ($p=0.0018$) in 100% of mice tested (Fig 5.4, B). This result suggests that anti-capsular immune responses were not required to prevent subsequent carriage of encapsulated pneumococci.

5.5 Protection against future invasive disease

After finding that prior colonisation in mice was able to provide protection against future carriage events, the next logical step was to assess whether colonisation of the nasopharynx could improve the outcome of subsequent invasive pneumococcal disease.

Once again, 28 days after the inoculation of attenuated serotype-2 carriage (PlnA$^-$), precolonised and control naive mice were challenged with an acute dose (1×10^6

CFU/50 µl) of serotype-2 WT-D39 pneumococci, which normally results in lethal bacteraemia and septicaemia in MF1 mice within 3 days. During the acute challenge there was no statistical difference in nasopharyngeal CFU between controls and pre-colonised mice at time zero, however there were significantly fewer CFUs of WT-D39 in the nasopharynx of pre-colonised mice at 24 (p=0.0167) and 48 hours (p=0.0187) post infection, and at all times excluding time zero the infection rate of the nasopharynx was lower in pre-colonised mice (Fig 5.5, A). Similarly there was less evidence of lung infection as pre-colonised mice had lower mean lung CFUs at 24, 34 (p=0.0421), and 48 hours post infection, with one mouse at each of the time points completely free of pneumococci (Fig 5.5, B). The biggest differences in bacterial numbers however were observed in the blood. Septicaemia was present in 100% of control mice at 24 hours and 34 hours post infection, but only 42.85% (p=0.0116) and 20% of precolonised mice at those time respectively showed evidence of pneumococci (Fig 5.5, C). In line with the detection of septicaemia, at all time points post infection there was consistently less severe signs of disease in pre-colonised mice, with the difference most pronounced at 34 hours (p=0.0004) and 48 hours (p=0.0115) post infection (Fig 5.5, D), and only 30% of pre-colonised mice developed any outward signs of disease throughout the invasive challenge. The survival rate in pre-colonised mice was statistically greater than that of naive control mice (p=0.0010) (Fig 5.6), with 62.5% of mice pre-colonised with PlnA$^-$ surviving the acute pneumococcal challenge until 7 days post infection, (the limit of observation in this study), at which point they showed no evidence of bacteraemia or septicaemia (data not shown). In contrast <10% of control mice survived past 48 hours post challenge.

5.6 Contribution of capsular serotype to protection against subsequent invasive challenge

Because the carriage of serotype-2 pneumococci did not affect subsequent re-colonisation with a serotype-3-pneumococcus (Fig 5.2), it was sought to identify whether this was also true for an invasive challenge following pre-colonisation with a heterologous serotype. MF1 mice were colonised with attenuated PlnA$^-$ (serotype-2), and challenged 28 days later with WT-A66 (serotype-3). At 24 and 48 hours post acute infection there were less colonies of A66 in the nasopharynx, lungs and blood of pre-colonised mice compared to naive controls (although not significant in this case) (Fig 5.7, A, B, C). Although both naive and pre-colonised mice had colonies in both

the nasopharynx and lungs at 24 hours, colonies had only started to reach the blood of infected mice at 48 hours post infection, and lung CFUs comparatively fell at 24 and 48 hours compared to time zero in both groups of mice (Fig 5.7). The progression of pneumococcal disease in A66 was therefore slower than observed in D39, with significantly less colonies in the lungs ($p=0.0095$), and blood ($p=0.095$) at 24 hours. This concurs with the observation that mice infected with A66 exhibited a slower onset of the outward signs of disease than those infected with D39 at early time points, which were normally delayed until the third day of infection in A66, (data not shown) compared to just 24-34 hours with D39 (Fig 5.5). In this acute model the signs of disease were also assessed by measuring the body temperatures of infected mice. At 24 hours post infection there was a significant difference in core body temperature between pre-colonised (mean=34.65 °C ± 0.1555), and control mice (mean=33.58 °C ± 0.2689, $p=0.0286$). Body temperature continued to fall in both groups after 48 hours as the disease progressed (Fig 5.7, D). Although mortality rates in A66 control mice were not significantly different to those in the D39 invasive experiment (Fig 5.6), the mean time of death was substantially delayed to an average of 83 hours compared to just 30.5 hours respectively. Survival during the A66 challenge was significantly increased in mice pre-colonised with PlnA$^-$ (77.77%) vs. naive controls (25%) ($p=0.0228$) (Fig 5.8). The survival rate in pre-colonised mice was not however significantly different to that induced by homologous serotype colonisation and invasive challenge with WT-D39 which was 72.43% (Fig 5.6), indicating that there may be no additive effect of capsule in protection against invasive disease.

To clarify the immunising potential of the polysaccharide capsule, in protection against subsequent encapsulated invasive pneumococcal disease, the carriage with non-encapsulated pneumococci (110.58) which is cleared by antibiotics before re-infection, (described in Fig 5.4), was repeated. but instead the 110.58 carriage was followed with an acute challenge of WT-D39. In this instance there was no clear statistical difference in survival between the pre-colonised and naive mice as 100% of controls and 75% of pre-colonised mice had succumbed to the infection by 55 hours and were culled (Fig 5.9). In contrast to the previous result where anti-polysaccharide capsular antibodies did not appear to have an additive effect on the clearance of subsequent invasive disease with WT-A66 (Fig 5.8), this result suggests the involvement of capsule based immunity in the prevention of subsequent acquisition invasive disease, which was not

demonstrated in the prevention of subsequent carriage by 110.58 (Fig 5.4). The strain specific and condition specific differences in the protection observed so far, are summarised in table 5.19.

5.7 Contribution of PspA to protection against subsequent invasive disease

In chapter 4, it was shown that systemic antibody was raised against the surface protein PspA during both long term carriage with WT-D39 and the clearance of its isogenic mutants, and also showed that a mutant deficient in PspA was cleared from the nasopharynx faster than WT-pneumococci in <14 days compared to >28 days in the WT. In order to test whether a natural carriage of the PspA mutant could also prevent subsequent invasive disease, mice which had cleared the $PspA^-$ colonisation were challenged with WT-D39, at least 2 weeks after the last detected colonies in the nasopharynx. At 24 hours after the acute infection, mice pre-colonised with $PspA^-$ had lower CFUs in the lungs and blood with 80% of mice exhibiting no signs of septicaemia, compared to just 20% in control mice (Fig 5.10). 60% of mice that received the $PspA^-$ pre-colonisation survived to 7 days post invasive challenge compared to just 20% of naive controls (Figure 5.10c, not significant), however, if we assume that all mice which develop moderate septicaemia at 24 hours will eventually succumb to the disease then 70% of pre-colonised and 20% of controls would survive to day 7 ($p=0.0076$), almost identical to the protection observed using the PspA sufficient $Plna^-$ (Fig 5.6).

In order to identify any additive effect of the polysaccharide capsule, the experiment above was repeated but substituting serotype-3 strain A66, as the challenge strain. Differences in CFU between pre-colonised and control mice could not be identified in the nasopharynx, lungs or blood at 48 hours post infection (Fig 5.11, A, B, C), however survival in the immunised group was just 50% (compared to 20% of controls, not significant) (Fig 5.11, D). This result suggests that protection may still be observed in the absence of two immuno-dominant antibodies (PspA and functional capsular antibodies), and indicates there may be additional important prot+ection inducing antigens.

Pneumococcal PspA can be subdivided into three families; family 1 consisting of clades 1 and 2, family 2 which contains clades 3, 4, and 5 and family 3 containing just clade 6 (Hollingshead *et al*., 2000). Similarity in structural homology of the N-terminal regions

of PspA has been shown to increase cross-reaction of anti-PspA antibodies however cross-protective responses in mice challenged with strains possessing different clades may be limited to those within the same clade (Miyaji *et al*., 2002). The homology between strain A66 and D39 which are both clade-2, family one members, would have been expected to have contributed to the cross-serotype protection against future carriage, but this was not found to be the case (Fig 5.2). In contrast, cross-serotype protection against invasive disease was observed in these strains (Fig 5.7 & 5.8). In order to investigate this further it was necessary to repeat this protective study with an alternative challenge strain; 23F which possesses clade-4, family 2, PspA. MF1 mice were pre-colonised with PlnA$^-$ as described previously, and challenged with 1 x 10^6 CFU of 23F. During the acute challenge there were lower bacterial loads, in the nasopharynx and lungs after 3 days, of pre-colonised mice compared to naive mice (Fig 5.12, A, B). However, colonies of 23F were cleared from the lungs and nasopharynx of both control mice and pre-colonised mice at day 14, and septicaemia did not develop in any mice tested, thus survival in both groups was 100% (Fig 5.12, D). Although this challenge was not lethal at this acute dose in MF1 mice, the results from the earlier points in the carriage indicate that cross-reactive antibodies other than those directed against PspA or capsular polysaccharide may have contributed to the lower CFU's observed in precolonised mice.

5.8 Systemic immunity during secondary invasive pneumococcal disease

Further to the discovery of antibodies raised against capsule and conserved pneumococcal protein antigens in chapter 4, it was necessary to describe the humoral response during carriage and subsequent invasive challenge. Mice which had cleared attenuated carriage with PlnA$^-$ (Fig 3.5) were subjected to an invasive WT-D39 challenge at day 28 (Fig 5.5). Both the titre of serum IgM anti-type-2-capsule antibodies and IgG anti-PspA specific antibodies increased throughout carriage (Fig 5.13) as previously described (Fig 4.2). However, at 24 hours post acute challenge there was a reduction in serum type-2 antibody titre compared to day 28 of colonisation (the day of the challenge) ($p=0.0320$), which may be indicative of functional antibody leaving the serum compartment in order to engage antibody in the mucosal surfaces and the lungs (Fig 5.13, A). At 34, and 48 hours the level of antibodies increased in the serum, in line with the recovery of these animals, and pre-colonised mice that survived the acute challenge had constitutively high levels of anti-capsular IgM titre (Fig 5.13,

A) (but not significantly higher than observed during the primary colonisation with PlnA$^-$, Fig 4.3). Levels of IgG anti-PspA titre also initially fell compared to pre-challenge levels in the serum, at 24 hours post infection, and recovered over the next two times points assayed (not significant) (Fig 5.13, B). One week after the challenge all survivors of the PlnA$^-$ colonisation and acute challenge of WT-D39 had levels of anti-PspA that were significantly higher than those at day 28 of the initial colonisation ($p=0.0189$) (Fig 5.13, B). In contrast, no anti-PspA IgG titre could be detected in control mice that received the acute challenge only (data not shown). As the antibody titres after the carriage of PlnA$^-$ were high, the temporal disappearance of antibody from the serum at 24 hours post acute infection in these mice, suggests that antibody may have had an impact on bacterial numbers in the nasopharynx and lungs, which were lower in pre-colonised mice at 24 hours post acute challenge with WT-D39 (Fig 5.5) and WT-A66 respectively (Fig 5.7). Whilst IgM anti-capsular antibody titre correlated directly to nasopharyngeal CFU ($p=0.0083$) during the acute challenge, the anti-PspA titre correlated strongly to both the nasopharyngeal CFU ($p=0.0006$) and lung infection ($p=0.0370$) (Fig 5.14). Although not significant, there was also a trend towards correlation between both antibody titres and disease sign severity scores (data not shown).

Humoral immunity during the cross-serotype acute challenge with A66 (Fig 5.7 & 5.8) was also assessed as described previously. Titres of anti-PspA IgG in pre-colonised mice were higher than control mice at all time points measured, with the greatest difference observed in survivors of the A66 acute challenge one week post infection ($p=0.0275$) (Fig 5.15). Levels of IgG anti-PspA did not correlate with CFU in the nasopharynx, lungs or blood in this instance (data not shown), however 80% of pre-colonised mice that survived the challenge had high titres of PspA, whereas pre-colonised mice that succumbed to the challenge had no detectable anti-PspA titre (data not shown).

5.9 The effect of antibody only on subsequent invasive disease

The introduction of live pneumococci into the nasopharynx has the potential to stimulate many innate and adaptive immune processes which could potentially affect the outcome of subsequent encounters with the organism. In order to assess the effect of antibody alone in protection against an acute pneumococcal challenge, pooled

immune serum from mice that had survived carriage and invasive infection with serotype-2 pneumococci, were administered intraperitoneally (I.P.) one hour prior to an I.P. challenge with WT-D39. Although 100% of mice eventually succumbed to this challenge, the mean survival in passively protected mice was significantly longer (42 hours, compared to 33 hours in control mice, p=0.0039) (Fig 5.16, A). Signs of disease were also delayed in passively protected mice by an average of >9 hours (data not shown, p=0.0256), and there was a trend towards higher CFU's in the blood of control MF1 mice recorded at 24 hours post infection, although this was not significant (Fig 5.16, B).

5.10 Local immunity during secondary invasive pneumococcal disease

In chapter 4, antibodies were identified during the course of nasopharyngeal carriage, both systemically, in serum, and locally in nasal draining cervical lymph nodes of the subclass IgA (Fig 4.9). During the acute challenge with WT-D39 which followed prior colonisation with attenuated PlnA$^-$, IgA could be detected in the nasal mucosa, of pre-colonised but not control mice (Fig 5.17). This antibody was specific for type-2 polysaccharide capsule (Fig 5.17). At time zero, (day 28 of the experiment) there were low amounts of pre-existing IgA antibody levels in some pre-colonised mice, which increased after the acute infection so that all mice at 48 hours had IgA above background levels. In contrast, no capsule specific IgA could be detected in cervical lymph nodes at the same time points (data not shown).

5.11 The detection of IL-17 during carriage and subsequent invasive disease

It has been previously shown that IL-17A secretion is stimulated by pneumococci *in vitro* and *in vivo* during carriage, where high levels of the cytokine correlated to low density of CFU in the nasopharynx (Malley *et al*., 2006; Lu *et al*., 2008). Mice that lacked the IL-17A receptor were also not protected against subsequent carriage of pneumococci following immunisation (Lu *et al*., 2008). The data in this thesis shows that levels of IL-17A were higher in the cervical lymph nodes of mice pre-colonised with PlnA$^-$ pneumococci at 28 days post infection, but not significant) compared to control naive mice (Fig 5.18), and that the concentration of IL-17A (100-300 pg/ml) was comparable to previous studies (Lu *et al*., 2008). Post-invasive challenge, levels of IL-17A increased for the first 24 hours post-challenge in both pre-colonised and naive control mice, but had decreased by 48 hours post infection (Fig 5.18). Pre-colonised

mice, which survived an invasive pneumonia challenge with D39 and were subsequently re-infected with an additional acute (A66) challenge, and showed further increases in levels of IL-17A ($p=0.0498$) (Fig 5.18). In contrast, significant levels of IFN-γ could not be detected above background levels at any time point in the CLN (data not shown).

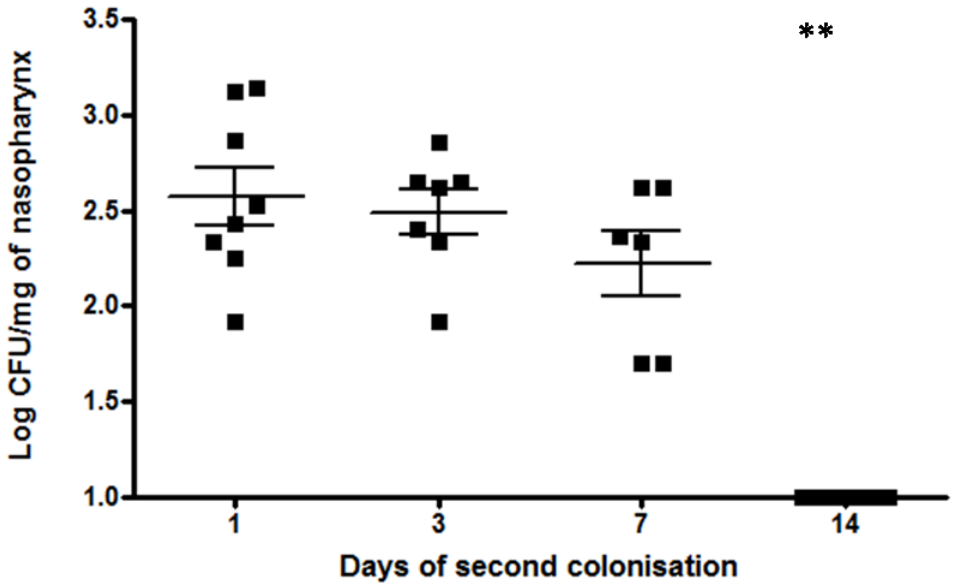

Figure 5.1 The effect of prior colonisation on future carriage. MF1 mice which had cleared PlnA$^-$ serotype-2 carriage from the nasopharynx were re-colonised, on day 28 post infection, with the same carriage dose of parental WT-D39. Shown is the second carriage only, which was monitored for a further 14 days post infection. The density of colonisation is expressed as mean Log CFU/mg of total homogenised nasopharynx ± S.E.M. Each closed square represents one individual mouse that was culled at that time point and data is n=6 animals for each group at all time points, and 3 independent experiments. The lower limit of detection was 1 bacteria per 10^{-1} (Log 1). Statistical significance of CFU compared to day WT-D39 carriage in naive mice was assessed using the Mann-Whitney test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001).

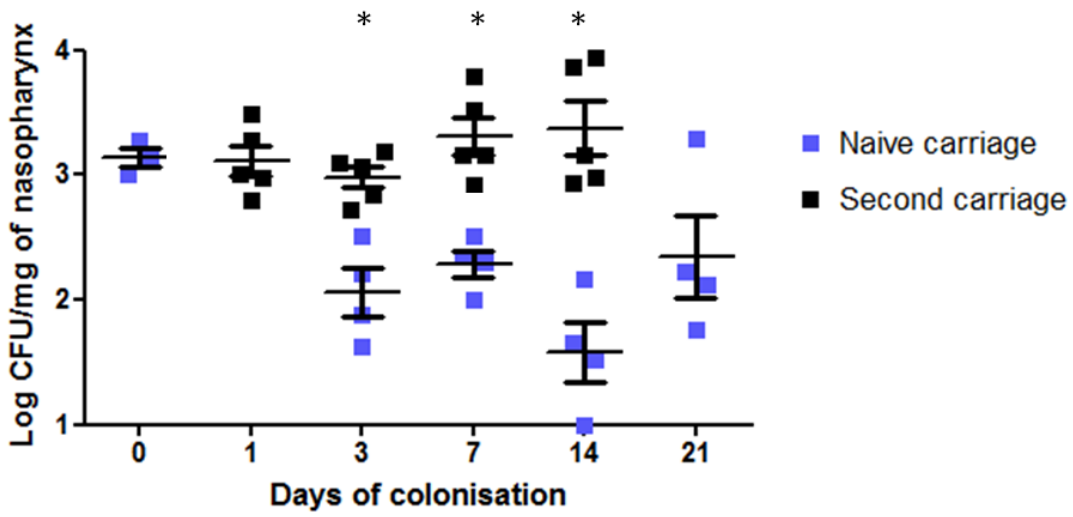

Figure 5.2 The effect of prior colonisation on future (heterologous-serotype) carriage. MF1 mice which had cleared PlnA$^-$ serotype-2 carriage from the nasopharynx were re-colonised, on day 28 post infection, with the same carriage dose of serotype-3 (A66) which was monitored for a further 14 days post infection. Shown is the second carriage (black squares), and the naive carriage of A66 (blue squares), The density of colonisation is expressed as mean Log CFU/mg of total homogenised nasopharynx ± S.E.M. Each closed square represents one individual mouse that was culled at that time point and data is n=5 animals for each group at all time points. The lower limit of detection was 1 bacteria per 10^{-1} (Log 1). Statistical significance was calculated using the Mann-Whitney test, as compared to naive A66 colonisation. * (p=< 0.05).

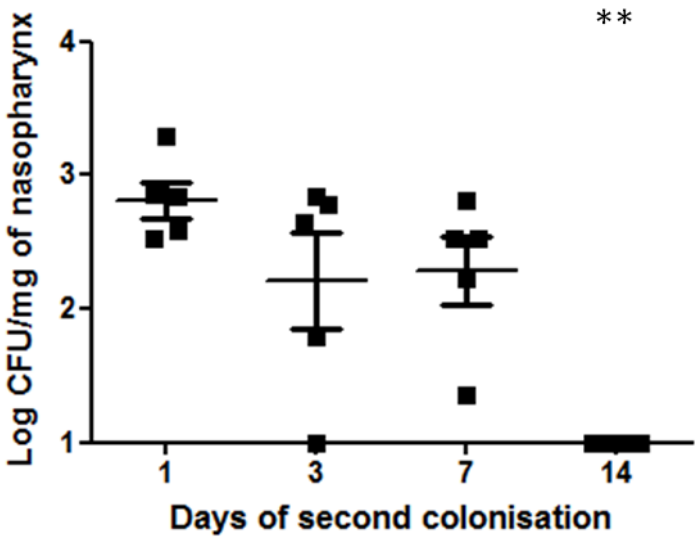

Figure 5.3 The effect of encapsulated carriage on subsequent non-encapsulated carriage. MF1 mice which had cleared PlnA$^-$ serotype-2 carriage from the nasopharynx were re-colonised, on day 28 post infection, with the same carriage dose of non-encapsulated (110.58). Shown is the second carriage only, which was monitored for a further 14 days post infection. The density of colonisation is expressed as mean Log CFU/mg of total homogenised nasopharynx ± S.E.M. Each closed square represents one individual mouse that was culled at that time point and data is n=5 animals for each group at all time point. The lower limit of detection was 1 bacteria per 10^{-1} (Log 1). Statistical significance was assessed using the Mann-Whitney test as compared to naive 110.58 colonisation. ** (p=<0.01).

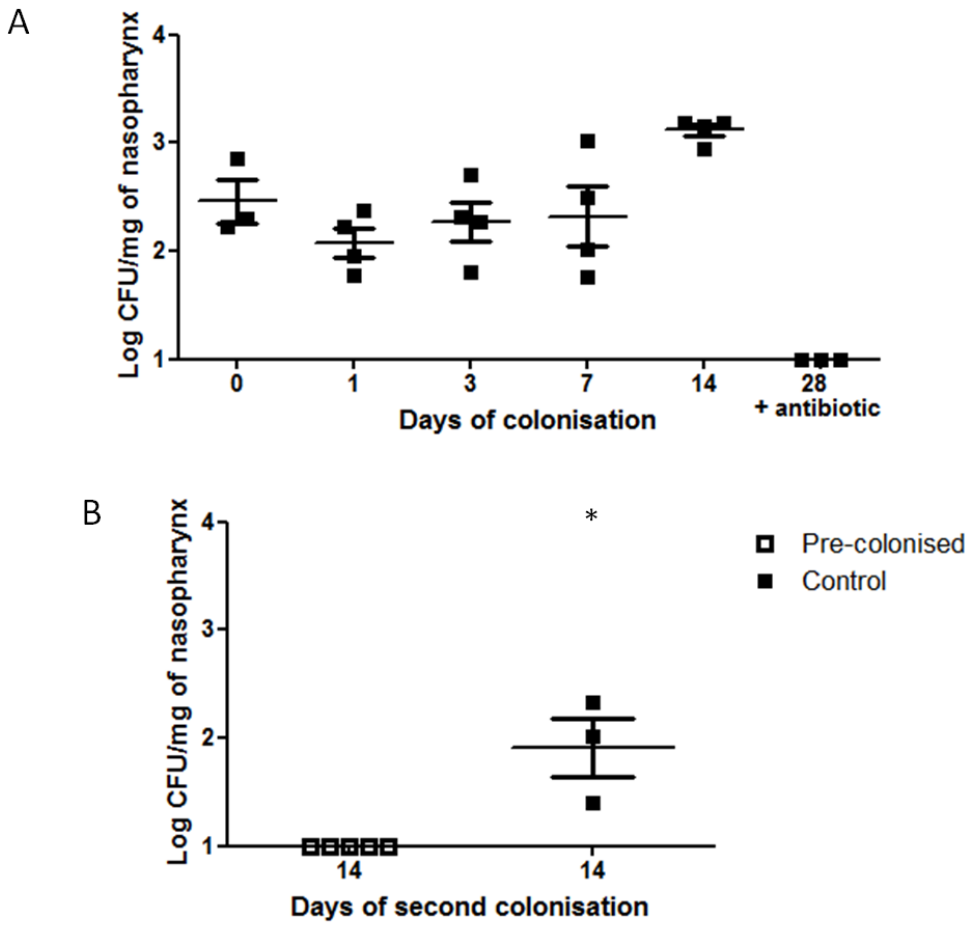

Figure 5.4 The effect of non-encapsulated pneumococcal carriage on subsequent encapsulated carriage. (A) Nasopharyngeal carriage of the non-encapsulated strain, 110.58 (filled squares) was monitored by assessing CFU/mg nasopharyngeal tissue, over 14 days, after which time all colonies in the nasopharynx were cleared by the addition of rifampin by I.P. injection on days 15 and 16. No pneumococci were present before the second colonisation on day 28. Mice which had received 110.58 + rifampin (open squares), and rifampin alone (filled squares) were re-colonised with WT-D39 on day 28 post infection, and the density of colonisation was assessed after a further 14 days (day 42) (B). The density of colonisation is expressed as mean Log CFU/mg of total homogenised nasopharynx ± S.E.M. Statistical differences between pre-colonised and control mice was calculated using the Mann-Whitney test, * ($p =< 0.05$).

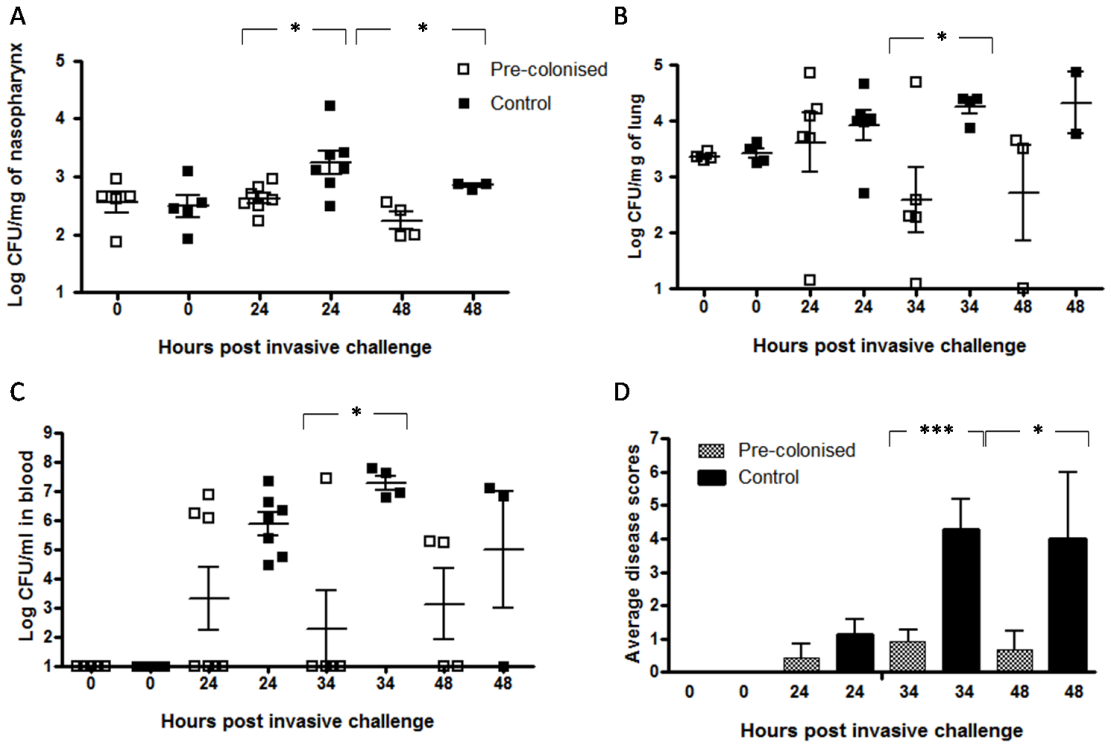

Figure 5.5 Colonisation is protective against subsequent invasive disease. MF1 mice which had cleared PlnA$^-$ serotype-2 carriage from the nasopharynx were re-infected on day 28 post infection, with an acute challenge dose of parental WT-D39 (1 x 10^6 CFU/50 µl. CFU of WT-D39 in control (Filled squares) and precolonised MF1 mice (open squares) were measured in the (A) nasopharynx, (B) lungs and (C) blood. The density of pneumococci is expressed as mean Log CFU/mg of total homogenised tissue, or CFU/ml of blood ± S.E.M. Each closed square represents one individual mouse that was culled at that time point and data is n=7 mice at 24 hours, n=5 mice at 34 hours and n=4 mice at 48 hours. A measure of the severity of infection was given by recording the outward signs of disease of pain score (D), the disease score is represented ± S.E.M. The lower limit of detection was 1 bacteria per 10^{-1} (Log 1). The statistical differences between naive and pre-colonised mice were assessed using the Mann-Whitney test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001).

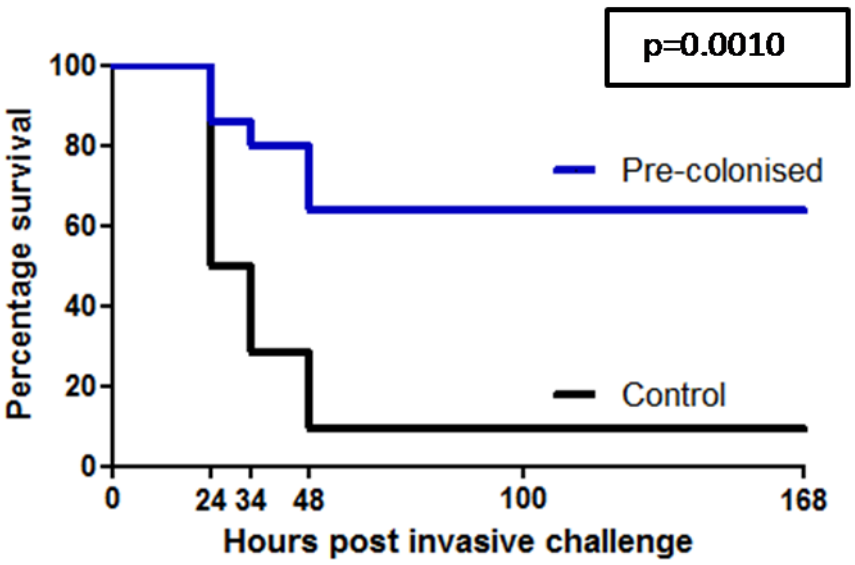

Figure 5.6 Survival of naive and precolonised mice during an invasive pneumococcal challenge. MF1 mice that had been precolonised with PlnA$^-$ (blue line) and naive control mice (black line) were challenged with a normally fatal invasive dose of WT-D39 pneumococci, and were monitored for 7 days post challenge. Statistical significance of the difference in survival curves was assessed using the Log-rank (Mantel-Cox) test.

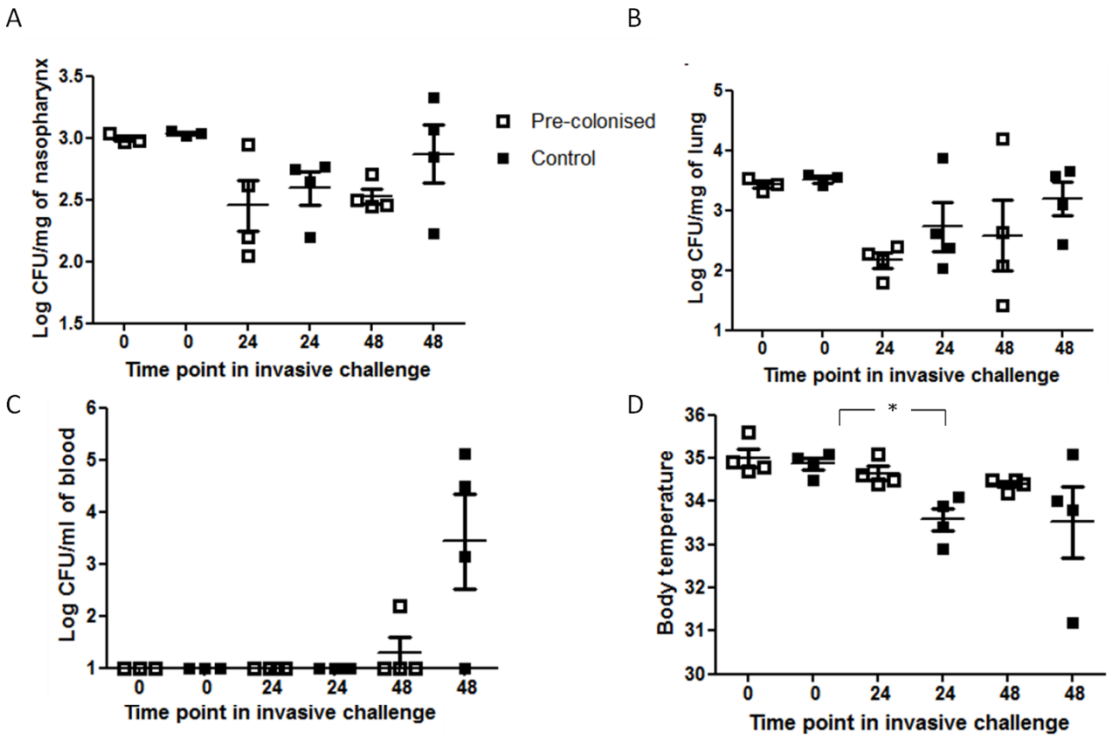

Figure 5.7 Cross serotype colonisation is protective against subsequent invasive disease. MF1 mice which had cleared PlnA$^-$ serotype-2 carriage from the nasopharynx were re-infected on day 28 post infection, with an acute challenge dose of parental WT-A66 (1 x 10^6 CFU/50 µl. CFU of WT-A66 in control (Filled squares) and precolonised MF1 mice (open squares) were measured in the (A) nasopharynx, (B) lungs and (C) blood. The density of pneumococci is expressed as mean Log CFU/mg of total homogenised tissue, or CFU/ml of blood ± S.E.M. Each closed square represents one individual mouse that was culled at that time point and data is n=4 mice at all time points except time zero n=3. A measure of the severity of individual mice infection was given by recording core body temperature at each of the time points (D), which is presented as the mean temperature °C ± S.E.M. .A measure of the severity of infection was given by recording the outward signs of disease of pain score (D), the disease score is represented ± S.E.M. The lower limit of detection was 1 bacteria per 10^{-1} (Log 1). The statistical differences between naive and pre-colonised mice were assessed using the Mann-Whitney test, *(p=<0.05), ** (p=<0.01), *** (p=<0.001).

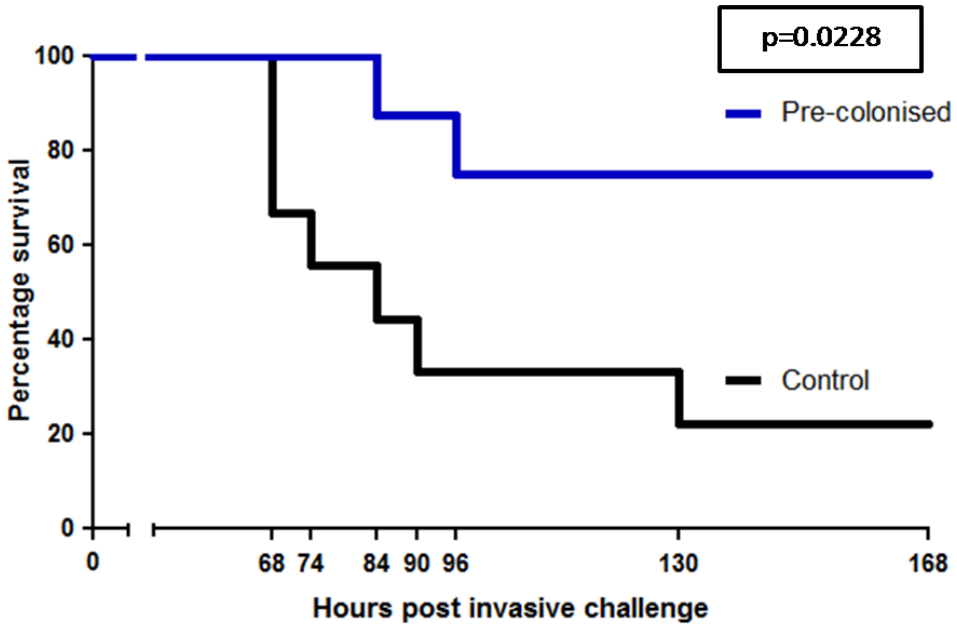

Figure 5.8 Cross serotype protection against invasive pneumococcal disease. MF1 mice that had been pre-colonised with PlnA$^-$ (blue line) and naive control mice (black line) were challenged with a normally fatal invasive dose of WTA66 pneumococci, and were monitored for 7 days post challenge. Statistical significance of the difference in survival curves was assessed using the Log-rank (Mantel-Cox) test.

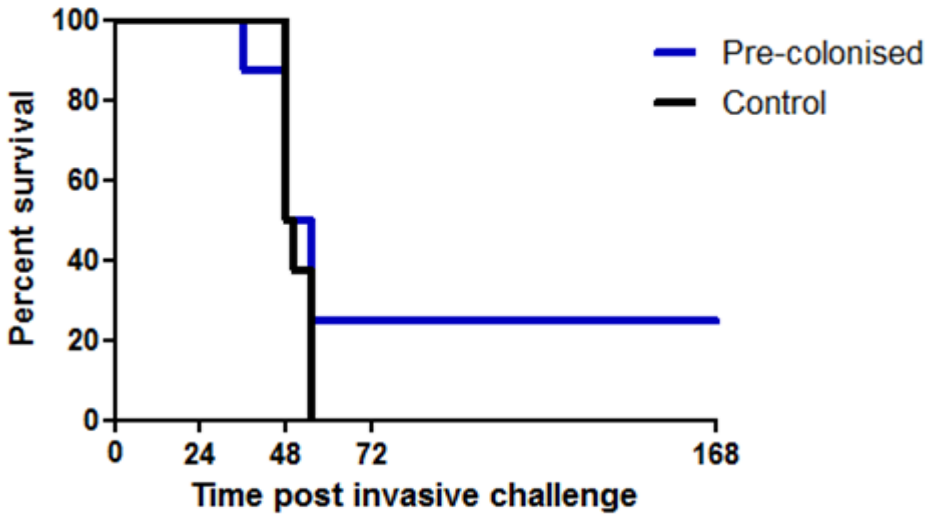

Figure 5.9 Non-encapsulated protection against invasive disease. Nasopharyngeal carriage of the non-encapsulated strain, 110.58 was monitored by assessing CFU/mg nasopharyngeal tissue, over 14 days, after which time all colonies in the nasopharynx were cleared by the addition of rifampin by I.P. injection on days 15 and 16. No pneumococci were present before the invasive challenge initiated on day 28. Shown is the survival of MF1 mice after receiving an invasive dose of WT-D39 for 7 days post infection, which had received either 110.58 colonisation + rifampin (blue line), or rifampin alone (black line), n=5 pre-colonised mice and n=4 naive mice. Statistical significance of the difference in survival curves was assessed using the Log-rank (Mantel-Cox) test.

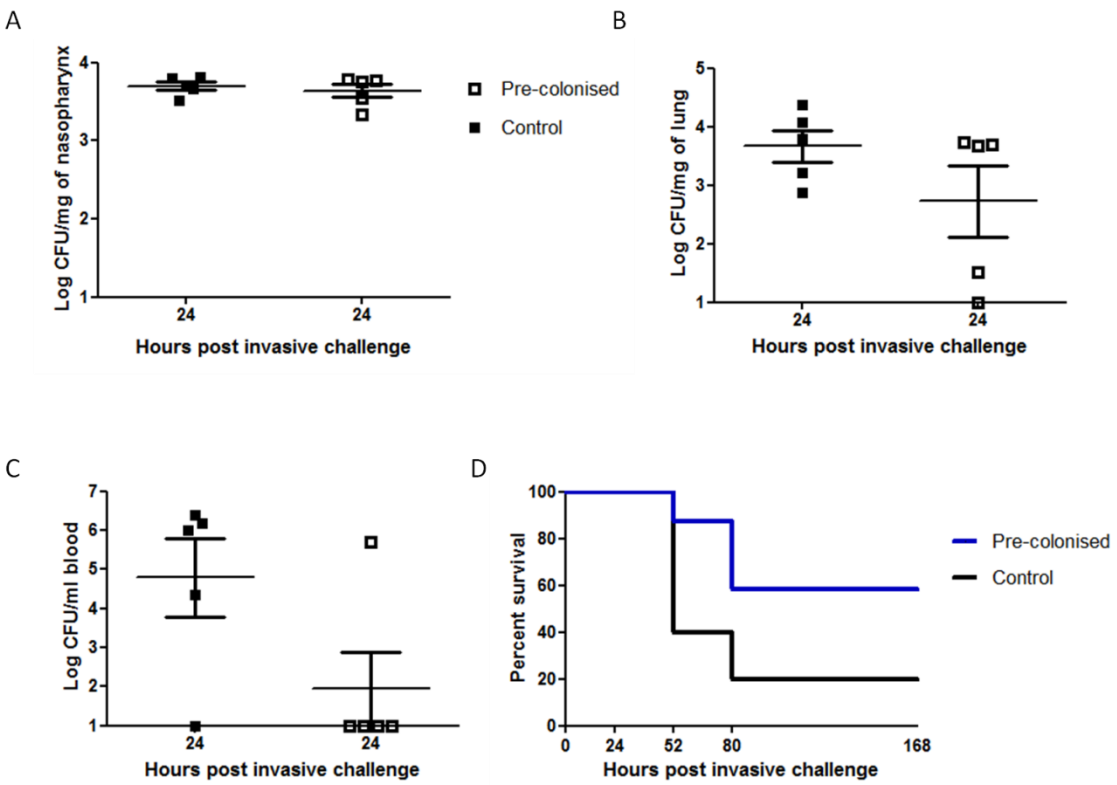

Figure 5.10 Protection against invasive pneumococcal disease with PspA$^-$. MF1 mice which had cleared PspA$^-$ serotype-2 carriage from the nasopharynx were re-infected on day 28 post infection, with an acute challenge dose of parental WT-D39 (1 x 10^6 CFU/50 µl. CFU of WT-D39 in control (Filled squares) and precolonised MF1 mice (open squares) were measured in the (A) nasopharynx, (B) lungs and (C) blood. The density of pneumococci is expressed as mean Log CFU/mg of total homogenised tissue, or CFU/ml of blood ± S.E.M. The lower limit of detection was 1 bacteria per 10^{-1} (Log 1). Each closed square represents one individual mouse that was culled at that time point and data is n=5 mice at 24 hours in each group. n=5 additional mice from each group were monitored for a further 7 days for survival (D). The statistical differences between naive and pre-colonised mice were assessed using the Mann-Whitney test, and statistical significance of the difference in survival curves was assessed using the Log-rank (Mantel-Cox) test.

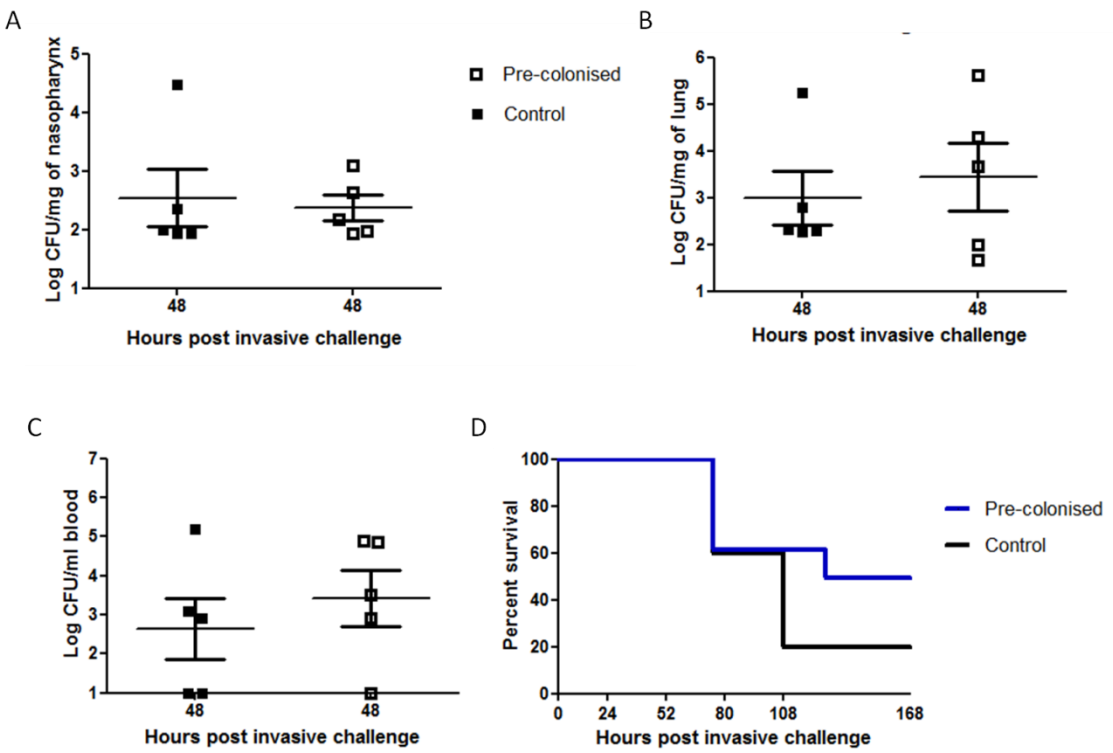

Figure 5.11 Protection against invasive pneumococcal disease with PspA$^-$ heterologous serotype challenge. MF1 mice which had cleared PspA$^-$ serotype-2 carriage from the nasopharynx were re-infected on day 28 post infection, with an acute challenge dose of WT-A66 (1 x 10^6 CFU/50 µl. CFU of WT-A66 in control (Filled squares) and precolonised MF1 mice (open squares) were measured in the (A) nasopharynx, (B) lungs and (C) blood. The density of pneumococci is expressed as mean Log CFU/mg of total homogenised tissue, or CFU/ml of blood ± S.E.M. The lower limit of detection was 1 bacteria per 10^{-1} (Log 1). Each closed square represents one individual mouse that was culled at that time point and data is n=5 mice at 48 hours in each group. n=5 additional mice from each group were monitored for a further 7 days for survival (D). The statistical differences between naive and pre-colonised mice were assessed using the Mann-Whitney test, and statistical significance of the difference in survival curves was assessed using the Log-rank (Mantel-Cox) test.

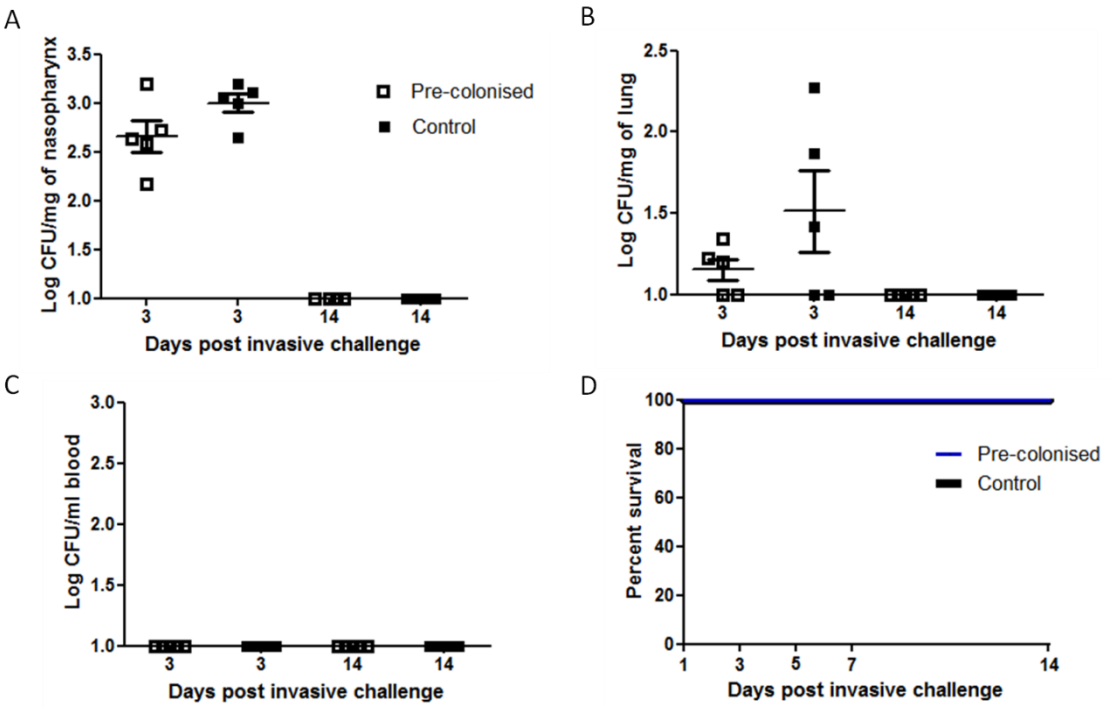

Figure 5.12 Cross serotype protection against invasive pneumococcal disease caused by 23F. MF1 mice which had cleared PlnA$^-$ serotype-2 carriage from the nasopharynx were re-infected on day 28 post infection, with an acute challenge dose of WT-23F (1 x 10^6 CFU/50 µl. CFU of WT-23F, in control (Filled squares) and precolonised MF1 mice (open squares) were measured in the (A) nasopharynx, (B) lungs and (C) blood. The density of pneumococci is expressed as mean Log CFU/mg of total homogenised tissue, or CFU/ml of blood ± S.E.M. The lower limit of detection was 1 bacteria per 10^{-1} (Log 1). Each closed square represents one individual mouse that was culled at that time point and data is n=4 mice at 3 days and 14 days in each group. n=4 mice from each group were monitored 4 days for survival (D). The statistical differences between naive and pre-colonised mice were assessed using the Mann-Whitney test, and statistical significance of the difference in survival curves was assessed using the Log-rank (Mantel-Cox) test.

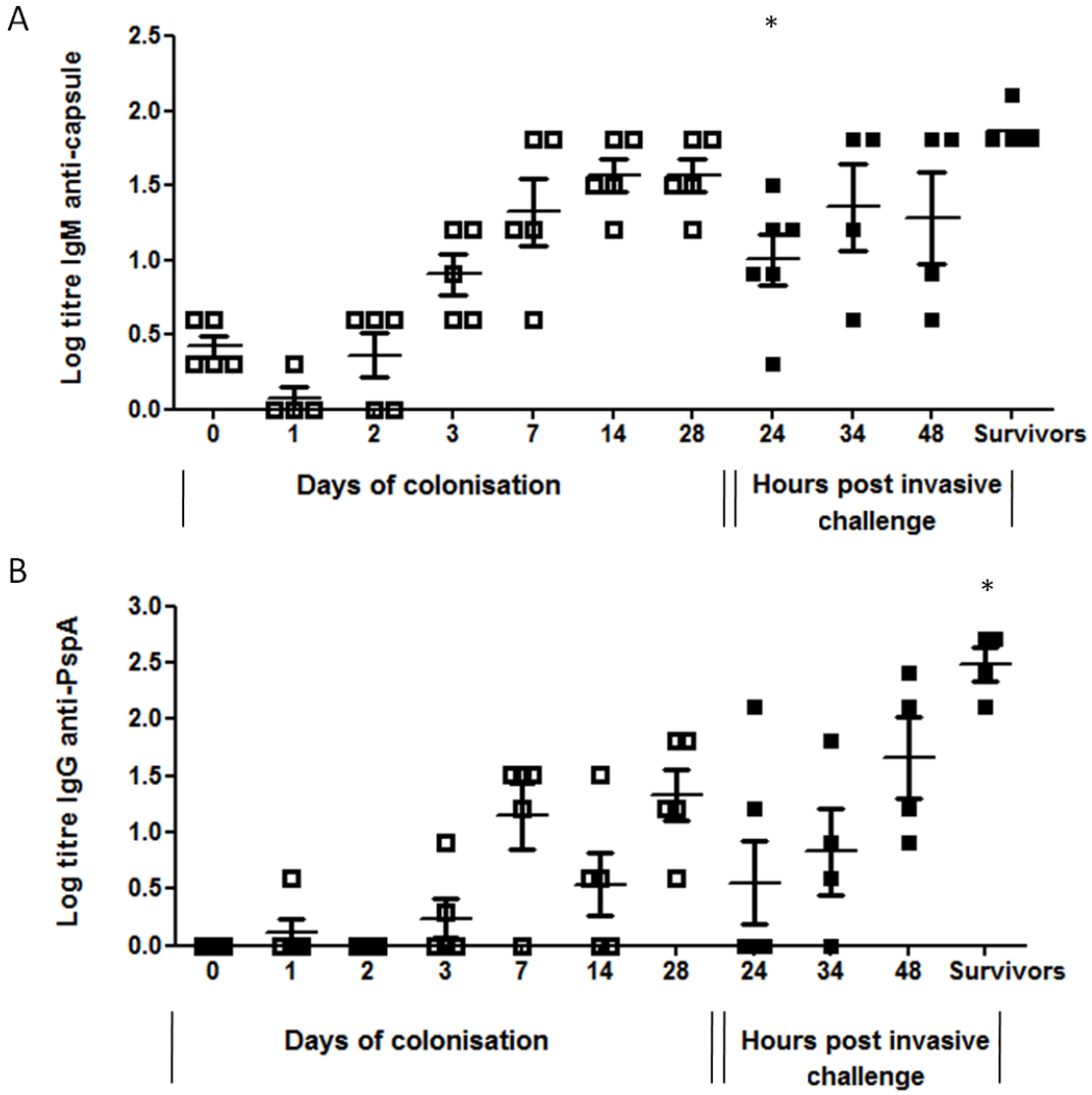

Figure 5.13 Systemic antibody during colonisation and subsequent invasive disease. Antibody titres of (A) IgM anti-capsular antibodies and (B) IgG anti-PspA antibodies were assessed by ELISA, in MF1 mice during pre-colonisation with PlnA$^-$ (open squares) and subsequent acute challenge with WT-D39 (filled squares). Titres of control naive mice are not shown. Each square represents the analysis of one mouse, n=4 for all time points. Mean log endpoint titres ± S.E.M. of duplicate wells analysed on two separate days, were calculated as the last dilution to reach 0.1 absorbance at 405 *nm*. Statistical significance of changes in antibody titres compared to day 28 of the initial colonisation were assessed using the Mann-Whitney test, *(P =<0.05).

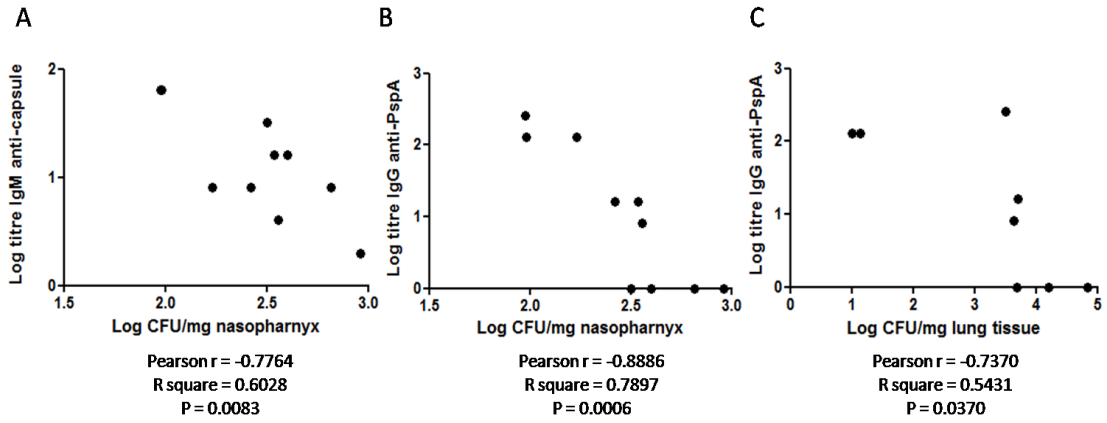

Figure 5.14 Correlation between pneumococcal CFU in the nasopharynx and lungs, and serum antibody titre following colonisation and invasive D39 challenge. MF1 mice challenged pre-colonised with PlnA$^-$ and challenged with and acute dose of WT-D39. Anti-capsular antibody titre as a function of nasopharyngeal CFU during the invasive challenge is represented in (A). Anti-PspA IgG titre as a function of nasopharyngeal CFU and lung CFU are shown in (B) and (C) respectively. Pearson correlation (which assumes Gaussian distribution) and statistical significance was calculated using Graphpad prism software.

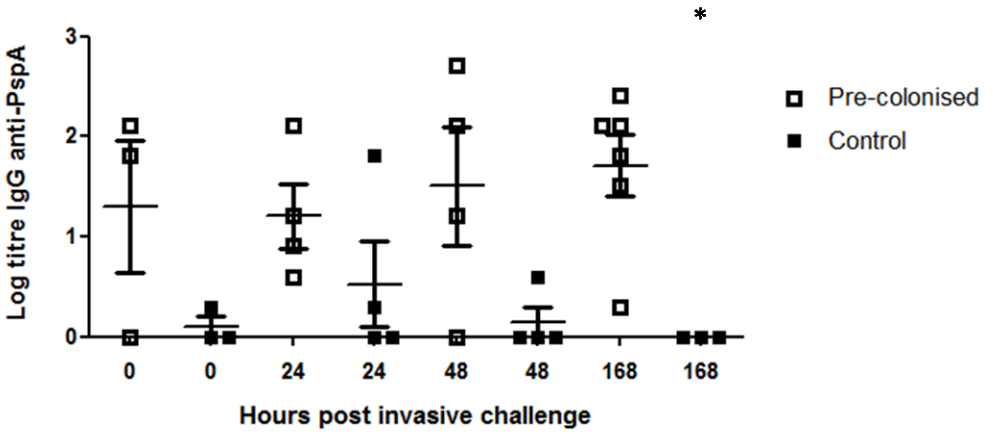

Figure 5.15 Systemic antibody during colonisation and subsequent cross serotype invasive disease. Antibody titres of IgG anti-PspA antibodies were assessed by ELISA, during an acute challenge with WT-A66, after pre-colonisation with PlnA$^-$ (open squares) and naive mice (filled squares). Each square represents the analysis of one mouse. Mean log endpoint titres ± S.E.M. of duplicate wells analysed on two separate days, were calculated as the last dilution to reach 0.1 absorbance at 405 *nm*. Statistical significance of differences between pre-colonised and control mice were assessed using the Mann-Whitney test, *(p=<0.05).

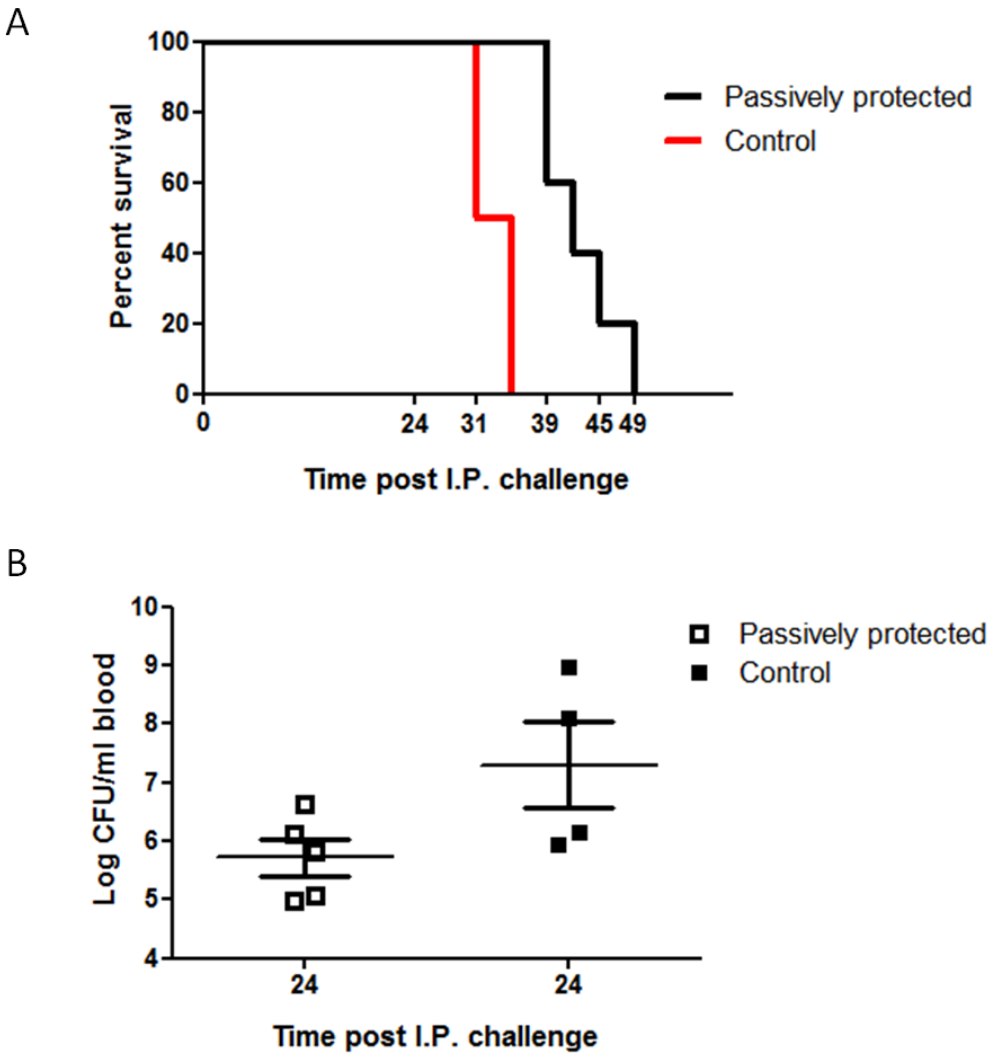

Figure 5.16 Passive protection with immune serum. MF1 mice receive 100 µl of pooled immune serum intraperitoneally (or PBS in control mice), followed one hour later by I.P. infection of 5 x 10^5 CFU of WT-D39. n=5 mice that received immune serum (black line) and control mice (red line) were monitored for survival (A). Additional control mice (filled squares, n=4) and immunised mice (open squares n=5) were culled at 24 hours post infection and the density of pneumococci in the blood is expressed as mean Log CFU/ml ± S.E.M. (B). The statistical differences in CFU between the two groups were assessed using the Mann-Whitney test, and the difference in survival curves was assessed using the Log-rank (Mantel-Cox) test.

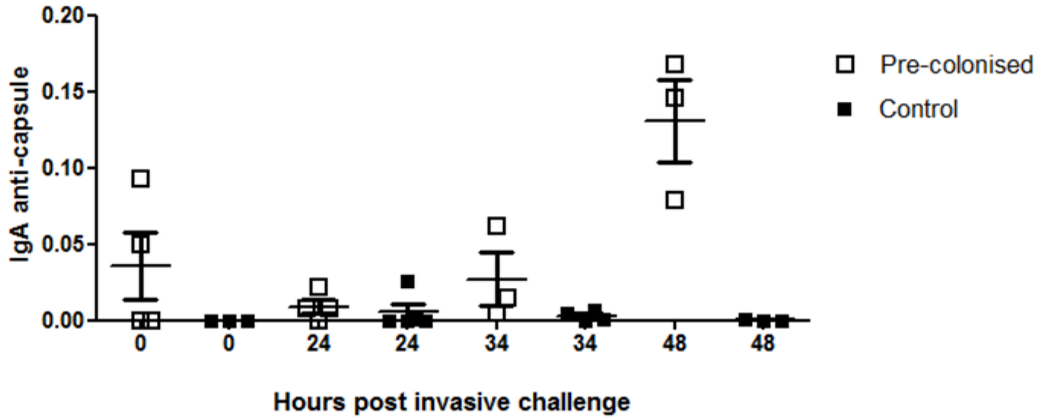

Figure 5.17 IgA from nasal mucosal tissue during invasive WT-D39 challenge. MF1 mice which had cleared colonisation of PlnA$^-$, were re-infected with WT-D39 pneumococci 28 days later, as described previously. Equal volumes (50 µl) of supernatants from nasal mucosal tissue homogenates taken during the acute infection in pre-colonised mice (open squares) and control naive mice (which had the same acute challenge but no pre-colonisation) (filled squares) were used as primary antibody source for ELISA, on microtitre plates coated with type-2 polysaccharide. Relative antibody concentrations are shown as the absorbance, measured at 405 *nm*. The statistical differences between the two groups were assessed using the Mann-Whitney test.

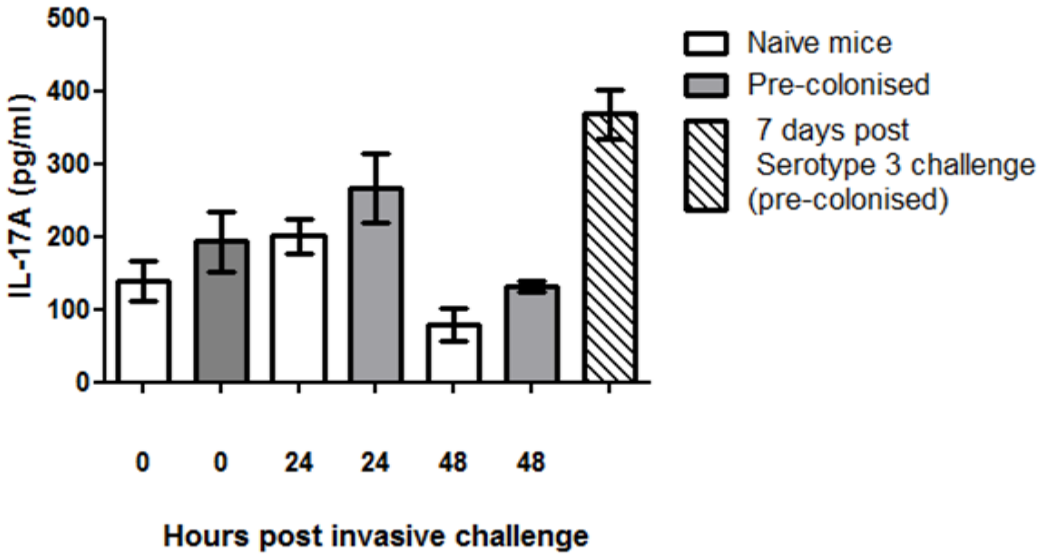

Figure 5.18 IL-17A from CLN following colonisation and during invasive WT-D39 challenge. The levels of IL-17A were detected by ELISA, from cervical lymph node homogenate-supernatants made after the colonisation of PlnA$^-$, and during invasive challenges with WT-D39 and WT-A66. The concentration of IL-17A was compared to a standard curve made from purified IL-17A and shown is the mean pg/ml of IL-17A ± S.E.M. for n=5 mice per time point for each group. Control naive mice (open bars) received the acute infection of WT-D39 only, whereas mice precolonised with PlnA$^-$ (filled bars) received both the WT-D39 challenge at time zero (day 28 of the experiment), and a further A66 challenge 7days later (day 35 of the experiment), with samples of CLN taken after a further 7 days (diagonal hatched bars) (Day 42). The statistical differences between the two groups and the different days during the acute infection were assessed using the Mann-Whitney test.

Initial carriage strain	Duration of naive carriage	Challenge strains WT-D39	WT-A66	23F	110.58
WT-D39	>28 days				
WT-A66	>21 days				
PlnA-	<14 days	✓ ✓	✓ ✗	✓	✓
NanA-	<7 days	✓			
NanB-	<14 days	✓			
PspA-	<14 days	✓	✓		
110.58	>14 days	✗ ✓			
Δ4	<14 days				

✓✗ Protection against future invasive disease

✓✗ Protection against future carriage

Table 5.1

Summary of carriage and its protective responses. Carriage strains used in naive mice appear on the 1st column, next to the duration for which they were detected in the nasopharynx of MF1 mice (column 2). The challenge strains used for subsequent carriage or invasive disease are listed in columns 1-4. The outcome of initial carriage strain and challenge strains can be found by matching the columns and rows with protection against carriage (red x or tick) or invasive disease (blue x or tick) shown for all combinations used in this thesis.

5.12 Conclusions

Nasopharyngeal carriage of the PLY negative D39 mutants were chosen as the preferred initial colonisation event to model the protection against subsequent pneumococcal challenges, as they were cleared naturally from the nasopharynx in a convenient timescale (<14 days), and broadly displayed the same level of host immune responses as in WT carriage. Due to the Home Office regulations it was not permitted to re-infect any mice which already had existing ongoing pneumococcal infection, and therefore $PlnA^-$ was able to substitute the eventual clearance of a sufficient strain which may only have disappeared naturally from the nasopharynx after a period of months.

The data in this thesis is the first description of the timeframe of pneumococcal clearance during a subsequent carriage event in mice, following a single immunising dose of natural carriage. Whilst serotype-2 colonisation was not able to prevent future acquisition of carriage of the same serotype, it significantly shortened the length of the second carriage, reducing its duration from >28 days in naive mice, to <14 days after pre-colonisation (Fig 5.1). This result concurs with carriage in humans, that are often repeatedly colonised <2 years of age (Hill *et al*., 2008). Although carriage is highest in this population, invasive pneumococcal disease remains a relatively rare occurrence. One reason for this could be that the amount of anti-capsular antibody produced in human carriage which is required to protect against subsequent homologous colonisation of serotype 14 was shown to be 5 µg/ml (Goldblatt *et al*., 2005b), which is over 10 times greater than the level required to protect against invasive disease (Jodar *et al*., 2003). This would allow individuals to be repeatedly colonised but still be protected from invasive disease, and explains why carriage events in adults (who produce superior anti-capsular antibody responses) are shorter in duration than in children, and is one reason why carriage rates in the adult population may often be underestimated (Goldblatt *et al*., 2005b). The high levels of IgG antibody needed to protect against carriage of *S. pneumoniae* and another common coloniser of the nasopharynx, *H. influenza* (Fernandez *et al*., 2000), is speculated to arise from the difficulty of antibody leaving the serum and entering mucosal secretions (Goldblatt *et al*., 2005b). This may explain why the initiation of carriage is not simply prevented altogether and clearance may occur only once a boost in titre occurs upon second exposure to pneumococci. From the analysis of immune responses following acute

challenges with pneumococci it is clear that only the IgG anti-PspA titre receives a significant boost response, and not IgM anti-capsular antibody, and thus capsule seems unlikely to play a significant role in future encounters. However, contrary to this and results from other studies, these data have shown that the fate of future carriage events are dependent on capsular type, as homologous serotype-2 carriage is cleared following pre-colonisation with the same serotype, but serotype-3 successfully re-colonised 100% of mice which had carried serotype-2. Although there is human epidemiogical evidence that natural carriage events of certain serotypes offer specific protection (Weinberger *et al*., 2008), protection against carriage in mice is also complex, and was shown not to depend on serotype alone, as both homologous and heterologous serotypes could re-colonise, although carriage rates were significantly different between the serotypes used (Malley *et al*., 2005b). The protection against re-colonisation in rats was also slightly enhanced with homologous compared to heterologous carriage (Malley *et al*., 2001). As protection against carriage can also be elicited with vaccines that lack polysaccharide capsule completely (Malley *et al*., 2001) it is unlikely that capsular antibody is the sole determinant of protection in the nasopharynx. Indeed, I have shown that both the non-encapsulated carriage could prevent encapsulated carriage, and *vice versa* (Fig 5.4 & 5.3 respectively) and thus other non-capsular antigens must be considered to have an effect on the outcome. PspA is one such antigen which has long been considered to have vaccine potential (Briles *et al*., 2000b), as antibodies produced by immunisation of PspA are passively protective against sepsis, and colonisation in mice (Briles *et al*., 2000c; Briles *et al*., 2000a). Furthermore, in a model of human experimental carriage it was pre-existing antibodies against PspA in both the serum and saliva, and not anti-capsular antibodies that were predictive of protection against subsequent carriage (McCool *et al*., 2002). When PspA from different serotypes belong to the same clade, protection between them may be even stronger (Miyaji *et al*., 2002), however, despite both D39 and A66 expressing clade-2 PspA, protection against future carriage was not observed in this scenario (Fig 5.2). This finding is in line with a recent study which found that although PspA was the immuno-dominant antibody detected during carriage, mice that carried PspA deficient pneumococci were still protected from subsequent colonisation with a heterologous strain (Roche and Weiser, 2010), which suggests that more minor pneumococcal antigens also generate cross reactive antibodies which contribute to this effect.

It is notable that the non-encapsulated strain (110.58), which produced comparatively poor humoral immune responses (Fig 4.4), offered significant protection against a subsequent encapsulated carriage (Fig 5.4), whereas the clearance of PlnA$^-$, which elicited a strong humoral response could not protect against encapsulated serotype-3 carriage. This result is perhaps surprising, as it might be expected that cross-reactive antibodies to surface proteins would be more readily created in the absence of the polysaccharide capsule, and similarly, for antibodies to bind subsequently better to non-encapsulated bacteria. It has been shown previously that resistance to subsequent infections in the presence of pre-existing antibodies was not reliant on the presence of antibodies raised against protein virulence factors such as PspA, but was dependent capsular polysaccharide type, and that it was the susceptibility to the deposition of complement on the surface of the bacteria and not antibody binding that was crucial to protection (Abeyta *et al*., 2003). In this study a type-3 capsule switched mutant with a type-2 capsule bound less complement than the type-2 parent, but was still less virulent than a WT serotype-3 strain, WU2, therefore the genetic background of the strain is also vital, as it affects complement deposition differently even in non-encapsulated pneumococci (Abeyta *et al*., 2003). It may also be the case that anti-protein antibodies raised against non-capsulated strains simply do not bind well to encapsulated bacteria and *vice versa*, because different epitopes are accessible during first encounter with the pneumococcus which are then obscured either by the presence of capsule, or simply a thicker capsule (as is the case with serotype-3, A66), during secondary exposure. This hypothesis might explain why the non-encapsulated colonisation could not prevent subsequent invasive disease with WT-D39, and may also explain why PlnA$^-$ colonisation could not prevent carriage with A66 despite cross reactivity between their PspA types.

In contrast to the effects observed during the re-colonisation studies, both the cross-serotype and same serotype pre-colonisation protected mice equally well against subsequent invasive challenges (Fig 5.5 – Fig 5.8), and this is supported by the fact that the titre of anti-PspA antibody and not anti-capsular antibody correlated with the subsequent pneumonia in the lung (Fig 5.14). Protection against subsequent invasive disease in mice has been previously shown by diverse immunisation methods which are not dependent on pneumococcal capsule components and show levels of protection which are not dissimilar to those presented in this thesis (Basset *et al*., 2007; Malley *et*

al., 2001; Malley *et al*., 2006; Malley *et al*., 2005b; Roche *et al*., 2007). However, standard intranasal immunisation protocols in mice usually require multiple doses of the immunogen, even when using live pneumococci, in order to show the same level of protection as observed in this thesis (Malley *et al*., 2005b; Lu *et al*., 2008). Furthermore, non-live vaccines often require the addition of cholera toxin as a mucosal adjuvant which by itself offers non-specific protection (Malley *et al*., 2001). It is unclear how the dynamics of these immunisation protocols could affect local immunity, as it is clear that the immune response to a single dose of natural carriage itself is relatively non-stimulatory event. These factors may bias the immunisation to be more dependent on cellular rather than antibody based immunity, and thus the experiments described by Malley *et al* may not be extrapolated easily to natural carriage in humans, as human carriage must develop from small numbers of bacteria. It is interesting that the proposed mechanism for protection in the majority of the above mentioned studies is independent of antibodies but dependent on CD4+ T-cells and in particular the cytokine IL-17A (Malley *et al*., 2005b; Malley *et al*., 2006; Basset *et al*., 2007; Lu *et al*., 2008). In contrast, protection against both carriage and invasive disease with a single dose of attenuated carriage, (which is most similar to the results presented in this chapter), showed that humoral immunity (although not capsular specific) was indispensible to protection (Roche *et al*., 2007). In general the antibody response to attenuated pneumococci was better than WT strains, which is speculated to be a consequence of their clearance from the nasopharynx (Roche *et al*., 2007). This provides an explanation for the comparatively poor IgG and IgM response in the non-encapsulated 110.58, which is carried in the nasopharynx in increasing amounts >14 days, and cleared by artificial means (antibiotics), and thus was the least effective immunising strain against the invasive challenge (Fig 5.9).

The impact of antibody alone on the protection against pneumococcal disease can be measured easily by the passive protection of immune sera from colonised mice. The I.P. injection of serum from mice that had survived colonisation and invasive challenge was able to significantly delay (although not prevent) the time of death, with just a single dose given before the challenge (Fig 5.16). However, despite being identified as the immune-dominant antigen in this and other studies (Roche and Weiser, 2010), live carriage of a PspA deficient mutant was able to protect against subsequent invasive disease. As homologous protection with the PspA mutant was only moderately more

effective than heterologous protection (Fig 5.10, Fig 5.11), this suggests that like the protection against carriage, cross-reactive antibodies other than capsule and PspA are also good correlates of protection.

IL-17A produced locally in the CLN was enhanced by the presence of pneumococci *in vivo* (Fig 5.18) which concurs with previous studies (Lu *et al*., 2008; Malley *et al*., 2006). Sources of IL-17A in the CLN may be from a subset of CD4+ T-cells designated Th17 (Harrington *et al*., 2006) or gamma delta (γδ) T-cells (Sutton *et al*., 2009). γδ T-cells are part of the innate immune system contributing IL-17A expression initiated by pathogens on toll-like receptors (Martin *et al*., 2009), but in addition to IL-1β, IL-21 and IL-23 (Langrish *et al*., 2005) IL-17A can also drive the expansion and production of IL-17A from Th17 CD4+ T-cells (Sutton *et al*., 2009). In the upper airway IL-17A can recruit macrophages (Sergejeva *et al*., 2005) and neutrophils (Kolls and Linden, 2004), which have been identified to be important in reducing colonisation in naive mice, and subsequent carriage respectively (Zhang *et al*., 2009). The relative lack of IL-17A seen after the colonisation only, compared to naive mice may be due to the low levels of bacterial load during carriage, and the absence of a robust inflammation identified during asymptomatic carriage (van Rossum *et al*., 2005). However it may also be due to the lack of PLY ($Plna^-$ was used as the initial coloniser or immunisation in this model), as PLY has been shown to induce IL-17A expression both *in vitro*, and *in vivo* (McNeela *et al*., 2010). PLY deficient bacteria cause less inflammation in the lungs and do not cause death in MF1 mice (Kadioglu *et al*., 2000). The higher levels of IL-17A observed during the invasive challenges may therefore reflect both the presence of PLY, and the higher bacterial load. It may ultimately be desirable to prevent a large infiltration of neutrophils into the upper respiratory tract during carriage, which are responsible for significant tissue lung injury (Kadioglu *et al*., 2000), and allow this semi-commensal bacteria to stay within the nasopharynx and not cause progression towards invasive disease.

Chapter 6: Discussion

The work described in this thesis is part of a broader aim to inform vaccine development against all forms of pneumococcal disease, which carry a significant burden of morbidity and mortality, and a considerable financial cost to society. As is the case with many bacterial pathogens, renewed interest in pneumococcal research has come from the advent of antibiotic resistance strains. Currently as many as 15-30% of all pneumococcal strains display some form of antibiotic resistance, commonly penicillin or erythromycin resistance as well as those strains which show multi-drug resistance (Lynch and Zhanel, 2009a). Antibiotic resistance, pneumococcal vaccination and nasopharyngeal carriage are linked, as the initial impact of penicillin antibiotics changed the landscape of pneumococcal serotypes in the population, reducing the incidence of so-called 'epidemic strains' (serotypes 1, 2, 3, and 5), which were rarely associated with nasopharyngeal carriage, leading to the emergence of serotypes 4, 6, 9, 14, 18, 19, and 23, which are commonly associated with both invasive disease and carriage (Feikin and Klugman, 2002). The greater amount of pneumococcal exposure to antibiotics which is the case in carriage strains (antibiotics have varying degrees of efficacy against carriage) provided selective pressure which favours the appearance of resistant strains (Nuermberger and Bishai, 2004). Furthermore, whilst pneumococci are in the upper respiratory tract they take on a transparent phenotype, expressing lesser amounts of polysaccharide capsule, as capsular material can be a barrier to bacterial competence and it is likely that pneumococci have become adept in exchanging DNA (and potential antibiotic resistant genes) with other commensal strains whilst in the nasopharynx (Weiser and Kapoor, 1999). The current polysaccharide conjugate vaccine has helped reduced the burden of antibiotic resistance among the serotypes contained in the vaccine, however, one major weakness of the polysaccharide specific immunisations is that whilst they may reduce the prevalence of serotypes contained in the vaccine they inevitably induce the subsequent replacement of non-vaccine serotypes in the population, so that although vaccine serotypes are reduced, the overall rate of carriage is unchanged compared to non-vaccinated individuals (Mulholland, 2000). This is also the case with antibiotic resistant strains, and the introduction of the polysaccharide conjugate vaccine has led to the emergence of multidrug resistant (non-vaccine type) pneumococci such as serotype 19A, which is an increasingly problematic

serotype today (van Gils *et al*., 2010). Despite plans to extend the scope of the current PCV-7 vaccine to include 10 or even 13 valent formulas, the emergence of non-vaccine serotype, multi-drug resistant pneumococcal strains will remain a constant issue until all (>90 serotypes) are covered by vaccination, a fact which has led to greater urgency to find a better immunisation strategy. PREVNAR, the current polysaccharide conjugate vaccine is composed of polysaccharide from the 7 most common serotypes conjugated to a non-toxic diphtheria toxin analogue CRM_{197}, and whilst it is the most efficacious vaccine to date, especially in children <5 years of age, it remains inefficient and expensive to produce and to include available serotypes in the vaccine would be untenable. Observational studies of herd immunity offered by the conjugate vaccine, which allows the non-vaccinated population to benefit from the reduced prevalence of nasopharyngeal carriage (the reservoir of bacterial transmission) in the population, has led to a particular scrutiny of the effects of vaccination on carriage. Long term follow up on trials with PCV-7, are limited, and show only modest protection against vaccine type incidence of carriage after 2 years, 10% *vs* 17%, which again was offset by an increase in non-vaccine serotypes compared to controls (Millar *et al*., 2006). There is also evidence that the dynamics of protection against carriage with the conjugate vaccine are different from both the data presented here and human studies of natural carriage in unimmunised adults, where subsequent carriage is permitted for some time but then prematurely (compared to naive or earlier carriage events) cleared from the nasopharynx (Goldblatt *et al*., 2005b). In contrast, the vaccine prevents reacquisition of carriage (Dagan *et al*., 2005). It is arguable which one of the two situations is optimal for protection, as the persistance of an attenuated carriage strain may stimulate mucosal immunity, as was demonstrated here and eslewhere, which could provide cross-serotype protection against both vaccine and non-vaccine serotypes. Furthermore, the protection offered by the conjugate vaccine may be more effective against invasive pneumococcal disease (with accompanying septicaemia) than against bacterial non-invasive pneumonia, in which the route of immunisation (intra-muscular) may be a factor in the compartmentalisation of the immune response (Cutts *et al*., 2005). Intranasal immunisation as demonstrated here and in many other mouse models, can induce a protective response to both carriage and systemic challenges which in some cases is more effective than immunisation by other routes (Hanniffy *et al*., 2007). However a poor mucosal and systemic antibody response observed by an inhaled 23-valent polysaccharide vaccine in humans highlights both the poor immunogenicity of

polysaccharides alone, and the need for a mucosal adjuvant to stimulate a protective response (Gordon *et al*., 2008). In mice such an adjuvant could be cholera toxin, (Malley *et al*., 2004), or IL-12 (Lynch *et al*., 2003), but it was shown here that the advantages of the live attenuated carriage as a vaccination is that it does not require additional adjuvant and protection against both carriage and invasive challenges are comparable to those studies previously mentioned with just a single exposure. Ultimately, the development of a mucosally delivered vaccine would have great advantages, as it would be easier to administer where it is most needed without the need for medical training. Currently the major contenders for new vaccine candidates are a whole cell vaccine, using an ethanol inactivated non-encapsulated pneumococcal strain which is both autolysin negative and non-haemolytic, or vaccination with individual or combinations of pneumococcal protein virulence factors. The whole cell vaccine goes against the tendency towards well characterised individual components that can be chemically standardised and therefore progress with protein candidates is more advanced to date. Immune serum from human volunteers immunised with a family 1 fragment of the surface expressed PspA, reacted with 37 serotypes tested which expressed a variety of PspA and capsule types, and it is thought that protection against almost all serotypes could be achieved with a small number of PspA fragments (Nabors *et al*., 2000). Clinical vaccine trials of PspA were recently stalled when it was found that the molecule possessed homology to a human factor and may have the potential to create auto-reactive antibodies. However it is hoped that these difficulties can be avoided by modification of the molecule which is still protectively immunogenic but not cross-reactive. Clearly the work undertaken in this thesis provides support for immunisation using the whole cell method, as protection against both subsequent carriage and disease after carriage of live pneumococci was observed. The protection against both carriage and disease was associated with, but not dependent on systemic IgG against PspA as carriage with a PspA negative mutant still prevented deaths from invasive pneumococcal disease and colonisation with $PlnA^-$ could not prevent a subsequent carriage with A66 despite the cross-reactivity between PspA in these two strains. However capsular antibodies were also protective against invasive disease, and may have a strain specific role in the re-colonisation of the nasopharynx, as although a serotype-3 carriage was not prevented after serotype-2 immunisation, carriage of encapsulated strains could also be prevented by the pre-colonisation of a non-encapsulated strain. One of the strongest observations made during this thesis was that

the protection against future encounters with pneumococci were not dependent on the presence of PLY, as protection against both carriage and invasive disease was observed using the PLY negative mutant. Although PLY may be responsible for the accumulation of granulocytes observed in the CLN, and of CD4+ T-cells *in vitro*, this did not contribute to the clearance of WT bacteria and neither were they responsible for the generation of humoral immunity to the pneumococcus, as levels of antibody against capsule and PspA were if anything higher in the PlnA$^-$ mutant colonisation. Despite the previously identified immuno-stimulatory properties of PLY which suggested that it could act as a suitable mucosal adjuvant it seems likely that *in vivo* other pneumococcal components can compensate for the presence of PLY.

The results in this thesis add to the knowledge that both nasopharyngeal carriage and its subsequent effects on future pneumococcal disease in mice are multi-faceted. Asymptomatic carriage of the nasopharynx can be observed in WT strains in MF1 mice and requires functional PLY, neuraminidases, PspA but not polysaccharide capsule in order to persist long term, although naturally isolated CPS negatative strains differ from laboratory generated strains in more than just the expression of capsule. Carriage induces both a humoral immune response (characterised by serum immunoglobulin and mucosal IgA) and a cellular immune response which involves macrophages, T-cells and IL-17A. These factors may contribute to the clearance of carriage of attenuated pneumococci and also contain the carriage of WT strains which are ultimately carried long term, preventing the transition to invasive disease. The local and systemic antibody response which is generated against a plethora of targets can then prevent subsequent carriage events and invasive pneumococcal disease. These results indicate that there is a strong justification for the inclusion of PLY, polysaccharide capsule and PspA in any future vaccine

Appendices

Appendix 1

Publications completed during the course of this thesis:

Richards, L., Ferreira, D.M., Miyaji, E.N., Andrew, P.W., and Kadioglu, A. (2010). The immunising effect of pneumococcal nasopharyngeal colonisation; protection against future colonisation and fatal invasive disease. Immunobiology 215, 251-263.

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