The use of protein antigens in immunisation against infection caused by *Streptococcus pneumoniae*

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By

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STATEMENT OF ORIGINALITY

The accompanying thesis submitted for the degree of PhD entitled 'The use of protein antigens in immunisation against infection caused by *Streptococcus pneumoniae'* is based on work conducted by the author in the Department of Infection, Immunity and Inflammation at the University of Leicester mainly during the period between October 2007 and October 2010.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other University

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Abstract

Streptococcus pneumoniae (the pneumococcus) is a major human pathogen which causes pneumonia, septicaemia and otitis media infections. The pneumococcus asymptomatically colonises the upper respiratory tract, which may act as a reservoir for subsequent infection of the lower respiratory tract. Current vaccines available include a polysaccharide vaccine (Pneumovax), and a conjugate vaccine (Prevenar). However, problems with both vaccines exist; Pneumovax does not elicit protection in children under the age of 2 years, the elderly or people who are immunocompromised, and Prevenar is an expensive vaccine that has limited serotype coverage. Serotype replacement has also become a growing problem. Therefore, new vaccine targets which can elicit broad cross-serotype protection are required. Pneumolysin and neuraminidase A are proteins that are highly conserved, in all serotypes of pneumococci.

Throughout the research presented in this thesis, MF1 outbred mice and Balb/c inbred mice were immunised with PdB or neuraminidase A protein. Following I.P. or subcutaneous immunisation the mice were challenged using a variety of doses of virulent passaged pneumococci by the intraperitoneal, intravenous or intranasal routes.

Immunisation with neuraminidase A elicited specific anti-neuraminidase antibodies. However, these antibodies were unable to protect the mice from acute pneumococcal challenge. Immunisation with PdB elicited a high titre of anti-PdB antibodies which were able to inhibit PLY activity *in vitro*. However, the antibodies were unable to protect the mice from acute pneumococcal challenge, or invasive pneumococcal challenge. Following colonisation challenge a significant drop in pneumococci recovered from nasopharynx of PdB immunised mice was seen. This significant drop in bacterial numbers correlated with a significant increase in the titre of specific IgG antibodies, as well as a significant increase in the number of specific IgG and IgA producing B cells, present in the cervical lymph nodes of PdB immunised mice, in comparison to that of the controls.

Immunisation with neuraminidase A was unable to protect the mice from intraperitoneal and intranasal pneumococcal challenge, therefore, may not be necessary to include in future protein vaccines developed. Immunisation with PdB was able to significantly reduce nasopharyngeal colonisation, but was unable to clear colonisation. Therefore, PdB should be considered, along with other protective pneumococcal proteins, for inclusion in any future protein vaccine developed against pneumococcal colonisation.

Abbreviation

%	Percentage
ºC	Degrees centigrade
G	Gravity force
Amps	Amperes
BAB	Blood agar base
BHI	Brain heart infusion
CLN	Cervical lymph nodes
CFU	Colony forming units
CFU/ml	Colony forming units per millilitre
CNS	Central nervous system
CRP	C-reactive protein
dH_2O	Distilled water
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immuno spot
FACS	Fluorescence activated cells sorting
FCS	Fetal calf serum
GFP	Green fluorescent protein
HBSS	Hanks balanced salt solution
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
I.N.	Intranasal
I.P.	Intraperitoneal
I.V.	Intravenous
IgA	Immunoglobulin A
IgG	Immunoglubulin G
IPD	Invasive pneumococcal disease
kDa	Kilodaltons
LA	Luria Agar
LB	Luria Broth
LPS	Lipopolyssacharide
LytA	Autolysin A
ml	millilitres
mg	milligrams
µl	Microlitre
MHC	Major histocompatibility complex
mg/ml	Milligrams per millilitres
mM	Millimoles
mins	Minutes
M	Moles
NaCl	Sodium chloride
NanA	Neuraminidase A
NanB	Neuraminidase B
NanC	Neuraminidase C
NALT	Nasal associated lymphoid tissue
Pav A	Platelet activating factor A
PBS	Phosphate buffered saline
PdT	Genetically detoxified pneumolysin
PdB	Genetically detoxified pneumolysin
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear leucocytes
PsaA	Pneumococcal surface antigen A
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
MBL	Mannan binding lectin
ml	Millilitres
PLY	Pneumolysin
Psi	Pounds per square inch
O.D.	Optical density
RPM	Revolutions per minute
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
TBST	Tris buffered saline with tween
TEMED	Tetramethylethylenediamine
TMB	Tetramethylbenzidine
Tris	Tris base
µg	Microgram
µg/ml	Microgram per millilitre
µm	Micrometer
v/v	Volume by volume
WHO	World Health Organisation

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I. Introduction

A. *Streptococcus pneumoniae* and its history

Streptococcus pneumoniae, the pneumococcus, is a gram positive "lancet shaped" bacteria. The pneumococcus is a major human pathogen, which is the leading cause of community acquired pneumonia and can also cause meningitis, otitis media and septicaemia.

S. pneumoniae was first isolated by George Sternberg in 1881. Sternberg, a member of the army, was initially performing experiments to investigate malarial fever, when for a control in his experiment he injected his saliva into a rabbit. The rabbit died around forty eight hours later. Sternberg then isolated pneumococci from saliva and recognised it as the cause of the rabbit's septicaemia and death (Sternberg, 1881). Sternberg also observed that pneumococci were present in pairs; one of the first to identify that pneumococci are diplococcic. Later, in 1887, he recognised that the bacteria isolated were streptococcus and that it also grew in chains of differing lengths. However, it was not until 1974 that the pneumococcus was named *Streptococcus pneumoniae* due to its ability to cause pneumonia (Deibel, 1974). Louis Pasteur also isolated the pneumococcus from saliva in the same year as Deibel (Pasteur, 1881). Pasteur noticed, under the microscope, the capsule surrounding the bacteria but, as yet, did not have a clear understanding of the importance of the pneumococcal capsule.

It was not until 1887 that the pneumococcus was shown to be the major cause of bacterial pneumonia by Carl Friedlander (Austrian, 1960), as there had been much controversy over the cause of pneumonia between scientists at that time. During these years *S. pneumoniae* was one of the first bacteria to be stained using the Gram stain. It was this technique, which was being developed in the late 1800's,

that helped stain the pneumococcus and allowed it to be identified as the causative agent of pneumonia (Austrian, 1960). Following this discovery, the pneumococcus was then shown to also cause septicaemia, otitis media and meningitis (Austrian, 1981).

Soon it was found, that *S. pneumoniae* antiserum, recovered from infected animals, was useful in protecting the animals after passive immunisation with the serum. In the 1890's humans were injected with pneumococcal antiserum. However, little was known of the number of differing pneumococcal capsular serotypes so the experiments had little success. It was in 1902 that Neufeld discovered the Quelling reaction and was able to determine differences in capsular serotypes (Austrian, 1981).

S. pneumoniae also played a major role in one of the first known genetic engineering experiments carried out in the laboratory in 1928. Frederick Griffiths showed, that when unencapsulated heat killed pneumococci were injected into mice with a virulent strain, the unencapsulated pneumococci could transform to a virulent strain and cause infection in the mouse (Griffiths, 1928). This showed that the pneumococci were able to exchange capsule genes and transform into a virulent type.

B. Epidemiology

The burden of pneumococcal disease throughout the world is great in both the developed and developing world. WHO figures showed that in the year 2000 there were 14.5 million pneumococcal cases worldwide (WHO, 2009). In 2008 WHO estimated that 14% of child mortality in the world was from pneumonia and,

of these pneumonia cases, *S. pneumoniae* was responsible for 50% (Wardlaw, 2006).

S. pneumoniae causes around two thirds of community acquired pneumonia cases (Thibodeau and Viera, 2004). In developed countries, the incidence of community acquired pneumonia due to *S. pneumoniae* is estimated to be 45-60 cases per 100,000 people (Fedson and Scott, 1999). Child mortality due to pneumonia in developed areas of the world such as Europe and the Americas is 11-12%.

Pneumonia is a major cause of infant mortality in the developing world; 14% of all child deaths in Africa are due to pneumonia (WHO, 2010). However, it is difficult to estimate the figures for the developing world, since accurate records on pneumococcal infections in these populations are lacking. Data from high risk groups, such as the elderly and immuno-compromised patients who have taken part in vaccine strategies, suggests that 50-75% of pneumonia is attributable to *S. pneumoniae* (Fedson and Scott, 1999). The high risk groups also include patients with HIV, where the incidence of invasive pneumococcal disease is estimated to be 97 cases per 100,000 people (Fedson and Scott, 1999).

S. pneumoniae makes up part of the human microflora and it is estimated that up to 20-30% of adults carry *S. pneumoniae* as a commensal (Ghaffar et al., 1999). Colonisation of the nasopharynx by *S. pneumoniae* is an important first step in pneumococcal infection. Nasopharyngeal colonisation in humans is very common and may lead to disease, although in most cases it does not. It is estimated that 40-50% of children are carriers of the pneumococcus whilst those under the age

of two years are mostly likely to be carriers. Furthermore, colonisation of the nasopharynx in children under two may occur repeatedly (Malley, 2010).

Since the introduction of the pneumococcal 23 valent polysaccharide vaccine, protection from invasive pneumococcal infection has increased to 60-80% of the young healthy population (Lynch and Zhanel, 2009b). However, as discussed later, this vaccine is unable to protect children under 2 years or immunocompromised patients. Since the introduction of the 7 valent conjugate vaccine (Prevenar) in the United States, there has been a fall in pneumonia, meningitis and otitis media due to serotypes included in the vaccine (Lynch and Zhanel, 2009b). However, there has been a 61% increase in meningitis due to non vaccine serotypes (Lynch and Zhanel, 2010). The vaccines currently available and their impact on epidemiology of pneumococcus is discussed later in more detail.

C. *Streptococcus pneumoniae* virulence factors

The pneumococcus has many virulence factors which allow the bacteria to invade and modulate host defences. The major virulence factors of the pneumococcus and the contributions they make to the pathogenesis of disease are discussed below.

Pneumococcal virulence factors range from the polysaccharide capsule to cell surface proteins. These virulence factors have a multitude of differing roles in protecting the bacteria from the host immune system, mediating attachment to host surfaces and promoting tissue invasion as well as enabling nutrient acquisition. An illustration of some of the major virulence factors of *S. pneumoniae* is shown below.

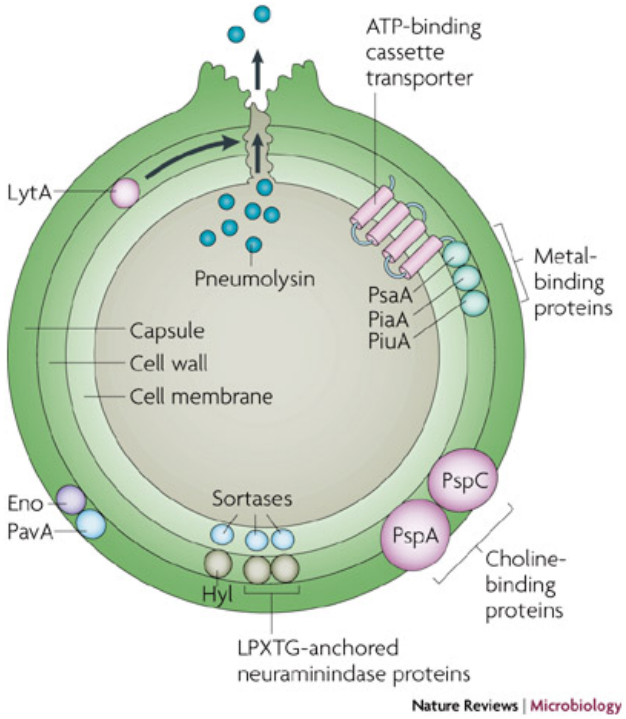


Figure 1- taken from Kadioglu et al., (2008) shows some of the major *Streptococcus pneumoniae* virulence factors.

1. The pneumococcal capsule

Arguably, the most important virulence factor of the pneumococcus is the polysaccharide capsule. There are over 90 different polysaccharide capsule varieties or serotypes (Henrichsen, 1995, Park et al., 2007). Serotypes are determined by the order of monosaccharide chains and side branches which are attached to the peptidoglycan to make up the polysaccharide capsule (Sørensen et al., 1990). The polysaccharide capsule is the antigen, which is used in current vaccine strategies, employed to provide protection against *S. pneumoniae* infection. The current vaccines available and new approaches to vaccination are discussed later in the chapter.

The capsule is essential for pneumococcal virulence in invasive disease models of infection (Watson and Musher, 1990, Morona et al., 2006). In this study

one group of mice were intraperitoneally infected with pneumococci serotype 3 that had a functioning capsule, and another group of mice were intraperitoneally infected with pneumococci type 3 without a functional capsule. The results showed that pneumococci without a functional capsule was significantly less virulent (Watson and Musher, 1990). In anther study, mice were intranasally infected with serotype 2 pneumococci, again, with and without a functioning capsule. The mice challenged with pneumococci that had a non functional capsule, had significantly less pneumococci present in the lungs and blood at both 24 and 48 hours after infection (Morona et al., 2006). This again showed that the capsule is important for invasive pneumococcal infection. However, pneumococci without a capsule are still able to colonise the nasopharynx (Nelson et al., 2007).

Although previous work has shown that the presence of the pneumococcal capsule inhibits attachment during colonisation (Cundell et al., 1995c), recent *in vitro* work shows that the capsule prevents mucus mediated clearance of the bacteria (Nelson et al., 2007). In the study by Nelson et al. 2007, the ability of both capsulated and unencapsulated pneumococci, to bind to mucus samples from human upper airways, was measured. Unencapsulated pneumococci were unable to escape the binding of mucus to the cell surface. Whereas, encapsulated pneumococci were able to escape from mucus binding to the cell surface. This ultimately allows the pneumococcus to bind to epithelial cells promoting nasopharyngeal colonisation (Nelson et al., 2007). However, the expression of the capsule and the expression of cell wall proteins, responsible for mediating attachment to host cell membranes, is a fine balance. This hypothesis explains the need for two different types of capsule expression in the same strain; opaque (O) variants and transparent (T) variants (Weiser et al., 1994). These variants, recovered

from clinical isolates, have differing amounts of capsule expression, as well as other surface proteins (Kim and Weiser, 1998). O variants have been shown to be more virulent due to a larger amount of capsule expression. This variant unlike the T variant was able to cause sepsis in a mouse model of infection (Kim and Weiser, 1998). The pneumococcus, it seems, is able to vary between these two types of capsule expression which facilitate the bacteria to mediate attachment and evade host defences in different environments of the host (Tong et al., 2001).

The capsule has many functions, and evading the host defences is an important function for the pneumococcus. The polysaccharide capsule is able to prevent phagocytosis by inhibiting the build up of C3 complement proteins on the cell surface of the pneumococcus (Brown et al., 1983). This inhibition of complement proteins, prevents the bacteria from being phagocytosed by neutrophils, as shown in an invasive guinea pig model of infection (Brown et al., 1982). Guinea pigs were intravenously infected with unencapsulated pneumococci or encapsulated pneumococci; the results showed that neutrophils from guinea pigs infected with unencapsulated pneumococci were able to clear pneumococci from the blood stream. The neutrophils from guinea pigs infected with encapsulated pneumococci were unable to clear the bacteria from the blood stream. More recent work shows that the classical complement pathway is inhibited by the capsule by preventing the deposition of antibody on the surface of the bacteria. Antibody begins the classical pathway of complement leading to C3b deposition on the surface of the pneumococcus. However, this complement mediator has been shown to be degraded by the capsule, which in turn prevents phagocytosis by neutrophils (Hyams et al., 2010).

These studies show that it is necessary to activate the classical complement pathway for efficient clearance of the pneumococcus. Confirmation of the importance of an efficient classical complement pathway was shown with research into human patients deficient in classical complement pathway proteins. It was found that, because of an inefficient classical complement pathway, these patients were more susceptible to pneumococcal infection (Yuste et al., 2008).

Furthermore, the capsule inhibits the alternative complement pathway. Although the alternative or MBL pathway, is not the dominant pathway necessary for the phagocytosis of the bacteria by neutrophils, it is able to contribute to C3 deposition on the pneumococcal cell surface (Hyams et al., 2010). The alternative pathway is inhibited by the capsule, which prevents the attachment of MBL proteins to the pneumococcal cell surface, and in turn inhibits the cascade of proteins, such as C3b, being deposited onto the pneumococcal cell surface.

Recent work *in vitro* has also shown that the capsule is able to protect the pneumococcal cell from intracellular killing by microglia in mice. Mice were infected with unencapsulated pneumococci or encapsulated pneumococci which were able to cause meningitis. The results showed that, although the capsule does not protect the pneumococcus from phagocytosis by mouse brain cells, it is, however, able to prevent intracellular killing. The mechanism of this prevention of intracellular killing is unclear. However, the authors suggest the capsule may inhibit phagolysosome maturation which prevents the production of bactericidal molecules (Peppoloni et al., 2010). This shows that the capsule is also an important virulence factor in meningitis models of disease.

2. Autolysin A

Autolysin A (Lyt A) is a member of the N-acetylmuramoyl-l-alanine amidase (NAM-amidase) family of proteins (Romero et al., 2007). Lyt A, the enzyme which cleaves peptidoglycan in the cell wall of pneumococci, breaks open the cell to release its contents. Lyt A is initially present in the cell in a low activity form. However, in the presence of choline (a substance that partly forms the pneumococcal cell wall) it becomes a highly activated form (Romero et al., 2007). It is this highly activated form that is able to cleave peptidoglycan and break open the cell wall. However, Lyt A is also involved in normal cell processes such as cell separation after division (Giudicelli and Tomasz, 1984). Berry et al. 1989, showed that an encapsulated strain of pneumococci that was deficient in Lyt A had significantly decreased virulence, in a mouse model of infection, when compared to the wildtype strain.

Lyt A is essential for the pathogenesis of pneumococcal infection as it mediates the release of pneumolysin and other inflammatory molecules after bacterial cell apoptosis. This was shown when mice were intranasally and intraperitoneally infected with either a lyt A knock out strain or a wild type strain. Mice infected with the lyt A gene knocked out survived significantly longer than mice infected with wild type pneumococci (Berry et al., 1989). Autolysin is also essential for the virulence of pneumococci in meningitis. Pneumococci, deficient in Lyt A, were unable to cause meningitis in a rat model of infection (Romero et al., 2007). These studies all show that Lyt A is an important virulence factor of pneumococci.

LPxTG anchored proteins are anchored to the cell wall by sortase transpeptidases which are able to recognize the sequence LPxTG (Kadioglu et al., 2008). This group of proteins includes IgA1 protease, hyaluronate lyase, and neuraminidase.

3. IgA1 Protease

IgA1 protease is produced by all pneumococci (Bergmann and Hammerschmidt, 2006). The enzyme is able to neutralize the action of secretory IgA by cleaving the antibody's Fc fragment (Romanello et al., 2006). IgA1 is present on the mucosal surfaces of the host and when cleaved is able to assist in pneumococcal binding to epithelial cells (Weiser, 2003). Therefore, this enzyme is not only a protease that is able to cleave host antibodies but it is also able to promote pneumococcal binding, playing a role in colonisation.

4. Hyaluronate lyase

Hyaluronate lyase (or hyaluronidase) is an enzyme which breaks down hyaluronan present in the extracellular matrix of the host. Although it is anchored to the peptidoglycan of the cell wall, it has been suggested that hyaluronate lyase is also released by the pneumococcus during infection, allowing the enzyme to further facilitate bacterial invasion (Jedrzejas, 2001). Hyaluronidase maybe an important virulence factor and its role in different models of infection is being discovered. There is debate about whether the protein contributes to virulence in a meningitis model of infection. In one study, mice intranasally infected with pneumococci and purified hyaluronidase developed meningitis, whereas, mice infected with pneumococci without purified hyaluronidase did not develop meningitis. The authors suggest that the protein contributes to virulence, as it is able to disrupt

epithelial cells in the host, allowing pneumococci to cross the mucosal barrier and gain access to the CNS (Zwijnenburg et al., 2001). However, another study where pneumococci were directly inoculated into the CNS of mice showed that hyaluronidase does not contribute to virulence in a meningitis model of infection (Wellmer et al., 2002).

Hyaluronidase activity, coupled with the action of pneumolysin, has been shown to have a detrimental effect on ciliated epithelium. Epithelial cells, pre-treated with hyaluronidase and subsequently treated with pneumolysin, became dysfunctional and damaged. Hyaluronidase alone did not cause damage or disrupt epithelial cell function. The authors suggest that the action of hyaluronidase on the intracellular matrix of epithelium, allows easier access for pneumolysin and its pore forming activities (Feldman et al., 2007).

As well as the activity of hyaluronidase on epithelial cells, it also has immuno-modulatory effects on the host immune system. The enzyme is able to bind to CD44 on host immune cells, causing inflammatory cytokine secretion (Mitchell and Mitchell, 2010).

Hyaluronidase was considered as a novel vaccine target. However, when the recombinant purified hyaluronidase was used as an immunogen, it did not afford any protection in a mouse model of infection (Paton et al., 1997).

5. Pneumococcal Surface Protein A

Pneumococcal surface protein A (PspA) is a surface exposed protein which has variable sequence and variable molecular weights ranging from 66kDa to 96kDa (Waltman et al., 1990). PspA has been found in all pneumococcal serotypes

characterised so far (Crain et al., 1990). The surface exposed region of PspA is highly electronegative, preventing complement proteins binding to the surface of pneumococci (Jedrzejas et al., 2000). C3, an important complement protein, is prevented from attaching to the pneumococcal cell surface, which in turn inhibits the activation of the alternative complement pathway and subsequent clearance by phagocytosis (Jedrzejas et al., 2000). PspA is also a lactoferrin binding protein. Lactoferrin is a bactericidal component of the host's innate immunity. PspA protects the pneumococcus from the action of lactoferrin by binding the iron depleted form of the molecule, called apolactoferrin (Shaper et al., 2004). Thus far, it is unclear whether PspA is required for virulence, since there are conflicting data on the subject. This depends on the method used for PspA loss of function (Kadioglu et al., 2008). One study, which used the same genetic background for the wildtype and knock out mutant of PspA, suggested that the capsule serotype, together with PspA, is important in preventing complement deposition on the surface of pneumococci (Abeyta et al., 2003). PspA has been shown to be important for colonisation of the nasopharynx. In a mouse model of colonisation, where the PspA gene was knocked out, the numbers of PspA negative pneumococci recovered from the nasopharynx of mice was significantly decreased (Ogunniyi et al., 2007b). This study also showed that PspA was important for lung infection and bacteraemia, since pneumococci recovered from the lungs of mice infected with the PspA negative mutant was significantly decreased. The PspA negative mutant was also unable to cause bacteraemia (Ogunniyi et al., 2007b). These studies show that PspA is indeed required for virulence.

Although PspA has sequence variability, it has been used as an antigen in many immunisation studies which will be discussed later.

6. Pneumococcal Surface Protein C

Pneumococcal surface protein C (PspC) is a 75kDa cell surface exposed protein, which has many functions as a virulence factor (Jedrzejas, 2001). PspC is also known as choline binding protein A (CbpA) and is involved in attachment, colonisation and invasion of the host. Attachment to host cell surfaces, through the human polymorphic immunoglobulin receptor, is mediated by PspC and this interaction leads to translocation of pneumococci across the epithelial cell layer (Zhang et al., 2000).

PspC is also able to inhibit the host immune system by a range of different methods. Firstly, PspC is able to bind secretory IgA, present on mucosal surfaces of the host, therefore preventing complement activation by the classical pathway (Hammerschmidt et al., 1997). Secondly, PspA inhibits alternative pathway complement activation by binding factor H, which is a protein that usually prevents tissue damage upon complement activation (Dave et al., 2004). Thirdly, PspC is able to directly bind the complement component C3, which prevents the production of C3a and subsequently opsonisation and phagocytosis of pneumococci (Cheng et al., 2000).

PspC is required for prolonged colonisation of the nasopharynx but is not required for virulence in lung infection or bacteraemia (Ogunniyi et al., 2007b). Due to PspC's role in colonisation, it is a novel vaccine target and has been used in immunisation studies, which will be discussed later in the chapter.

7. Pneumococcal Surface Antigen A

Pneumococcal surface antigen A (PsaA) is a 37kDa protein attached to the cell membrane of pneumococci (Lawrence et al., 1998). PsaA was originally thought to be a pneumococcal adhesion. However, structure studies showed that the protein did not protrude from the pneumococcal capsule, therefore, the protein could not be an adhesion (Johnston et al., 2004). PsaA's function as metal binding protein, that binds manganese ions, has now been elucidated (Johnston et al., 2004). PsaA is an ABC transporter which transports manganese or zinc ions across the pneumococcal cell membrane (Dintilhac et al., 1997). These ions are required for normal growth of pneumococci. This function of PsaA also protects pneumococci from superoxide production, either by the bacterial cell or by the host cells, making it essential for the virulence of *S. pneumoniae* (Tseng et al., 2002, Johnston et al., 2004). Knock out mutants of PsaA were avirulent in both an intranasal and intraperitoneal mouse model of infection (Berry and Paton, 1996). PsaA is also required for virulence in colonisation of the nasopharynx and otitis media infections (Marra et al., 2002).

PsaA is an important virulence factor and, when it is knocked out, renders pneumococci avirulent. Due to its importance in pneumococcal infection and a conserved sequence in one domain of the protein, it is a novel vaccine target. PsaA has been used as an antigen in pneumococcal immunisation studies which will be discussed later in the chapter.

8. Pneumococcal iron acquisition A and pneumococcal iron uptake A proteins

Pneumococcal iron acquisition A (PiaA) and pneumococcal iron uptake A (PiuA) proteins are ABC transporters which bind metal ions. The function of these cell surface proteins is to acquire iron required for pneumococcal cell growth (Brown et al., 2001a). The proteins are able to acquire iron from haemoglobin (Brown et al., 2001a). At least one of these iron uptake proteins is required for virulence of pneumococci, as a double knock out mutant of these proteins was significantly less virulent than the wild type in a pneumonia and sepsis model of infection (Brown et al., 2001a). A PiaA knock out showed some decrease in virulence in a mouse model of pneumonia and sepsis. However, the proteins are able to compensate for one another, in acquiring iron, if one of the proteins is knocked out (Brown et al., 2001a).

In the study by Tai et al. 2006, PiaA and PiuA were not surface exposed in a serotype 2 strain. However the authors state that further exploration of surface eexposure of these proteins in other pneumococcal strains is required before a conclusion can be made. The presence of PiaA and PiuA proteins in most *S. pneumoniae* strains, and previous immunisation studies in mouse models of infection, make these proteins promising vaccination candidates.

9. Pneumococcal adherence and virulence factor A

Pneumococcal adherence and virulence factor A (PavA) is a 62kDa plasminogen binding protein, present on the pneumococcal cell surface (Holmes et al., 2001). PavA is able to bind to fibronectin, present in host cell tissues, to mediate pneumococcal cell attachment (Bergmann et al., 2001). PavA is essential

for virulence in colonisation and sepsis (Kadioglu et al., 2010). PavA deficient pneumococci were cleared from the nasopharynx of infected mice within 24 hours post infection. In a sepsis model of infection, PavA deficient pneumococci were unable to be detected in the blood of infected mice, as they were unable to translocate across the alveolar capillary barrier (Kadioglu et al., 2010). However, this mutant was able to persist in the lungs of mice, showing this protein is not important in lung infection. PavA is also an important virulence factor in a meningitis mouse model of pneumococcal infection, as mice infected with PavA deficient pneumococci did not develop meningitis, in an intracranial challenge model (Pracht et al., 2005, Kadioglu et al., 2010).

Noske et al., (2009), showed PavA has an immunomodulatory function which prevents phagocytosis of pneumococci by dendritic cells (an important cell for antigen presentation and phagocytosis). Expression of PavA was able to interfere with the recognition of pneumococci by dendritic cells *in vitro* and prevent phagocytosis, which in turn prevented dendritic cell maturation and activation of an optimal adaptive immune response. Although PavA has been shown to be an important virulence factor in pneumococcal infection, and may be a vaccine candidate, it has not yet been used in immunisation protection studies.

10. Enolase

Enolase is another surface protein of *S. pneumoniae* that is able to bind plasminogen. This protein plays a major role in mediating the attachment and transmigration of pneumococci across host cell surfaces (Kolberg et al., 2006). The protein is essential for pneumococcal virulence, as demonstrated by a knock out mutant which was unable to transmigrate through the host cell extra cellular matrix

(Bergmann et al., 2005). However, this protein is not a likely vaccine candidate, despite it playing a major role in pneumococcal virulence, due to low expression on the cell surface, (as measured by monoclonal antibody binding and subsequent flow cytometric analysis) (Kolberg et al., 2006).

Pneumolysin and neuraminidase are also important virulence factors in pneumococcal disease. Since these proteins are important in this work, they are discussed in more detail in this chapter.

Pneumococcal virulence factors, their functions and important publications describing their functions are summarised in Table 1. *S. pneumoniae* has many virulence factors, which all work together to contribute to its ability to infect the host. Most of the virulence factors described are necessary for full virulence of the bacteria. The role these factors play in colonisation, pneumonia and septicaemia will be discussed later.

Table 1- pneumococcal virulence factors, their functions and important publications

Virulence factor	**Function**	**Important publications**
Polysaccharide Capsule	• Prevents mucus mediated clearance • mediates surface protein attachment to host cells • prevents opsonophagocytosis	• Nelson et al., 2007 • Weiser et al., 1994 • Hyams et al., 2010
Pneumolysin (Ply)	• Induces pore formation in host cells • Inhibits lecocyte chemotaxis • Inhibits respiratory burst of neutrophils • Activates complement pathways • Activates CD4+ T cells • Induces cytokine synthesis	• Tilley et al., 2005 • Paton and Ferrante, 1983 • Paton et al., 1984 • Kadioglu et al., 2004 • Houldsworth et al., 1994 and McNeela et al., 2010
Neuraminidase A, B and C (Nan A, Nan B and Nan C)	• Exoglycosidase which cleaves terminal sialic acids mediating host cell adhesion • May be involved in providing a carbohydrate source	• King et al., 2006 • Manco et al., 2006
Autolysin A (LytA)	• Cell separation • Apoptosis leading to release of inflammatory molecules	• (Giudicelli and Tomasz, 1984) • (Romero et al., 2007)
IgA1 protease	• Cleaves secretory IgA released from host mucosal surfaces	• Romanello et al., 2006
Hyaluronidase	• Breaks down hyaluronan present in the extracellular matrix	• Jedrzejas, 2001
Pneumococcal surface protein A (PspA)	• Prevents deposition of complement proteins on pneumococcal cell surface • Lactoferrin binding protein	• Jedrzehas et al., 2000 • Shaper et al., 2004

Virulence factor	Function	Important publications
Pneumococcal surface antigen A (PsaA)	• Metal binding protein • Protects pneumococci from action of superoxide molecules	• Lawrence et al., 1998 • Johnston et al., 2004
Pneumococcal iron aquistion A (PiaA) and Pneumococcal iron uptake A (PiuA)	• Iron acquisition and uptake proteins	• Brown et al., 2001a
Pneumococcal adherence and virulence factor A (Pav A)	• Plasminogen binding protein mediating pneumococcal attachment to host cell surfaces	• Holmes et al., 2001 and Kadioglu et al., 2010
Enolase	• Plasminogen binding protein mediating pneumococcal attachment to host cell surfaces • Mediates translocation of pneumococci across epithelial cell barrier	• Kolberg et al., 2006 • Bergmann et al., 2005

11. Pneumolysin

Pneumolysin (PLY) is a 52 kDa soluble protein that is released into the extracellular milieu when pneumococci are grown in culture. Pneumolysin is a conserved protein that, although initially thought to be released from the pneumococcal cell following the enzymatic action of Lyt A, is now known to be released throughout the growth cycle of the bacteria.

Pneumolysin is a member of the cholesterol dependant cytolysin (CDC) family. Once pneumolysin is released from the pneumococcal cell the soluble monomers then oligomerize to form pores in the hosts cell membrane (Tilley et al., 2005). There are three stages in pneumolysin pore formation; firstly, monomers insert into the phospholipid membrane, secondly, these monomers oligomerise to form a prepore and thirdly, full pore formation occurs.

PLY has four domains; domains 1, 2, 3 and 4. Domain 1 consists of β sheets, which are concave and convex in shape on either side of the domain, and these are important for monomers to oligomerize. Domain 1 is also electronegative, which defines the orientation of other monomers, once inserted into the membrane, allowing prepore formation. Domains 2 and 3 consist of β sheets and α helices. Domain 4 consists of β sheets and the end of the domain is tryptophan rich. This tryptophan rich area forms a loop and is where the cholesterol binding site is thought to be. When these monomers oligomerise, domains 1, 2 and 3 make up a long stalk shape and domain 4 acts as flexible arm; the final shape of the monomer is an L shape. The stalk of the molecule, containing domain 1, is concave in shape and allows other PLY monomers to ionically bond to the convex face of domain 1 of the neighbouring monomer (Tilley et al., 2005). The monomers firstly insert into

the membrane, following cholesterol binding, and then begin to oligomerise inside the membrane to form a prepore (Rossjohn et al., 1998). Another conformational change happens when domain 4 is pushed up into the membrane by domain 3. Movement of domain 3 causes domain 4 to wedge into the phospholipid bilayer where, it is thought to disrupt it and break down the permeability barrier (Gilbert et al., 1999, Bonev et al., 2001). A pore may consist of 30 to 50 PLY monomer subunits. The structural changes that take place during pneumolysin induced pore formation are illustrated in figure 2 (Rossjohn et al., 1998, Tilley et al., 2005).

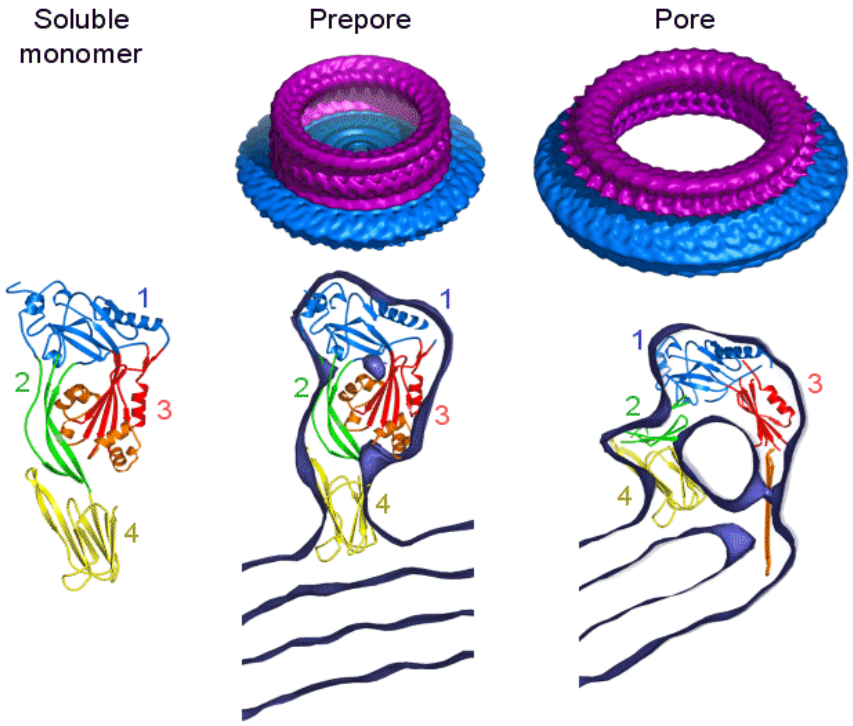


Figure 2- shows the different structural stages in pore formation of pneumolysin. Taken from http://people.cryst.bbk.ac.uk/~ubcg16z/pore/pore.html. Firstly, the soluble monomers oligomerize on the membrane to form the prepore, and then puncture the membrane to form the full pore of 30-50 subunits of pneumolysin.

As well as its ability to form pores in host cells, pneumolysin has many other immunomodulatory effects. Firstly, pneumolysin inhibits the ciliary beat of epithelial cells in the respiratory tract. *In vitro* studies showed, when purified

pneumolysin was added to epithelial cells, the ciliary beat was slowed or even halted at high concentrations of the protein. This is due to pneumolysin pore formation in the epithelium (Feldman et al., 2002). Slowing or halting of respiratory epithelial cells allows a more advantageous environment for the bacteria to colonise the nasopharynx (Steinfort et al., 1989). Inhibition of the ciliated epithelium allows the pneumococcus to adhere and mediate attachment to epithelial cells. This function of pneumolysin further facilitates bacterial invasion by causing epithelial cell disruption and invasion of epithelial cell sub layers (Steinfort et al., 1989).

Pneumolysin is also toxic to lower respiratory tract epithelium (Rubins et al., 1993) and vascular endothelial cells (Rubins et al., 1992), leading to the break down of the alveolar and capillary barrier (Rubins et al., 1996). The breakdown of this alveolar capillary barrier may lead to alveolar flooding or haemorrhage, which then allows optimal growth conditions for the pneumococcus to replicate (Rubins et al., 1993). Consequently, breakdown of the endothelium and alveolar capillary barrier allows the bacteria to translocate easily to the blood stream causing bacteraemia.

Another effect of pneumolysin is its ability to inhibit leukocyte chemotaxis (Paton and Ferrante, 1983). *In vitro* studies showed that, when purified pneumolysin was added to polymorphnuclear cells (PMN's), they were inhibited from migrating to the site of infection. Inhibition of PMN migration allows the bacteria to grow unimpeded by the host immune system, causing lung infection as well as tissue damage.

Pneumolysin also inhibits the respiratory burst of PMN's (Paton and Ferrante, 1983). Respiratory burst is the generation of reactive oxygen species

(ROS) by an NADPH oxidase pump, present in the phagosome membrane. Respiratory burst happens once the bacteria have been phagocytosed by the cell. Following ingestion of bacteria, a series of changes inside the phagosome occurs, generating superoxide, hydrogen peroxide and other ROS which contribute to bacterial cell killing (Segal, 2005). It was shown that pneumolysin inhibits hydrogen peroxide production, which may lead to the survival of the pneumococcus inside the phagosome (Paton and Ferrante, 1983). However, a recent study shows that pneumolysin induces reactive oxygen species formation in neutrophils by activating the NADPH oxidase pump to generate ROS in intracellular vacuoles which do not contain bacteria (Martner et al., 2008). Therefore, the respiratory burst by neutrophils is activated before the cell has an opportunity to phagocytose pneumococci. Following NADPH oxidase activation, the neutrophil response is altered. Martner et al., (2008) showed neutrophil response to PMA (a known leukocyte activator) was decreased after stimulation with purified PLY protein. Consequently, neutrophils are unable to mount an effective immune response to clear pneumococci from the site of infection (Martner et al., 2008). Functional neutrophils are especially important in pneumococcal infections, as these cells are the first major population to mediate an immune response along with resident macrophages.

Furthermore, pneumolysin activates both the classical pathway and alternative complement pathways (Paton et al., 1984). The classical complement pathway is activated by PLY binding the Fc region of antibodies present at the site of infection (Mitchell et al., 1991). Complement activation leads to inhibition of opsonphagocytosis of pneumococci, as the host innate response is diverted by pneumolysin release. C3a and C5a are complement mediators that contribute to an

inflammatory response and are produced after the complement cascade is activated. These molecules are also chemotactic and attract neutrophils and macrophages to the site where pneumolysin is released which, in turn, inhibits any phagocytosis of pneumococci by host effecter cells. Activation of C3a and C5a contributes to the inflammation and tissue damage which occurs in pneumonia as the host cells try and mediate the infection (Paton et al., 1984).

Pneumolysin is able to cause activation and migration of CD4+ T cells (Kadioglu et al., 2004). These cells are important in controlling the pneumococcal infection in mice. Kadioglu et al., (2004), showed that MHC class II knock out mice, which were deficient in CD4+T cells, were incapable of controlling and limiting pneumococcal infections. Pneumolysin was shown to be essential for the migration of CD4+ T cells to the site of infection, as pneumolysin negative mutants showed significantly less CD4+ T cell migration *in vivo* and *in vitro* (Kadioglu et al., 2000). This study also showed the important role of CD4+ T cell recruitment in the early clearance of pneumococci from the nasopharynx and will be discussed later in the chapter.

Additionally, pneumolysin induces cytokine synthesis. It was first shown by Houldsworth et al., (1994), that blood monocytes release tumour necrosis factor alpha (TNF-α) and interleukin-1β in response to pneumolysin. Release of pro inflammatory cytokines may contribute to the inflammation seen in pneumococcal infections. Furthermore, pneumolysin is essential for epithelial cells, in the upper respiratory tract, to release IL-8 in order to attract neutrophils to the site of infection (Ratner et al., 2005). This activity is limited to active pore forming pneumolysin, as PdB protein (a genetically detoxified derivative of pneumolysin) is unable to activate cytokine synthesis in epithelial cells. However, new research shows that

endotoxin free pneumolysin alone, was unable to promote cytokine production in dendritic cells (McNeela et al., 2010). Pneumolysin, in combination with heat killed pneumococci, was able to induce the release of the proinflammatory cytokines interferon gamma (IFN-γ) and IL-17. In addition, pneumolysin and heat killed pneumococci were able to stimulate the release of the cytokines IL-6, IL-12p40, IL-23 and TNF-α from dendritic cells. These cytokines have been shown to be important for the differentiation of T cells, which are able to protect the host from pneumococcal disease (McNeela et al., 2010). The differentiation of T cells necessary for the protection from pneumococcal infection is discussed later in the chapter.

Pneumolysin has recently been shown to activate the NLRP3 inflammasome (McNeela et al., 2010). The NLRP3 inflammasome belongs to a family of pattern recognition receptors (PRR) known as nucleotide binding domain leucine-rich repeat (NLR) containing receptors (Bauernfeind et al., 2011). NLR's are intracellular receptors which survey for intracellular microbes and danger signals (Bauernfeind et al., 2011). NLR's activate caspase-1, which in turn is able to cleave cytokines to an activated form that can be secreted. Caspase-1 cleaves an important cytokine called IL-1β. IL-1β is an important cytokine in an inflammatory response and has many functions, such as activating lymphocytes and epithelial cells as well as upregulation of adhesion molecules, which allow the infiltration of immune cells to the site of infection (Dinarello, 2009). Pneumolysin has been shown to activate the NLRP3 inflammasome, leading to the release of IL-1β, by forming pores in the host cell membranes. Pore formation allows an influx of potassium ions into the cell, which leads to the release of lysosomal molecules (acting as intracellular danger signals) which then activates the NLRP3 inflammasome. However, PLY

activation of the inflammasome may be a disadvantage to pneumococci, as the NLRP3 inflammasome has been shown to be required for the protection of mice from intranasal infection (McNeela et al., 2010).

Pneumolysin has been shown to be toxic to cells in the central nervous system (Braun and Sublett JE, 2002). During meningitis, mitochondria inside the cells in the central nervous system, are damaged by pneumolysin in combination with hydrogen peroxide. This damage triggers the release of an apoptotic molecule called apoptosis inducing factor (AIP), which leads to major tissue damage and programmed cell death. In the study by Braun and Sublett, (2002), it was shown that, when pneumolysin forms pores in the neurons of the hippocampus, it causes an increase in intracellular calcium ions which, in turn, leads to the release of AIP and subsequent cell death. Non pore forming pneumolysin was unable to induce neuronal cell death.

Previously, it was thought that pneumolysin was able to activate toll like receptor 4 (TLR4), which is a pattern recognition receptor of the innate immune system (Malley et al., 2003). TLR4 is normally activated by LPS from gram negative bacteria. It was thought that the activation of TLR4 by pneumolysin contributed to the overall proinflammatory response by the host. Malley et al., (2003), suggested that this proinflammatory response may contribute to causing invasive disease, after asymptomatic carriage of the pneumococcus. However, new research shows that pneumolysin is able to promote cytokine secretion and expression of costimulatory molecules independently of TLR4 activation (McNeela et al., 2010). This study showed that the immuno modulatory effects of PLY were not different in mice which were defective in TLR4. Another study showed that

mice deficient in TLR4 were protected from intraperitoneal and lung pneumococcal infection as well as mice with functioning TLR4 (Branger et al., 2004).

Pneumolysin has been shown to be important for the survival of bacteria in the upper and lower respiratory tracts (Kadioglu et al., 2002) and for survival in blood (Canvin et al., 1995). Mice were intranasally infected with wildtype of pneumolysin deficient (PLN-A) pneumococci. The results showed that mice infected with PLN-A were able to clear bacteria from the upper respiratory tract within twelve hours post infection. PLN-A pneumococci were also cleared from the lungs within 48 hours. Pneumolysin is also essential for pneumococcal seeding from the lungs to the blood stream (Rubins et al., 1995). PLN-A pneumococci were unable to invade the bloodstream to cause bacteraemia. The authors suggest that this is because pneumolysin deficient pneumococci are unable to halt the mucociliary beat and separate epithelial cell tight junctions. Interestingly, when purified pneumolysin was added to PLN-A in the lungs, virulence was restored to a similar level to the wildtype (Rubins et al., 1995). Pneumolysin is essential for the virulence of pneumococci in mouse models of meningitis (Hirst et al., 2008, Wellmer et al., 2002). PLN-A was unable to cause meningitis in mice infected with the bacteria. Pneumolysin is important in allowing pneumococci to cross the blood brain barrier. Mice infected with PLN-A were unable to breach the endothelium which forms the blood brain barrier (Zysk et al., 2001).

Pneumolysin is highly conserved throughout all pneumococcal serotypes tested (Kanclerski et al., 1987), which makes this protein a key vaccine target. Recently, it has been shown that pneumolysin is present on the cell surface of the pneumococcal cell (Tilley et al., 2005) even though it has no cell wall anchor peptide. The cell surface expression of pneumolysin, combined with the

conservation of the protein in most pneumococcal serotypes, is important when considering vaccine targets. Many pneumolysin immunisation studies have been carried out so far and show that the protein provides protection in a variety of models and the results of these experiments are discussed later.

Since pneumolysin has many immunomodulatory effects and is cytotoxic to host cells, other derivatives of this protein have been explored for immunisation studies. PdB protein is a genetically detoxified derivative of pneumolysin. The protein has one amino acid change at position 433 where the wildtype pneumolysin has a tryptophan residue; the mutant has a phenylalanine residue (Paton et al., 1991). The PdB protein has only 0.1% of the hemolytic activity compared to that of the wildtype pneumolysin (Paton et al., 1991).

Another derivative of pneumolysin protein is known as PdT. PdT has three amino acid substitutions (W433F, D385N and C428G) (Alexander et al., 1998) which cause the protein to be non toxic, by knocking out the complement binding and cytolytic functions of the protein. PdT has 0.001% hemolytic activity when compared to the wildtype PLY.

Pneumolysin is a major virulence factor of pneumococci that greatly contributes to the pathogenesis of infection with so many of its functions. The conservation of the protein and surface exposure makes it a key vaccine target. The PdB derivative of pneumolysin is used throughout this work as an antigen, to investigate the basis of the protective immune response that is mounted in a mouse model of infection.

12. Neuraminidases

Neuraminidase is a protein covalently linked to the pneumococcal cell wall by an LPxTG motif. *S. pneumoniae* has three neuraminidase genes *nanA, nanB* and *nanC.* Nan A and Nan B are two distinct proteins and Nan C is a homologue of Nan B. Nan A has been shown to be present in all pneumococcal serotypes, Nan B is present in 96% of serotypes and Nan C in 51% of serotypes (Pettigrew et al., 2006). Neuraminidase is highly conserved throughout pneumococcal serotypes and its surface expression makes it an ideal vaccine target. Some experiments into protein vaccinations have used pneumococcal neuraminidases as an antigen and the results of these studies will be discussed later in the chapter.

Nan A is a surface exposed exoglycosidase which cleaves galactose that is β1-4 linked to N-acetlyglucosamine and subsequently removes the N-acetlyglucosamine from β1 linked mannose sugars on the host cell surface (King et al., 2006). This exoglycosidase enzyme activity allows the pneumococcus to cleave terminal sialic acids from cell surface glycans such as mucin, glycoproteins and glycolipid (Jedrzejas, 2001). Neuraminidase activity exposes host cell surface receptors for aiding pneumococcal cell adhesion (Anderson et al., 1983).

Structural analysis of Nan B has shown that it is able to cleave the reaction product of Nan A. Nan B cleaves α2-3 linked terminal sialic acids (Gut et al., 2008). The three structural domains of Nan B are shown in Figure 3 (picture taken from (Gut et al., 2008)). This shows that Nan A and Nan B have different specificities and play different roles in the pathogenesis of pneumococcal infection. Earlier work by Berry and Paton, (2000), showed that the enzymes Nan A and Nan B work at different optimum pH ranges of 6.5 and 4.5 respectively, again

suggesting a distinct role for each enzyme (Berry and Paton, 2000, Yesilkaya et al., 2006).

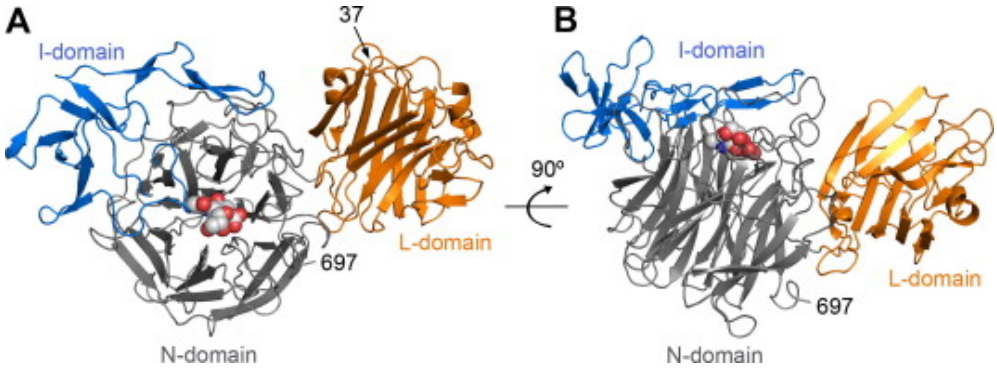


Figure 3- Structure of Nan B taken from (Gut et al., 2008). (A) shows cartoon representation of the NanB structure. (B) Same as in (A) but rotated around 90°. 2,7-anhydro-Neu5Ac bound to the NanB active site is shown as spheres.

The role of Nan C in pathogenesis and virulence has yet to be identified. However a few observations have been made for example, Nan C was found to be much more common in cerebrospinal fluid isolates than in carriages isolates (Pettigrew et al., 2006); Nan C has around 50% sequence homology to Nan B but its structure has yet to be elucidated (Pettigrew et al., 2006).

Nan A and B have a large role in virulence and have been shown to be essential for colonisation of the upper respiratory tract and survival in the bloodstream (Manco et al., 2006). Knock out studies in mice showed that pneumococci deficient in Nan A were cleared from the upper respiratory tract within 12 hours of infection. However, Nan B deficient pneumococci persisted but did not increase in numbers in the upper and lower respiratory tracts. Both of these mutants also showed inability to transfer from infected lungs to the blood after intranasal infection. Implying that Nan A and B are essential for pneumococcal survival in the blood. When Nan A and Nan B deficient pneumococci were

administered intravenously, both were cleared within 48 hours of infection, unlike the wildtype, where there was no clearance of bacteria and infection progressed (Manco et al., 2006). The role of neuraminidases in bactereamia is unclear. However, the authors suggest their exoglycosidase activity may interfere with host proteins such as secretory IgA, lactoferrin and secretory component. Secretory component is a glycoprotein derived from the polymeric antibody receptor and is present in a free form bound to mucosal surfaces or it is bound to polymeric IgA and IgM (Manco et al., 2006).

Wellmer et al., (2002), showed that neuraminidase A and B do not have a role in meningitis. In a mouse model of meningitis, there was no difference in the pathogenesis of infection compared to wild type pneumococci, when neuraminidase A or B function was knocked out. However, the authors did not rule out a compensatory effect of the neuraminidase protein that was not knocked out. Conversely, Uchiyama et al., (2009), showed that Nan A promotes the invasion of brain endothelial cells, which is the first step in meningitis, therefore showing an important role in the pathogenesis of the disease for Nan A.

It has recently been shown, that, after neuraminidase cleaves host cell sugars it is able to utilize these sugars as a carbohydrate source (Burnaugh et al., 2008). Yesilkaya et al., (2008), showed that the pneumococcus is able to use mucin (which is present in high amounts in the host nasopharynx) as a carbon source.

Neuraminidase has a role in biofilm formation (Parker et al., 2009). Biofilms are organised and complex communities of bacteria which grow at solid liquid interfaces (Allegrucci et al., 2006). Pneumococci change from free diplococci cells to the complex community of a biofilm, where there are many changes in

expression of virulence factors. Biofilms have been shown to be important in the development of otitis media infections (Ehrlich et al., 2002). Biofilms have also been shown to make it incredibly difficult to treat infection, as they are resistant to antimicrobial agents (Costerton et al., 1999). Nan A has been shown to be important in biofilm cluster formation and maturation and is highly expressed in biofilm formation (Parker et al., 2009). Biofilm formation has been correlated using *in vitro* and *in vivo* models, showing that pneumococci, causing pneumoniae and meningitis infections, grow in biofilm formations (Oggioni et al., 2006). Nan A gene expression is high in both types of experimental system, supporting the data that shows Nan A is essential for virulence in these models of infection.

Biofilm formation may happen in the nasopharynx during colonisation. Sialic acid is required for biofilm formation in the nasopharynx. Trappetti et al., (2009), have suggested that sialic acid cleaved by previous viral particles, such as influenza, leads to an upregulation and expression of Nan A in colonising pneumococci, causing biofilm formation. This in turn leads to an increase in pneumococci in the nasopharynx, which may seed to the lungs and switch to invasive disease (Trappetti et al., 2009).

NanA may also have a role in preventing complement deposition on the surface of the pneumococcus during pneumococcal colonisation (Dalia et al., 2010). This study showed that the exoglycosidase activity of Nan A was able to deglycosylate complement mediators, by either reducing their activity or allowing their degradation by serum proteases. This action allows the pneumococcus to resist phagocytosis by neutrophils and is another method by which the pneumococcus evades the host immune system.

Neuraminidase cleaves sugars from other bacterial species present in the nasopharynx (Shakhnovich et al., 2002). Nan A is able to cleave sialic acid, present on LPS, from both *Neisseria meningitidis* and *Haemophilus influenza.* This addition of sialic acid to LPS is a way that these bacteria are able to evade the host immune system. Sialic acid has a factor H binding site present within its structure. Factor H is a major complement mediator which converts C3b to C3bi. C3b is a complement protein which is deposited on bacterial cell surfaces and then opsonises, leading to phagocytosis. C3bi is an inactivator protein of the complement system and may be one way in which the immune system prevents complement deposition on host cell surfaces (Ram et al., 1998). However, neuraminidase is able to remove these sialic acids, leaving the bacteria open for recognition by the host immune system. This is a tool by which the pneumococcus prevents other bacteria from occupying its niche in the nasopharynx (Shakhnovich et al., 2002).

Neuraminidase is a multifunctional virulence factor which also plays a large role in the pathogenesis of pneumococcal infection. Neuraminidase A is a conserved protein that is surface exposed, making it an ideal vaccine candidate. Nan A is used as an antigen in this work to determine whether it may give any protection in different mouse models of infection.

D. Colonisation, pneumonia and sepsis

S. pneumoniae is spread horizontally in humans, which increases the rates of carriage in overcrowded environments such as hospitals, prisons and day care centres (Gray et al., 1980). Carriage of pneumococci can begin soon after birth and children under five years old have the highest carriage rates of any age group (Riley and Douglas, 1981). It has been shown that it is possible to have more than one

serotype of pneumococci carried in the nasopharynx at any one time (Gray and Dillon, 1989).

S. pneumoniae colonises the nasopharynx first before it causes invasive disease (Bogaert et al., 2004a). Invasive disease is an exception and, in healthy hosts, the pneumococcus does not tend to cause invasive disease and colonisation is asymptomatic. This may be due to the host immune system generating specific polysaccharide antibody to the serotype which colonises the nasopharynx (Musher et al., 1997). However, more recent work suggests that systemic antibody response levels have no effect on nasopharyngeal colonisation (Richards et al., 2010). Other research shows that, pneumococci are cleared from the nasopharynx by a CD4+ T cell response, which is dependent upon the presence of neutrophils (Malley et al., 2005). However, in immuno-compromised hosts such as the elderly and HIV infected individuals pneumococci can cause disease.

1. Colonisation

In the situation where invasive disease does occur, colonisation of the nasopharynx happens for around one month (Gillespie, 1989). Colonisation of the nasopharynx begins with adhesion of the encapsulated pneumococcus to the epithelial cell membrane. The polysaccharide capsule prevents the exposure of bacterial cell proteins and is down regulated to allow adhesion to epithelial cells (Magee and Yother, 2001). The capsule is required to prevent the deposition of complement factors on the bacterial cell wall from mucus secretions, present in the nasopharynx, and prevent phagocytosis of the pneumococcus (Nelson et al., 2007). Adhesion and attachment to the host cell membrane is mediated by many pneumococcal proteins such as phosphorylcholine, choline binding protein A and

neuraminidase A. Phosphorylcholine is present on the cell wall of pneumococci and binds to the host cell receptor for platelet activating factor (Cundell et al., 1995b). Choline binding protein A binds to the human secretory component, present on epithelial cells, and human secretory antibody (Rosenow et al., 1997). Neuraminidases are able to cleave host sialic acids and glycoproteins, to allow host cell receptors to be exposed for further adhesion and attachment of pneumococci to the host cell surfaces (King et al., 2006).

Following adhesion and attachment of the pneumococcus to host cell membranes, invasion of the basement membranes of the host begins. This is a switch from asymptomatic colonisation to invasive disease. The mechanism behind this change, from colonisation to infectious disease, is not yet completely understood. However, it has been shown that secondary infection of *S. pneumoniae* is common, following a viral infection with influenza or the common cold (Diavatopoulos et al., 2010). This may be due to the previous viral infection activating the receptors (Hakansson et al., 1994) needed for the pneumococcus to adhere and invade.

The mechanism, by which the switch from colonisation to invasive disease occurs, is thought to involve a change in phase variation. Phase variation of pneumococci, from an opaque colony morphology to a transparent morphology, causes the bacteria to be more virulent (Weiser et al., 1994). Pneumococci with transparent colony morphology are able to bind much more efficiently to epithelial cells (Weiser et al., 1994).

2. Pneumonia

Pneumococci move down to the lower respiratory tract. This is due to the airflow to the lungs, and is facilitated by the immuno-modulatory actions of pneumolysin. Pneumolysin prevents the cilliary beat of epithelial cells in the respiratory tract, allowing easy access for the bacteria into the lungs (Steinfort et al., 1989). Pneumococci then repeat the adherence and attachment to bronchoepithlial cells (Adamou et al., 1998).

The invasion of the epithelium and endothelium leads to pneumonia. The mechanism by which invasion happens has been largely studied using *in vitro* cell culture experiments. Invasion is facilitated by the pneumococcal proteins enolase A, PavA and hyaluronate lyase. Enolase A and PavA break down the fibronectin and plasminogen present in the extracellular matrix of the membranes (Kolberg et al., 2006, Bergmann et al., 2001), whilst hyaluronate lyase breaks down the hyaluronan found in connective tissues in the extra cellular matrix (Jedrzejas et al., 2002). Interestingly, an *in vivo* study using green fluorescent protein (GFP) labelled pneumococci, showed that no pneumococci were present in between tight junctions of broncho epithelial cells (Kadioglu et al., 2001). The authors suggest this is due to inflammatory conditions of epithelial cells *in vitro,* which may cause the expression of different receptors.

Pneumococci may then be internalised by activated endothelial cells, through the binding of phosphorylcholine to the platelet activating receptor (Geelen et al., 1993, Rosenow et al., 1997). Concurrently, *in vivo* studies using GFP labelled pneumococci, show that they are internalised by activated endothelial cells. However, transcellular migration of pneumococci through epithelial cells was not

dependent on attachment to the cell. Internalisation of pneumococci by epithelial cells occurs over time, allowing access to the peri –bronchiolar space. Pneumococci were located at the peri bronchiolar spaces *in vivo* within 24 hours of infection.

In cell culture experiments epithelial cells, known as A549's and endothelial cells, from the primary umbilical vein, have been shown to become activated. Activation of these cells occurs through the release of cytokines from host cells, as well as cell wall components present due to bacterial cell division (Cundell et al., 1995b). Activation of epithelial cells and endothelial cells may contribute to the upregulation of the platelet activating receptor, thereby, facilitating pneumococcal internalisation.

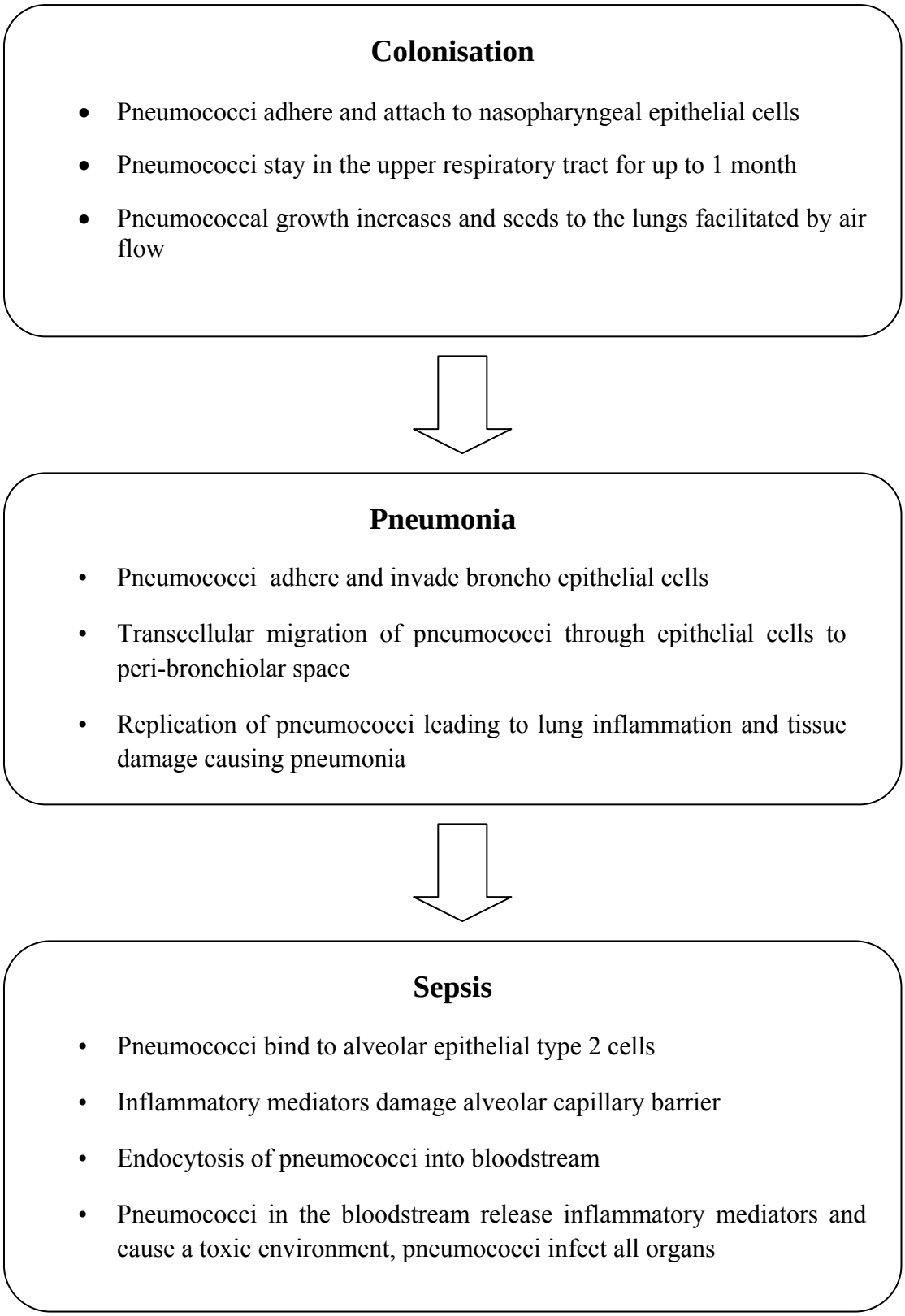
3. Sepsis

Pneumococci have been shown to bind to alveolar type 2 cells called A549 cells (Cundell and Tuomanen, 1994). Pneumococcal binding to alveolar type 2 cells leads to bronchopneumonia and transition of the bacteria from the lungs to the blood stream, which causes sepsis. The alveolar capillary barrier is damaged by pneumococcal proteins and cell wall products (Tuomanen et al., 1987). The host inflammatory response to the infection increases damage to the lungs through the influx of immune effecter cells.

It has been hypothesised that pneumococcal transition from the outer side of the alveolar cells to the blood stream through endocytosis (Cundell et al., 1995a). Phosphorylcholine, expressed on the surface of pneumococci, induces the epithelial cells to endocytose (Talbot et al., 1996) the bacteria, which then transits safely through the cytoplasm of the cell and is released whole on the other side

(Tuomanen, 1997). This is the mechanism by which it is thought pneumococci enters the blood stream and causes sepsis.

During sepsis, pneumococci proliferate in the bloodstream and infiltrate all organs. Pneumococci may also invade the lymphatic system to replicate (Boulnois, 1992). Pneumococci may then infiltrate the meninges and cause meningitis. Whilst pneumococci replicate, inflammatory factors such as cell wall products, pneumolysin, pspA and pspC all contribute to evading the host immune system (Quin et al., 2007). Infiltration of the blood stream creates an inflammatory toxic environment (Johnston, 1991) and the host may succumb to infection (Boulnois, 1992). However, if the host immune response is unable to clear the infection from the blood stream, treatment with antibiotics may also lead to clearance of pneumococci.


Colonisation
• Pneumococci adhere and attach to nasopharyngeal epithelial cells
• Pneumococci stay in the upper respiratory tract for up to 1 month
• Pneumococcal growth increases and seeds to the lungs facilitated by air flow
Pneumonia
• Pneumococci adhere and invade broncho epithelial cells
• Transcellular migration of pneumococci through epithelial cells to peri-bronchiolar space
• Replication of pneumococci leading to lung inflammation and tissue damage causing pneumonia
Sepsis
• Pneumococci bind to alveolar epithelial type 2 cells
• Inflammatory mediators damage alveolar capillary barrier
• Endocytosis of pneumococci into bloodstream
• Pneumococci in the bloodstream release inflammatory mediators and cause a toxic environment, pneumococci infect all organs

E. The host immune response to colonisation, pneumonia and sepsis

During colonisation, the innate and adaptive host immune response is activated. Many factors play a role in this activation. One of the most important factors in clearance of pneumococci is the activation of one or more of the complement pathways (Paterson and Mitchell, 2006). There are three complement pathways; the classical pathway, alternative pathway and the lectin pathway. Once one or more of these pathways is activated, it leads to the deposition of complement mediators on the surface of pneumococci, opsonising the bacteria to allow phagocytosis by macrophages and neutrophils. The classical pathway which is mediated by antibody deposition on the pneumococcal cell surface, is the dominant pathway in mediating pneumococcal clearance (Brown et al., 2002).

The innate immune response is triggered by pathogen associated molecular patterns (PAMP's), present on the pneumococcal cell surface, such as lipotechoic acid (Paterson and Mitchell, 2006). PAMP's trigger pattern recognition receptors (PRR's), which may be in soluble forms; some examples are C reactive protein (CRP) and mannan binding lectin (MBL), an alternative pathway complement protein. PRR's are also cell associated and are called toll like receptors (TLR's) (Janeway and Medzhitov, 2002). TLR2 recognises lipotechoic acids and peptidoglycan on the pneumococcal cell surface (Yoshimura et al., 1999). TLR 1 and TLR 6 are also activated by lipotectoic acid and peptidioglycan respectively. TLR 9 is an intracellular PRR and is thought to be activated by unmethylated cytosine-phosphate-guanosine (CpG) motifs present on bacterial DNA (Paterson and Mitchell, 2006). Activation of TLR's triggers a complex, intracellular, signalling cascade which activates the transcription factor NF-κB. NF-κB then

regulates the expression of genes, which the immune system needs to defend itself from infection, such as cytokines, chemokines, costimulatory molecules and antimicrobial peptides (Janeway and Medzhitov, 2002).

Although it is believed that the classical complement pathway is the most important way of clearing pneumococci, there is a limited role for antibody mediated clearance in colonisation. Van Rossum et al., (2005), showed that for clearance of colonisation, TLR2 needs to be activated along with an efficient CD4+ T cell response (Malley et al., 2005). Another group has shown that TLR2 is expressed on the surface of activated T cells and provides co-stimulation (Komai-Koma et al., 2004) This is an important determinant in understanding the mechanism of CD4+ T cell antibody independent clearance of pneumococci from the nasopharynx (Malley et al., 2005). More recent studies have shown those IL-17A producing CD4+ T cells are able to clear pneumococcal colonisation (Lu et al., 2008). This response is reliant upon the presence of neutrophils, which are activated by specialised IL-17A producing CD4+ T cells. These T cells are known as Th17A regulatory cells (Malley et al., 2005). This is a mechanism which does not require specific antibodies to clear colonisation (Lu et al., 2008). Although some of these studies used unencapsulated pneumococci, for induction of colonisation, this does not mimic colonisation in humans. These studies also show that antibody may not be required for clearance of pneumococci. However, it is unclear how pneumococci are phagocytosed by neutrophils, with out the activation of complement or the presence of specific antibodies for opsonisation.

The immune response to pneumococci in a mouse model of pneumonia was described in detail by Bergeron et al,. (1998). The immune response to pneumococci in the lung begins with activation of TLR's, which then initiates a

proinflammatory response. TLR activation leads to activation of alveolar macrophages which release proinflammatory cytokines IL-6 and tumour necrosis factor alpha (TNF-α). Following this, neutrophils and CD4+ T cells are recruited by cytokine and chemokine release. Infiltration of neutrophils is mediated by the expression of integrins, Mac-1 and $\alpha_4\beta_{1,}$ on the surface of the cells. T cell migration is mediated by the expression of $\alpha_4\beta_1$. These integrins bind to vascular cell adhesion molecule 1 (VCAM-1) on the bronchiolar epithelial cell surface (Kadioglu, 2011). This interaction allows T cell and neutrophil transmigration to the bronchioles to begin controlling pneumococcal infection.

Neutrophils are the main effector cell employed to limit the growth of pneumococci by phagocytosis and release of superoxide molecules. Release of superoxide molecules into the extracellular milieu can be detrimental to the host as the contents of neutrophils lead to lung tissue damage, facilitating transition of pneumococci from the lungs to the blood (Bergeron et al., 1998). It has been shown by Kadioglu et al., (2004), that pneumolysin is important in preventing neutrophils from clearing pneumococci in the lung. Pneumolysin negative mutants of *S. pneumoniae* are cleared by the host immune system within 48 hours of the first infection. Following neutrophil influx into the lung, lymphocytes surround the bronchioles. CD4+ T cells are also attracted to the site of infection by pneumolysin expression (Kadioglu et al., 2004).

The presence of CD4+ T cells have been shown to be important for the clearance of pneumococci in both early and late stages of pneumonia. Studies showed that MHC II deficient mice (mice that are CD4+ T cell deficient) were more susceptible to pneumonia and septicaemia (Kadioglu et al., 2004). CD4 + T cells have been shown to be crucial for the clearance of pneumococcal pneumonia

in humans. A decrease in circulating T cells, which happens in human immunodeficiency virus (HIV) infected individuals, makes the patient more susceptible to pneumonia and septicaemia (Torres et al., 1998). Previously, CD4+ T cells, with a type 1 cytokine profile, that were able to release interferon gamma (IFN-γ) were thought to be necessary for bacterial clearance of pneumonia (Rubins and Pomeroy, 1997). However, new data shows that IFN-γ, is released by natural killer cells (NK), in early pneumococcal infection (McNeela et al., 2010). Release of IFN- γ from NK cells drives a Th1 inflammatory T cell response (Martin-Fontecha et al., 2004). The cytokine profile of a Th 1 T cell includes IL-2, IL-3, IL-6, IL-12, TNF-α and IFN-γ (Gutcher and Becher, 2007). It has also been reported that, patients with an IL-12 deficiency are more prone to pneumococcal infection as they are unable to induce a type one cytokine profile (Haraguchi et al., 1998).

In vivo studies suggest T cells may also be driven to a Th17 cell subset, which release IL-17A, and have been shown to be an important cell in the protection from pneumococcal disease (Lu et al., 2008). Interestingly, these responses, driving IFN-γ and IL-17A production, are dependant upon the presence of pneumolysin. Pneumolysin synergises with endogenous danger signals to induce cytokine secretion (McNeela et al., 2010). Pneumolysin is also able to induce IL-1β expression in dendritic cells by activation of the NLRP3 inflammasome. Both IL-1β and the NLRP3 inflammasome are required for protection from pneumococcal disease (McNeela et al., 2010) as both drive an inflammatory response, which leads to neutrophil infiltration into the lungs.

During sepsis, there is a disappearance of circulating CD4+ T cells (Kemp et al., 2002). This may be due, in part, to pneumococci inducing apoptosis of T cells, both CD8+ and CD4+ or, it may be due to the cells infiltrating the tissues

where pneumococci are residing. Once pneumococci have entered the bloodstream, the most important host immune response is complement. Complement mediates clearance from the blood by CRP deposition on the bacterial cell surface, allowing blood circulating neutrophils and macrophage phagocytosis of pneumococci. CRP deposition on the pneumococcal cell surface also stimulates dendritic cell antigen presentation (Thomas-Rudolph et al., 2007). CRP binds pneumococcal C polysaccharide on the pneumococcal cell surface (Nakayama et al., 1983). Opaque phase variants, with less expression of C polysaccharide, survive for longer in the bloodstream (Cundell et al., 1995c). The spleen (Altamura et al., 2001) and liver (Hurlimann et al., 1966) are extremely important in clearance of pneumococci from the bloodstream. The liver is the organ that produces CRP for complement activation. The spleen is an immune system organ, which is able to mediate a T independent reaction to pneumococcal polysaccharide, as well as mounting an antigen specific response; all contributing to the clearance of pneumococci (Altamura et al., 2001). Patients with their spleens removed are more susceptible to pneumococcal sepsis (Iinuma and Okinaga, 1989).

The immune response to pneumococci is a balance between the innate and adaptive immunity. In most cases, invasive pneumococcal disease does not occur. However, in patients where invasive disease does occur, it is important to have pneumococcal specific antibodies. Specific antibodies play a large role in mediating and preventing colonisation, and mediate the clearance of pneumococci from the lungs and blood. Therefore, it is important to have a vaccine against *S. pneumoniae* which elicits T cell mediated immunity, and also leads to specific antibody production by B cells, in the host.

F. Vaccines currently available

Currently, there are two types of pneumococcal vaccine available. The first is a polysaccharide vaccine, containing 23 polysaccharides from the most commonly isolated serotypes responsible for causing invasive disease (Bogaert et al., 2004b). Over 80% of the world's cases of invasive pneumococcal disease can be attributed to just 20 serotypes out of 90 (Lynch and Zhanel, 2009a). The impact of the vaccination strategy, introduced in 1983, showed an overall decrease in invasive pneumococcal disease from serotypes included in the vaccine. However, there are some disadvantages of this vaccine; it is not protective in children under 2 years old and has decreased efficacy in the elderly (over 65's) (Huss et al., 2009), as it is poorly immunogenic in these groups (Bogaert et al., 2004b). Children, under the age of two, do not yet have a fully developed immune system and so, T cell independent antigens such as polysaccharides are poorly immunogenic and do not elicit the antibody response required to prevent pneumococcal disease. The normal response to T cell independent antigens also declines with age, which is why the elderly are affected. Immuno-compromised individuals, for example those who are HIV infected, also have a poor immune response to these T cell independent antigens (Ho and Lin, 2005). The 23 valent pneumococcal polysaccharide vaccine (23PPV) is most effective in young healthy adults. The disadvantages of 23PPV show a requirement for a different type of vaccine, which is effective in the young, elderly and immuno compromised.

The disadvantages of the 23PPV were taken into account and another vaccine was developed to combat invasive pneumococcal disease. The second vaccine, widely available, is known as Prevenar and is a protein-polysaccharide

conjugate vaccine, which contains 7 serotypes of the 90 of *S. pneumoniae.* The pneumococcal conjugate vaccine 7 (PCV-7) is conjugated to a diphtheria toxin. The conjugate vaccine is very effective in children under five years old (Black et al., 2000) and immuno compromised individuals, with the rate of invasive pneumococcal disease in the US falling by 63-74% (Lynch and Zhanel, 2010). The vaccination scheme for PCV-7 began in the United States in the year 2000. Currently, there is also a 9 valent pneumococcal conjugate vaccine, in use in Gambia, which is effective in reducing the number of cases of invasive pneumococcal disease (Cutts et al., 2005). The nine valent conjugate vaccine has also been shown to elicit protection in HIV infected individuals in South Africa (Klugman et al., 2003). Recently, a 10 valent conjugate vaccine and a 13 valent conjugate vaccine were brought on to the market (Lynch and Zhanel, 2010).

There are some disadvantages of vaccination with conjugate vaccines. Firstly, its high cost of production, means it is not used in the populations in developing parts of the world, like Sub Saharan Africa and South East Asia, where it is most needed. There is also the problem of serotype replacement (Mu et al., 2008). This is where serotypes, not included in that conjugate vaccine, are causing invasive pneumococcal disease. Serotype replacement has been noticed more in children in Native American and Alaskan populations, where the incidents of pneumococcal disease from non vaccine type pneumococcal serotypes has increased since the start of the PCV-7 vaccination program (Singleton et al., 2007). Serotype 19A (a non vaccine serotype) has been shown to be found in 70% of IPD cases in 2003-2004 in the US (Pai et al., 2005). Furthermore, the numbers of IPD cases attributable to this serotype, post vaccination, has more than doubled (Pai et al., 2005). Therefore, it is important to create a vaccine which will provide

protection against all pneumococcal serotypes and is also less expensive than Prevenar.

G. New approaches to pneumococcal vaccination

Many new approaches to pneumococcal vaccination are being considered. One idea is to use pneumococcal proteins that are conserved in all 90 serotypes, as antigens for immunisation. Several proteins are being tested for protective efficacy (Bogaert et al., 2004b). If pneumococcal protein vaccination could be developed, this would prevent the problem of serotype replacement (Bogaert et al., 2004b). Pneumococcal proteins are cheap and easy to produce (Paton et al., 1993) and so could be distributed to places with the most need for a vaccine. Many studies have been carried out on pneumococcal proteins and their protective roles in pneumococcal disease. Protein immunisation studies will be discussed in detail later in the chapter.

Another idea to prevent invasive pneumococcal disease, and which is still able to cover all serotypes of *S .pneumoniae,* is to use a heat killed whole cell vaccine (Malley et al., 2001). A study in rats and mice showed that immunisation, with an unencapsulated RX1 autolysin deficient mutant of *S. pneumoniae,* could be protective against colonisation and invasive disease, when administered with cholera toxin. The authors admit that although the vaccine was protective, this method of administering cholera toxin, with unencapsulated pneumococci, may not elicit the mucosal immunity needed. There may also be problems in producing exactly the same batches of unencapsulated bacteria, as different batches may not express all of the proteins required for protection in the correct conformation (Malley et al., 2001). Following this, investigations into better adjuvants and

inactivation of unencapsulated bacteria were carried out by Lu et al., (2010). Better adjuvants were found, such as heat labile toxin from *Escherichia coli,* which could illicit mucosal immunity (an important factor in preventing colonisation). However, unencapsulated pneumococci do not allow the host to generate immunity against the most important virulence factor: the polysaccharide capsule. This approach to pneumococcal vaccination only generates an immune response against protein virulence factors. The disadvantages of this type of vaccine are clear, therefore, further study is needed to produce the most protective whole cell vaccine.

Other new approaches to pneumococcal immunisation include trying to prevent colonisation of the nasopharynx. Preventing colonisation would stop all invasive pneumococcal disease. Clearance of colonising pneumococci relies on the vaccination being able to illicit mucosal immunity in the nasopharynx. Villena et al., (2010), have recently shown that *Lactococcus lactis,* expressing a pneumococcal protein, is able to induce mucosal immunity and protects from subsequent infection with pneumococci. Another group have shown that pneumolysin conjugated to green fluorescent protein and pneumococcal surface protein A is able to induce mucosal protection (Douce et al., 2010). However, there are difficulties with targeting mucosal immunity, as it is unclear whether or not the niche, that the pneumococcus occupies in the nasopharynx as a commensal, may be replaced with a different disease causing bacterium (Bogaert et al., 2004a).

It is clear there are many different approaches to producing a new and more widely protective pneumococcal vaccine. Most new approaches are attempting to address the issue of serotype replacement. Protein vaccines address these issues and are studied in this work.

H. Mouse models of *S. pneumoniae* infection

There are many models set up for infection of mice with *S. pneumoniae.* These models include colonisation, otitis media, pneumonia, meningitis and sepsis. The mouse models described here are relevant to this work and are the models used for challenge of mice within this project.

It is important to mention that mouse models of pneumococcal infection are dependant upon many factors, such as the strain of mouse. Previously it has been shown that different mouse strains have different susceptibilities to pneumococcal infection (Kadioglu and Andrew, 2005, Gingles et al., 2001). Other factors influencing the model of pneumococcal infection in mouse models, are pneumococcal strain, dose and volume.

S. pneumoniae infection first begins with colonisation of the upper respiratory tract and so a mouse model of colonisation was first set up by Wu et al., (1997). The study showed mice could be colonised with pneumococci by instilling 10µl into the nares of non anaesthetised mice. This model was tested in three different types of inbred mice BALB/cJ, CBA/N and A/J. The mice had carriage of pneumococci in the nasopharynx for up to seven days after infection, without any symptoms. A disadvantage of this model is that the infection did not spread from one mouse to another in the cage. Therefore, it is unable to completely mimic the way in which *S. pneumoniae* is spread in humans. Another model of colonisation was described by Richards et al., (2010), for MF1 outbred mice. This model is similar to the first model. However, mice are anaesthetised to instill pneumococci into the nares preventing them from sneezing out the bacteria. This model also uses outbred mice, which enables it to mimic genetic variation present in the human

population. This model allows asymptomatic, stable colonisation of the nasopharynx.

Mouse models of pneumonia were described in the late 1980's by Berry et al., (1989). Canvin et al., (1995), described the first, in detail, mouse model of intranasal infection with passaged pneumococci. Canvin et al., (1995), demonstrated that anaesthetized mice could be intranasally inoculated with 50µl of pneumococci, subsequently leading to pneumococcal pneumonia or bronchopneumonia. Mice, infected with pneumococci intranasally, develop pneumonia as the bacteria seed to the lungs. Following this, the infection can spread to the blood and cause bacteraemia and sepsis. This model is widely used for investigating lung infection in many studies, including immunisation protection studies. The method used for acute intranasal infection of MF 1 mice in this study has also been described in the work of Kadioglu et al., (2000).

One of the first models to induce sepsis in a mouse model of *S. pneumoniae* was described by Briles et al., in 1981. Briles et al., (1981), were investigating the importance of anti-polysaccharide antibodies in the protection of mice from intraperitoneal and intravenous infection with pneumococci. The model described the intravenous injection of 200µl of pneumococci into the mouse. The strains of mice used in this study were inbred CBA and BALB/c. The first study to describe the use of a lamp for vasodilatation before injection was Iannelli et al., (2004), and they used outbred MF1 mice, which are also used in the work presented here.

Another way of inducing systemic infection in a mouse model is intraperitoneal injection with pneumococci. This model was first described by Briles et al., in 1982, and used CBA and DBA inbred mouse strains. Mice were

intraperitoneally infected with pneumococci to cause sepsis. This model has also been described by Canvin et al., (1995), for the passage of pneumococci to increase virulence of the bacteria. Kadioglu et al., (2000), also showed the use of intraperitoneal infection with pneumococci type 2 strain, in outbred MF1 mice. This method is well established and is used for the passage of pneumococci in this study.

I. Pneumolysin immunisation studies

Pneumolysin has been used in many immunisation studies to date. However, since pneumolysin is toxic when it has hemolytic activity, pneumolysin toxoids, such as PdB or PdT, are generally used for immunisation studies.

The first protective immunisation, using pneumolysin, was carried out in 1983 by Paton et al. Native pneumolysin was purified from a high expressing colony of *S. pneumoniae*. Pneumolysin was mixed with Freunds complete adjuvant and injected intraperitoneally at three different time points, 10 days apart. Mice were then challenged, intranasally, with a virulent serotype 2 strain (D39) of *S. pneumoniae.* Pneumolysin immunised mice survived for 5.5 days, whereas, the control group survived for 2.4 days. Another outcome of this study was that it showed pneumolysin was immunogenic. When used as an antigen, the immunised group had a serum anti-pneumolysin level which was forty times higher than the control group.

In 1991 another protective immunisation study was carried out by Paton et al. This study was more sophisticated in its approach, as the authors created the pneumolysin toxoids PdB and PdA (now known as PdT), which had 0.1% and 0.001% hemolytic activity respectively, compared to the wildtype PLY. PdA and PdB were less toxic to mice. Balb/c mice were immunised, intranasally, with each

toxoid, separately, three times- fourteen days apart. Fourteen days after the last immunisation, mice were challenged with D39 intranasally or intraperitoneally. The results showed that the PLY, PdA and PdB toxoid protected the mice from intranasal challenge and the median survival time was significantly longer, compared to control immunised mice. However, there was no significant difference in survival between protein immunised groups. The PdB toxoid significantly increased survival time in mice, when compared to control, PLY and the PdA toxoid, after intraperitoneal challenge.

The next most noteworthy study using pneumolysin as an antigen was carried out by Alexander et al., in 1994. PdB was used as the antigen. The results showed, that immunisation, with this pneumolysin toxoid, conferred protection against challenge from nine different serotypes of *S. pneumoniae*. Mice were challenged intranasally and PdB immunised mice survived for 14 days, whereas, the control immunised group survived only 3.2 days on average. The antibody response was measured from serum samples, collected throughout the course of the experiment. ELISA results showed that there were high levels of IgG1 and IgG2a present. It is important for vaccines to elicit an IgG subclass response, such as IgG1 and IgG2a, as these antibodies are produced by a thymus dependant reaction. It has been shown that IgG2a is complement fixing, and promotes opsonophagocytosis as well as being able to bind to the high affinity macrophage receptor Fcγ (Unkeless et al., 1988). This response elicited immunological memory which was long lasting (van de Wijgert et al., 1991) compared to the weak interaction and short term memory that IgM antibodies are able to elicit.

Pneumolysin has been employed in many more immunisation studies. Ogunniyi et al., (2000), showed a combination of pneumococcal protein virulence

factors, including pneumolysin, conferred protection against invasive pneumococcal disease. However, immunisation with PdB plus alum adjuvant did not elicit a protective response, when Balb/c mice were challenged intraperitoneally with a type 2 strain of pneumococci. This is in contrast to the earlier work by Paton et al., 1991, in which PdB was protective when mice were intraperitoneally infected. The only difference between the studies by Paton et al., (1991), and Ogunniyi et al., (2000), were the adjuvants they each used. The combination of PdB, pneumococcal surface protein A (Psp A) and pneumococcal surface protein C (Psp C), as antigens, did provide a much higher protective response. When mice were immunised with all three proteins and subsequently challenged, their survival time was significantly increased, compared to the control immunised group. The authors suggest that PdB may be a better adjuvant than a protein vaccine candidate in this model of immunisation and infection.

Basset et al., (2007), studied the combination of three pneumococcal proteins as immunizing agents. PspC, PspA and PdT were the pneumococcal proteins used to immunise C57BL/6J mice (a mouse strain susceptible to pneumococcal infection (Gingles et al. 2001)) and the study also used cholera toxoid as an adjuvant. The authors demonstrated that CD4+ T cells also played some role in the protective response, showing protection in the mice is not always antibody mediated, as originally thought. T cell dependant protection was shown by depleting one group of immunised mice of CD4+ T cells before challenge. Colonisation of the nasopharynx, in immunised mice with a normal T cell response, was greatly reduced when compared to adjuvant alone. However, when the T cells were depleted the protective immune response, seen before, was lost.

The most recent immunisation study using pneumolysin was carried out by Wu et al. (2010). This group constructed a new pneumolysin toxoid ΔA146 Ply, which has a single amino acid change, leaving the protein with no haemolytic activity. Balb/c mice were subcutaneously immunised with ΔA146 Ply three times, 10-18 days apart. Mice were then challenged intraperitoneally 4 weeks after the last immunisation. Mice immunised with ΔA146 Ply survived significantly longer than the control group. However, there was no difference between the wildtype PLY and with ΔA146 Ply survival times.

Pneumolysin and its toxoids elicit protection in mice that are immunised. However, the protection is different in each study, as there is not one defined protocol for immunisation studies. The work discussed above is summarised in

Table 2.

These studies all show protection of mice is dependant on the immunisation route, challenge route and the strain of mouse used as well as the strain of pneumococci used. The underlying question is; what mediates this protection? Studies are conflicting about whether protection from colonisation is T cell mediated or antibody mediated and more work on pneumolysin immunisation needs to be carried out to clearly define which is accurate.

Table 2- summarises important pneumolysin immunisation studies carried out to date.

Immunising agent and route of administration	**Mouse strain**	**Challenge strain and site**	**Conclusions**	**Author and year of publication**
Native pneumolysin I.P.	Outbred Prince Henry	D39 serotype 2 I.N.	Increased survival time	Paton et al., 1993
PdB or PdT I.P.	Inbred Balb/c	D39 serotype 2 I.N. or I.P.	Both proteins increased survival time in I.N. challenge, PdB increased survival time in I.P. challenge	Paton et al., 1991
PdB I.P. or Subcutaneous	Outbred quakenbrush and MF1	9 different pneumococcal serotypes I.N.	Protection from 9 different strains of pneumococci. IgG1 and IgG2a antibody response	Alexander et al., 1994
PdB I.P.	Inbred Balb/c	D39 I.P.	Increase in survival time by one day compared to control group	Ogunniyi et al., 2000
PdB in combination with PspA and PspC I.P.	Inbred Balb/c	D39 I.P.	Increased survival time	Ogunniyi et al., 2000
PdT in combination with PspC and PspA I.N.	Inbred C57BL/6J	0603 serotype 6B I.N.	Reduced colonisation Protection is CD4+ T cell dependant and antibody independent	Basset et al., 2007
Ä146PLY Subcutaneous	Inbred Balb/c	D39 serotype 2 I.P. or I.N. 31614 Serotype 14 I.N.	Slight increase in survival time for I.P. D39 challenge Significant increase in survival following D39 I.N. challenge Decrease in lung colonisation after I.N. challenge with 31614	Wu et al., 2010

J. Neuraminidase immunisation studies

Recombinant neuraminidase A is the only pneumococcal neuraminidase protein to have been employed in immunisation studies. Yesilkaya et al., (2006), showed that recombinant Nan A gave outbred MF1 mice protection. Mice were intraperitoneally immunised three times with Nan A and subsequently intranasally challenged with virulent passaged pneumococci. The mean survival time for the control group in this study was 49 hours and the mean survival time for the Nan A immunised group was 97 hours. This study also showed that the protein was immunogenic in these mice and the protection was thought to be mediated by the antibody levels present in the mouse serum.

Recombinant Nan A has also been shown to afford protection against pneumococcal colonisation in a chinchilla model of intranasal infection (Tong et al. 2005). This study also showed that the protection from colonisation by immunisation led to a 50% decrease in otitis media in the chinchillas.

There have been few studies that use neuraminidase as an antigen. However, further investigation is required to understand the protection neuraminidase elicits in different mice and models of infection. The main mediators in protection elicited by Nan A also require further research.

K. Pneumococcal protein immunisation studies

Many other pneumococcal vaccine candidates have been identified because of their role as virulence factors, their surface exposure or conservation of the protein in all serotypes. Pneumococcal proteins that have been used in

immunisation protection studies, apart from pneumolysin and neuraminidase, are PspA, PspC, Lyt A, CbpA, PsaA, PiuA, PiaA, histidine triad proteins and pneumococcal protective protein A (PpaA) (Tai, 2006).

PspA is one of the most widely studied proteins used in immunisation experiments. The first study to discover immunisation with PspA elicited protection against pneumococcal challenge was carried out in 1991 by McDaniel et al. Inbred CBA/N mice were immunised with recombinant purified PspA twice, (fourteen days apart) and challenged intravenously seven days later. All of the mice in the immunised group survived challenge. This protection was cross reactive and protected the mice against two different pneumococcal serotypes.

Immunisation with *Lactococcus casei,* expressing PspA to induce mucosal protection was another approach to providing protection with PspA (Campos et al., 2008). C57 mice were immunised intranasally 5 times over a period of 28 days. 15 days later, mice were challenged intraperitoneally with a serotype 3 strain of pneumococci. Mice immunised with the vector expressing PspA survived significantly longer than the control group. The immunisation also induced mucosal and systemic anti PspA antibodies.

PspA has also been used in a DNA vaccine, which expresses the protein and induces protection against colonisation. The PspA gene was encoded into a DNA vaccine and C57/BL 6 mice were immunised through the intramuscular route three times (fourteen days) apart and subsequently challenged with a colonisation dose of serotype 6 pneumococci. Mice immunised with the PspA DNA vaccine had reduced carriage in the nasopharynx, when compared to the empty DNA vector vaccine (Ferreira et al., 2010).

PsaA is a highly conserved and surface exposed pneumococcal protein, making it an ideal vaccine candidate. The first study to show that PsaA was a protective antigen was carried out by Talkington et al., (1996). CBA mice were subcutaneously immunised twice, (fourteen days apart) with recombinant PsaA and complete Freunds adjuvant. Seven days later, mice were challenged with an intravenous dose of serotype 3 pneumococci. The results showed 18 out of 20 mice, immunised with PsaA, survived challenge compared to 2 in the control immunised group.

As well as PsaA being protective against invasive challenge, it is also protective in a colonisation model of infection (Briles et al., 2000). In the study by Briles et al., (2000) mice were intranasally immunised with purified PsaA 3-5 days apart, 6 times. Following this, mice were challenged intranasally with a colonisation dose of serotype 23 pneumococci. Mice immunised with PsaA had significantly less bacteria recovered from the nasopharynx, compared to the control immunised mice. However, the authors also combined PsaA and PspA, which induced more protection, as fewer bacteria were recovered from the nasopharynx of these mice compared to PsaA alone.

The PsaA gene has been encoded in to a live attenuated salmonella vector and expressed for use in an immunisation study (Wang et al., 2010). Live attenuated *Salmonella enterica* has previously been shown to induce long lasting immune responses (Li et al., 2008). *Salmonella enterica* was used to express PsaA. Following this, C57/BL6 and Balb/c mice were intranasally immunised at time zero and again six weeks later. Four weeks later, mice were challenged intranasally or intraperitoneally with serotype 2 pneumococci. Decreased numbers of pneumococci were recovered from the nasopharynx of immunised mice compared to the control

group. However, there was no difference in survival time between the control and immunised mice, following intraperitoneal challenge. Therefore, immunisation with the vector expressing PsaA was protective against colonisation of the nasopharynx but was not protective against invasive challenge.

Lyt A was shown to be a protective antigen by Lock et al., (1992). Balb/c mice were immunised intraperitoneally with purified LytA three times (fourteen days apart) and intraperitoneally challenged with serotype 2 pneumococci. Twenty three mice in the LytA immunised group survived challenge, whereas only 9 of the control immunised mice survived. This result is in contrast to an earlier published study by Berry et al., (1989). In this study, outbred MF1 mice were intraperitoneally immunised with purified LytA three times (10 days apart) and subsequently challenged intranasally with serotype 2 pneumococci. Mice immunised with LytA had an increased survival time of only one day, in comparison to the control immunised mice.

Choline binding protein A (Cbp A) and a serine protease of *S .pneumoniae* called caseinolytic protease (ClpP), which is involved in protein folding, have also been used in an immunisation study (Cao et al., 2009). Balb/c mice were immunised intranasally with a combination of the two antigens, with cholera toxin as the adjuvant, twice a week for three weeks. Fifteen days later, the mice were challenged intranasally with serotype 4 pneumococci. Mice immunised with a combination of the two antigens survived for significantly longer than the control group. However, five immunised mice did succumb to infection. This showed that immunisation was not completely protective in all the animals. The authors also showed that the protection from intranasal challenge was CD4+ T cell mediated. One group of immunised mice had depleted T cells and were subsequently

challenged. These mice did not have an increase in survival after challenge (Cao et al., 2009). This work is in concurrence with Basset et al., (2007), who also showed that protection from immunisation with a combination of pneumococcal proteins is CD4 + T cell dependant.

Immunisation of mice with recombinant PiaA and PiuA elicited some protection in a systemic model of infection with *S.pneumoniae* (Brown et al., 2001b). Balb/c mice were intraperitoneally immunised with single proteins or a combination of both proteins and then challenged intraperitoneally. The combination of both recombinant proteins gave the highest percentage of protection against the pneumococcal challenge with survival of 80% (Brown et al., 2001b).

Pneumococcal histadine triad proteins (Pht) are a family of proteins thought to be involved in metal and nucleoside binding (Adamou et al., 2001). There are four Pht proteins labelled PhtA, PhtB, PhtD and PhtE. These proteins are conserved in all of the serotypes tested so far and are present on the surface of pneumococci. Pht A, PhtB, PhtD and PhtE were used as an antigen in a protection study using Balb/c mice. Mice were immunised twice with one of the four proteins in combination with complete Freunds adjuvant three weeks apart. Mice were then challenged intraperitoneally with either pneumococcal virulent serotype 3, 4 or 6. PhtD immunised mice showed protection from all serotypes, PhtA and PhtB showed protection against serotype 6 but not serotype 4. There was no protection seen in mice immunised with PhtE.

Pneumococcal protective protein A (PppA), which is a surface exposed protein, has been used in immunisation studies by Green et al., (2005). This protein is conserved in all of the pneumococcal serotypes tested and makes a good vaccine

candidate. Green et al., (2007), intranasally immunised Balb/c mice with purified PppA and cholera toxin three times, two weeks apart. Mice were then challenged intranasally with a colonising dose of serotype 3 pneumococci. Mice immunised with PppA had significantly less bacteria recovered from the nasopharynx, compared to the control immunised mice. However, the authors suggest that this may not be the most protective protein antigen for immunisation, as PppA is expressed in small quantities on the pneumococcal cell surface and cross serotype protection with this antigen has yet to be shown.

Pneumococcal proteins have been tested for their antigenicity and protective efficacy in many different mouse models of immunisation and challenge. The studies discussed above are summarised in Table 3.

There is not yet a defined method used to show which pneumococcal proteins may be the most protective and need for further development or a standardised protocol. Mucosal immunity against pneumococci is one of the most important factors determining whether a protein should be studied further. Preventing colonisation is the key to preventing both otitis media and invasive infections in the host.

Table 3- summarises other protein pneumococcal factors used in protection studies

Immunising protein and route of administration	**Mouse strain**	**Challenge strain and route of administration**	**Conclusions**	**Author and year of publication**
PspA Subcutaneous	CBA/N (XID) inbred	WU2 serotype 3 EF-6796 serotype 6A I.V.	Increased survival and cross serotype protection	McDaniel et al., 1991
Lactococcus casei expressing PspA	C57BL/6 Inbred	A66 serotype 3 I.P.	Increased survival and induced mucosal immunity	Campros et al., 2008
PspA DNA vaccine	C57BL/6 Inbred	0603 serotype 6B I.N.	Reduced nasopharyngeal carriage	Ferreira et al., 2010
PsaA Subcutaneous	CBA/CaHNJ (XID) Inbred	Wu2 Serotype 3 I.V.	Increased survival	Talkington et al., 1996
PsaA I.N.	CBA/CaHNJ (XID) Inbred	L82016 serotype 23 I.N. E134 serotype 6B I.N.	Decreased colonisation of the nasopharynx from both challenge serotypes	Briles et al., 2000
Live attenuated salmonella expressing PsaA I.N.	Balb/c C57BL/6 Inbred	E134 serotype 23 I.N. Wu2 serotype 3 I.P. D39 serotype 2 A66 serotype 3 I.N.	Protection from colonisation with E134 No protection from I.P. challenge with WU2 No protection from pneumonia in D39 and A66 challenges	Wang et al., 2010

Immunising protein and route of administration	**Mouse strain**	**Challenge strain and route of administration**	**Conclusions**	**Author and year of publication**
LytA I.P.	Balb/c Inbred	D39 serotype 2 I.P.	Increased survival	Lock et al., 1992
CbpA and ClpP I.N.	Balb/c Inbred	TiGR4 Serotype 4 I.N.	Increased survival Protection was CD4+ T cell mediated	Cao et al., 2009
PiaA and PiuA I.P.	Balb/c Inbred	D39 serotype 2 I.P.	Combination of both proteins increased survival by 80%	Brown et al., 2001b
PhtA, PhtB, PhtD and PhtE Subcutaneous	C3H/HeJ BALB/cByJ Inbred	WU2 serotype 3 EF5668 and N4 serotype 4 EF6796 serotype 6A Sj2 serotype 6B I.P.	Protection from all serotypes with Pht D immunisation Protection from serotype 6 with PhtA and PhtB immunisation No protection from PhtE immunisation	Adamou et al., 2001
PppA I.N.	Balb/c Inbred	WU2 serotype 3 I.N.	Decreased nasopharyngeal colonisation	Green et al., 2005

L. Summary

S. pneumoniae has many virulence factors which contribute to the pathogenicity of infection. Current vaccines used against *S. pneumoniae* do not elicit immune responses in all of the population or are too expensive to be used in the populations, where pneumococcal infections are most common. There is also the major problem of serotype replacement, where the serotypes not included in the

current conjugate vaccine are causing invasive disease. Therefore, it is important to develop a vaccine which is able to encompass all serotypes of *S. pneumoniae*. Protein vaccines have been studied for their protective efficacy in many different models of infection. Both pneumolysin and neuraminidase proteins are important virulence factors and have previously been used in immunisation studies. However, they have not yet been used in immunisation studies which use outbred mice as well as colonisation of the nasopharynx.

M. Aims

There were three main aims of this project. Firstly, to determine whether immunisation with the pneumococcal protein Nan A, was protective in an outbred mouse model of pneumococcal infection. Secondly, to determine the protection that immunisation with PdB elicits, in three different outbred mouse models of pneumococcal infection 1) colonisation, 2) pneumonia and 3) sepsis. Thirdly, to determine which parts of the immune system mediate this protective response during colonisation, pneumonia and sepsis.

II. Material and Methods

A. Growth Conditions and Media

Streptococcus pneumoniae was grown on Blood Agar Base (BAB), containing 5% (v/v) defibrinated horse blood (Oxoid, UK). The medium was made according to Table 4, and then autoclaved at for 15 minutes, at 120°C, and 15-20 Psi. The medium was then allowed to cool, to 45-50°C, before 5% of defibrinated horse blood was added.

Liquid cultures of *S. pneumoniae* were grown, in 10ml of Brain Heart Infusion (BHI), overnight at 37 °C in a candle jar. Medium was made according to Table 4 and autoclaved, at 120°C for 15 minutes. Following overnight cultures, stock aliquots were grown for five to seven hours, in 80% (v/v) BHI and 20% (v/v) Foetal Bovine Serum (FBS, Sigma). These cultures were incubated statically at 37°C in a candle jar.

Escherichia coli strains were grown as outlined in Table 4. The agar was autoclaved, at 120°C for 15 minutes, and allowed to cool before pouring. Liquid cultures of *E.coli* were made according to Table 4. The medium was autoclaved at 120°C for 15 minutes. *E. coli* cultures were incubated, at 37°C, in a shaking incubator at 200rpm.

Table 4 – Media used in this study.

Medium	Recipe	Supplier
Blood agar base (BAB)	16g in 400ml dH_2O	Oxoid, UK
Brain heart infusion (BHI)	14.8g in 400ml dH_2O	Oxoid, UK
Luria Broth (LB)	4g NaCl, 2g Yeast extract, 4g Tryptone in 400ml dH_2O	NaCl – Fisher Scientific Yeast Extract – Oxoid, UK Tryptone – Oxoid, UK
Luria Agar (LA)	4g NaCl, 2g Yeast extract, 4g Tryptone, 6g Bacteriological agar in 400ml dH_2O	NaCl – Fisher Scientific, UK Yeast Extract – Oxoid, UK Tryptone – Oxoid, UK Bacteriological agar – Sigma, UK

B. Bacterial Strains and Plasmids

Bacterial strains and plasmids used, in this work, are listed in Table 5.

Table 5- Description of bacterial strains and plasmids.

Strain or plasmid	Description	Source
Streptococcus Pneumoniae (D39)	Virulent type 2	National Collection of Type Culture (NCTC) number 7466
Streptococcus pneumoniae (A66)	Serotype 3	National Collection of Type Culture (NCTC) number 7978
Streptococcus pneumoniae (TiGr 4)	Serotype 4	Clinical Isolate
Streptococcus pneumoniae (19F)	Serotype 19 Carriage strain	Clinical Isolate
Escherichia coli BL21 (DE3)	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pLysS (Cam^R)	Novagen
Escherichia coli BL21(DE3)pLysS	F- *ompT hsdS*B (rB-mB-) *gal dcm* (DE3) pLysS (CamR)	Invitrogen

Strain or plasmid	Description	Source
pSMB1	Recombinant NanA/PET23b (ampicillin-resistant) plasmid. *nan a* gene (Sp1536)	University of Siena
pSMB2	Recombinant NanB/PET23b (ampicillin-resistant)plasmid. *nan b* gene (Spr1531)	University of Siena
pSMB3	Recombinant NanB/PET23b (ampicillin-resistant) plasmid. *nan c* gene (Sp1326)	University of Siena
rPd1	Recombinant PLY genomic DNA from strain 472/96 (pd1) pAE vector containing a 6X His tag	Centro de Biotecnologia, Brazil
rPd2	Recombinant PdB Asp-380 to Asn substitution and a Trp-433 to Phe mutation (Pd2) pAE vector containing a 6X His tag	Centro de Biotecnologia, Brazil

C. Mouse Strains

Outbred MF1 mice and inbred Balb/c mice were obtained from Harlan Laboratories (Bicester, UK). Mice were at least 8 weeks old before use. Upon arrival, into the Biomedical Services Department (University of Leicester), animals were allowed to acclimatise for at least one week. Animals were kept in an individual ventilated cage system (IVC) and allowed unlimited food and water. All

animal procedures were carried out by a Home Office personal license holder. Following infection with pneumococci, animals were kept in separate IVC systems.

D. Neuraminidase A, B and C Expression

Neuraminidases were expressed for future purification. *E.Coli* vectors expressing the proteins Nan A, Nan B and Nan C were kindly provided by J.Athwal (University of Siena).

E.coli (BL21), for each of the recombinant proteins, were streaked onto LA, containing 100 μg/ml ampicillin (Sigma, UK) and 25 μg/ml chloramphenicol (Sigma, UK). The plates were then incubated overnight at 37°C. The following day, LB, containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol, was inoculated with the selected colonies from each expression vector. The culture was then grown at 37°C and was shaken at 200 rpm, until the absorbance reached 0.3-0.5. Following this, 500μl aliquots were made of the expression vectors and stored in 15% v/v glycerol at -80°C.

Stocks were thawed on ice and inoculated into auto induction medium (Appendix 1) containing 25μg/ml chloramphenicol and 50μg/ml ampicillin. The culture was grown for 14 to 16 hours at 37°C and was shaken at 200rpm. This was followed by centrifugation of the culture, at 4500 x*g,* for 20 min, at 4°C. The supernatants were discarded and the pellets were frozen at -20°C for future purification.

The pellets were thawed and resuspended in lysis buffer NPI-10 (appendix 1), and passed through a French Press (Sim Amnico), twice at 1280 psi. Afterwards, the suspensions were sonicated (Sanyo Sonicator), on ice, for bursts of 15 seconds,

with a 30 second rest period, for a total of 4 minutes. The cell suspensions were then centrifuged, for 40 minutes at 23708 x*g* and 4°C. Subsequently, the pellets were discarded, and the supernatants were stored at -20°C for future purification.

E. Neuraminidase Purification and Desalting

Neuraminidases were purified, for use in immunisation of MF1 mice. This was to evaluate any protection they may elicit, following challenge with virulent pneumococci. Purified neuraminidase was also used for ELISA plate coating to analyse antibody titre, produced by immunisation of MF1 mice.

A gravity flow purification column (prepacked 1.5ml Ni-NTA superflow, Qiagen), was equilibrated with 10ml lysis buffer, NPI-10 (Appendix 1). Following this, the supernatants containing the recombinant proteins were added to the column (a separate column was used for each protein). The column was washed twice, with 10ml lysis buffer, NPI-20 (Appendix 1). Protein was eluted with 3.5ml lysis buffer, NPI-250 (Appendix 1). The eluted proteins were collected in one fraction and desalted. For desalting, PD-10 disposable columns (containing 8.3ml sephadex G-25 medium from GE Healthcare) were equilibrated with PBS, and allowed to flow through by gravity (a different column was used for each protein). Next, 2.5ml of eluted protein was added to each column, and the proteins were eluted by adding 3.5ml of PBS (Oxoid,UK). The desalted eluted proteins were aliquoted, in 10% v/v glycerol, and stored at -80°C for future use.

F. Pneumolysin and PdB protein expression

Recombinant pneumolysin (PLY) and PdB protein, a genetically detoxified derivative of pneumolysin (with point mutations at Asp-380 to Asn substitution

and a Trp-433 to Phe mutation giving the protein a 0.1% activity of PLY), were expressed for future purification with Ni+ chromatography.

E.coli (Invitrogen) vectors, expressing the proteins pneumolysin and PdB, were kindly provided by Eliane Miyaji (Centro de Biotecnologia, Brazil.)

E.coli, containing the vectors for each recombinant protein, were streaked onto LA containing 100 μg/ml ampicillin, and were incubated overnight at 37°C. The following day, LB, containing 100 μg/ml ampicillin, was inoculated with the selected colonies from each expression vector. The culture was grown at 37°C, in a shaking incubator at 200 rpm, until the absorbance reached 0.3-0.5. Then, 500μl aliquots were made of the expression vectors and stored in 15% v/v glycerol at -80°C.

Stocks were thawed on ice. Once thawed, 100 μl of each vector was inoculated into 10 ml of LB, containing 100 μg/ml ampicillin, then the culture was grown overnight at 37°C, and was shaken at 200rpm. Following this, 50ml of culture was inoculated into 500 ml of LB, containing 100 μg/ml ampicillin, and grown until the absorbance reached 0.6, at wavelength 600nm. Protein production was induced by adding 1mM/ml of Isopropyl β-D-1-thiogalactopyranoside (IPTG, Promega) to the culture. The culture was then incubated for 5 hours at 37°C. After 5 hours the culture was centrifuged, at 14000 xg for 20 minutes, and the pellet was resuspended in 10mM of imidazole (Sigma) buffer (see Appendix 1). The solution was then stored at -20°C for future purification.

G. Pneumolysin and PdB purification

Pneumolysin was purified for future use as a positive control in hemolysis assays. PdB was purified for future ELISA plate coating, to allow evaluation of the antibody titre in the sera of PdB immunised mice. The PdB protein, for immunisation of the mice, was kindly provided by Germie van Doobbelsteen (Netherlands Vaccine Institute (NVI)).

A, C column (G E healthcare) was prepared by adding 10 ml of chelating sepharose fast flow (G E healthcare). The sepharose was allowed to settle for 30 minutes. Following this, the C column was connected to a peristaltic pump (G E healthcare). Then, 50 ml of distilled water was pumped through the sepharose to wash away any remaining storage buffer. Following this, 1 column volume of 0.2M of nickel chloride hexahydrate (Sigma) was added to the sepharose, as a source of nickel ions for metal ion affinity chromatography. Then, the sepharose was washed again, with 50 ml of distilled water, to remove any unbound nickel ions. Next, 50 ml of acidic buffer (see appendix 1) was added to the sepharose, to remove any loosely bound nickel ions. After this, 20 ml of equilibration buffer (see appendix 1) was added to the column. During preparation of the column, stored culture medium, containing the expressed protein of interest, was thawed, French pressed and sonicated. The culture medium was then centrifuged, at 14000 x*g* for 20 minutes, and the supernatant was retained. The supernatant was then filtered through a 0.45µm membrane (Acrodisc syringe filters, Pall Corporation). The filtered supernatant was then added to the column, at a flow rate of 1 ml/min. After the supernatant was added to the column, 50 ml of equilibration buffer was added, followed by 100 ml of 20 mM imidazole solution (see appendix 1). The column

was then eluted with 100 mM imidazole solution (see appendix 1), and the elutions were collected in 1 ml aliquots. Elution buffer was added to the column until no protein was detected by the Bradford assay. The Bradford assay was performed by adding 5 µl of each elution to 250 µl of Bradford reagent (Bio-rad), in a 96 well plate. When the Bradford reagent changed to a blue colour, protein was present in the elutions, if there was no change in colour, protein was no longer present and the elution buffer was stopped from running through the sepharose.

Elutions were then stored at 4°C for further SDS-PAGE and western blot analysis to confirm the protein purity and conformation.

H. Confirmation of expression and purification by SDS PAGE analysis and Western blot

In order to confirm the molecular weight of the purified proteins, and to confirm that the protein had no other contaminants in the eluates, a 12% v/v SDS-PAGE gels was used. Individual SDS PAGE gels were prepared using the reagents listed in Table 6. Crude cell extracts, washes collected from the columns, and the elutions were analysed for protein content. Samples were prepared with NuPAGE LDS sample buffer 4X (Invitrogen). The samples were then incubated at 100°C for 3 minutes. Then, 15µl of each sample was loaded into separate wells in the SDS gel. Also 4µl of precision plus protein standard (Bio-rad), was added to each SDS gel, for the molecular weight marker. TGS buffer (Appendix 1) was added to the tank, and the gel was run at 0.4 amps (for at least 1 hour).

Table 6- SDS PAGE gel reagents.

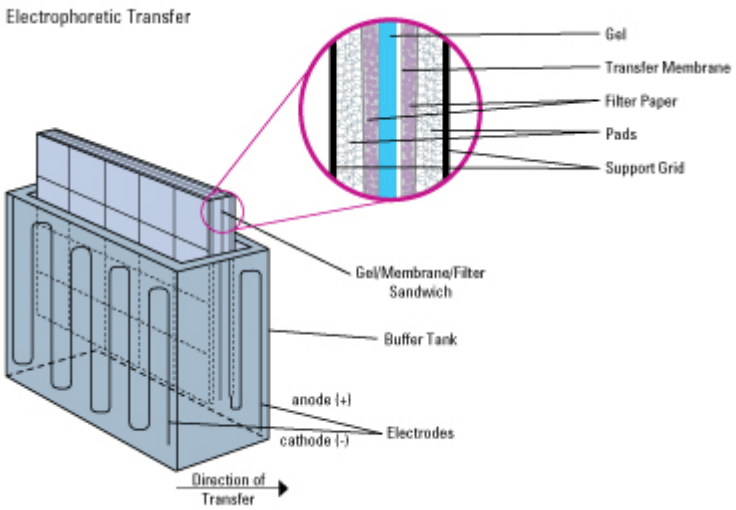
Reagents	**12% Separating Gel (15 ml)**	**5% Stacking Gel (5 ml)**
Nanopure Water	4.9 ml	3.4 ml
30% acrylamide (protogel, geneflow)	6 ml	0.83 ml
1.5M Tris HCl pH 8.8 (Sigma)	3.8 ml	0 ml
1M Tris HCl pH 6.8	0 ml	0.63 ml
10% SDS (Sigma)	0.15 ml	0.05 ml
10% Ammonium persulphate (Fisher Scientific)	0.15 ml	0.05 ml
TEMED (Sigma)	0.006 ml	0.005 ml

Following the SDS PAGE gel, western blotting was performed for each protein, in order to confirm that the purified proteins were in the correct conformation.

A western blot was begun by equilibrating the filter paper and sponges with transfer buffer (Appendix 1). The apparatus was assembled, as shown in Figure 4, and the proteins were transferred onto the nitrocellulose membrane (G E Healthcare), for 1 hour at 0.25 Amps. The membrane was then blocked overnight, with 5% v/v milk in TBST (Appendix 1) solution, at 4°C. The next day the nitrocellulose membrane was washed 3 times, with TBST solution. The membrane was then incubated for 2 hours, with primary rabbit anti-neuraminidase A or B

serum (obtained from the University of Siena, Italy), at a 1:1000 dilution. The serum raised against Nan B is also specific for Nan C (as Nan C is a homologue of Nan B). Subsequently, the membrane was washed with TBST, three times. Then the membrane was incubated for two hours, with the secondary antibody, (anti-rabbit IgG whole molecule, raised in goat) which was conjugated to alkaline phosphatase (Invitrogen). Following this, the membrane was washed with TBST, 3 more times. The bands were developed for 5-10 minutes by adding 5-7 ml of BCIP/NBT one step solution (Sigma). Finally, the reaction was stopped by adding 5ml of water to the membrane.

Figure 4- the organization of western blotting apparatus.



Picture from http://www.piercenet.com/media/PDetectFig24_400x.jpg

A western blot was also performed, as above, to confirm that the PdB protein was able to bind the specific antibody. However, the primary antibody used in this western blot was an anti-pneumolysin monoclonal antibody (Statens Serum Institute, Denmark). The secondary antibody used was the same as above.

Western blotting was also used to confirm the presence of serum antibodies, from mice immunised with PdB. The SDS PAGE gel, and transfer of each protein, was performed as described above. The membrane was then blocked and washed as explained above. Sera, from both PdB immunised mice and control immunised mice, were selected for primary antibody incubation. The membrane was then incubated, for 2 hours, with the primary antibody serum, which was diluted to 1:1000 in 5% v/v milk (Sigma). The membrane was washed three times with TBST. The secondary antibody, (goat anti-mouse antibody, conjugated to alkaline phosphatase (Invitrogen)) was added to the membrane, at a dilution of 1:3000, and incubated for a further 2 hours. After this the membrane was washed and developed as described above.

I. Bradford Assay

Neuraminidase protein quantification was performed by Bradford assay. Bradford reagent (Bio-rad) was first diluted to 1:5, and then 200µl was added to a well in a flat bottomed, 96 well plate (Nunc). Following this, 5µl of the protein solution was added to a well, and incubated for 5 minutes, at room temperature. Each protein solution was measured in triplicate. The absorbance was read, at 595nm, using an ELISA microplate reader (Bio-rad). Bovine serum albumin (BSA (Bio-rad)) was used, as a standard protein concentration marker, at 100 µg/ml to 1.5 mg/ml.

J. Protein quantification by Nanodrop spectrophotometer

PdB and PLY proteins were quantified by nanodrop 1000 spectrophotometer (Thermo Scientific). The nanodrop software was opened and

A^{280} was selected. Following this, 2 µl of nanopure water was dropped on to the detector and the machine was calibrated. Then the detector was wiped clean. Following this, 2 µl of the buffer that the proteins were being stored in, was added to the detector as a blank control. The protein elutions were then added to the detector, separately, and in between each elution measurement the machine was blanked. The protein concentration was determined and displayed on the software in mg/ml.

K. Neuraminidase Activity

Enzyme activity was measured using the synthetic substrate 2-O-(p-Nitrophenyl)-α-D-N-acetylneuraminic acid (2 OPN) (Sigma).The assay was carried out by adding 25 µl of purified Nan A, B or C, diluted 1:100, to 25µl of 0.3M 2,OPN, and incubating them together at 37°C. The reaction was stopped after 1 minute, by adding 100µl 1M Na_2CO_3, and the absorbance at 405nm was read. The standard curve used was 4-Nitrophenol (Sigma).

L. Pneumolysin and PdB activity

Purified pneumolysin and PdB proteins were assayed for hemolytic activity. The assay was performed in a 96 well plate (Nunc). Pneumolysin or PdB were serially diluted, by two, 24 times in PBS. The top row of the plate contained PBS only, to serve as a negative control. A 4% defibrinated sheep red blood cell (Oxoid, UK) solution was prepared, by centrifuging blood at 2360 x*g* for 15 minutes. After centrifugation 400 µl of the pellet, containing red blood cells, was removed and added to 10 ml of PBS. The red blood cell solution was then added to each well of the 96 well plate, containing serially diluted pneumolysin or PdB, and was

incubated for 30 minutes at 37°C. After incubation, the hemolytic activity was determined by eye. This was defined as the dilution at which pneumolysin or PdB protein lysed 50% of the red blood cells.

M. *Streptococcus pneumoniae* culture

Streptococcus pneumoniae (serotypes D39, TiGR4, A66, and 19F) were grown as described in Alexander et al., (1998). Pneumococci were grown for the challenge of mice following immunisation with the purified proteins.

Streptococcus pneumoniae serotypes were separately streaked onto BAB, and grown overnight in a candle jar at 37°C. The following day a sweep colonies were inoculated, into 10ml of BHI, and grown statically overnight at 37 °C in a candle jar. The culture was then centrifuged, at 900 x*g* for 15 minutes, and the pellet was retained. The pellet was resuspended in 1ml serum broth (80% BHI and 20% Fetal bovine serum). From this resuspension, a fresh 10 ml serum broth was prepared for growth. The starting absorbance at 500 nm was adjusted to 0.7. The culture was then allowed to grow, for 5-7 hours, until the absorbance 500 nm reached 1.4-1.6. The culture was then aliquoted and stored at -80°C. The viability was checked 24 hours later. The bacteria were thawed, and then centrifuged at 12470 x*g* for 2 minutes. After, the pellet was resuspended in 400 µl PBS. The culture was then serially diluted by ten, plated out onto BAB, and incubated overnight in a candle jar at 37 °C. Optichin sensitivity was confirmed by streaking the bacteria onto BAB, and adding an optichin disc (Oxoid). The plate was then incubated overnight, in a candle jar, at 37 °C. The next day the colonies were assessed for alpha hemolysis and optichin sensitivity. The viable colonies were

counted and calculated. Finally, the viability was expressed as colony forming units per ml.

N. Animal passage of pneumococci

Streptococcus pneumoniae (serotypes D39, TiGR4, A66, and 19F) were passaged as described by Canvin et al., (1995), to increase the virulence of the pneumococci, and to eliminate pale and transparent phase variants (Hackenback et al. 1948).

Pneumococci were streaked onto BAB and grown overnight, at 37°C, in a candle jar. The following day, a sweep of colonies were inoculated into BHI and grown statically overnight, at 37°C, in a candle jar. The culture was centrifuged, at 900 x*g* for 15 minutes, and the pellet was resuspended in 5ml sterile PBS. The $O.D_{500nm}$ was adjusted to 1.4-1.6 with PBS. Two MF1 mice (Harlan UK) aged ≥8 weeks were given 100 µl of the bacterial suspension by intraperitoneal (I.P.) injection. The viability of the suspension was then confirmed as described above. The mice were monitored for disease signs graded as outlined in Table 7. If the mice reached ++Starry, in 22-28 hours, blood was taken by cardiac puncture under deep anaesthesia with 2.5% (v/v) isoflurane in 1.6-1.8L O_2/min. The mice were then culled by cervical dislocation.

Following this, 50 µl of blood from the cardiac puncture was inoculated into 10 ml BHI, and allowed to grow statically overnight, at 37°C, in a candle jar. The following day, the supernatant was removed and centrifuged, at 12470 *xg* for 15 minutes. The supernatant was discarded and the pellet was resuspended in 1 ml serum broth as above, and adjusted to an O.D of 0.7 at 500nm. When the absorbance, at 500 nm, reached 1.4-1.6 the passaged bacteria were aliquoted and

stored at -80 °C for future use. The viability of the bacteria was confirmed 24 hours later as described above.

Table 7- outlines the symptom scores used to grade mice infected with pneumococci.

Disease Score	**Description**
Normal	Mouse is moving around the cage normally and has a normal coat.
Hunched +	Mouse is slightly arched over in the middle and walking on tip toes
Hunched ++	Mouse is very arched over and walking on tip toes
Starry +	Coat of the mouse is not groomed well around the neck area and fur is upright
Starry ++	Coat of the mouse is not groomed all over and fur is upright
Lethargic +	Mouse is slower at moving around the cage
Lethargic ++	Mouse is not moving around the cage unless encouraged
Moribund	Coat of the mouse is not at all groomed, mouse has stopped moving around that cage, mouse has laboured breathing

O. Virulence testing of *S. pneumoniae*

Passaged pneumococci, of each serotype, were virulence tested to asses whether the stocks grown were virulent. The virulence test was performed using five MF1 mice (Canvin et al. 1995). All pneumococci passages and virulence tests were carried out using Mf1 mice, as it is a well documented and published method.

An *S. pneumoniae* aliquot was thawed in the hand and centrifuged, at 12470 *xg* for 2 minutes. The supernatant was discarded and the pellet was resuspended in 400 µl of PBS. The bacterial dose was calculated from the viable

count. The required amount of bacteria from the pellet was added, to give an infection dose of $2x10^7$ per ml of suspension, to the calculated amount of PBS. The dose was then used within 20 minutes.

The mice were anaesthetized, with 2.5% (v/v) isoflorane in 1.6-1.8 L O_2/min, in an anaesthetic box. When the animals were fully anesthetized, 50µl of bacterial suspension, containing approximately $1x10^6$ CFU, was dropped into the nostrils of the mice. A viable count of the challenge dose was confirmed as described in section M. Mice were monitored for disease signs. If any mice reached a disease sign of ++ lethargic, they were culled by cervical dislocation. If 4 out of 5 mice succumbed to infection, within 44-52 hours, the bacteria was confirmed as virulent.

P. Toxicity tests of proteins

Active purified neuraminidase proteins Nan A, Nan B and Nan C as well as PdB (provided by NVI), were toxicity tested. This was to ensure no disease signs were caused by administering the proteins, by the I.P. route, in seven day successions.

Three MF1 mice were injected, by the I.P. route, with a single purified protein. Each protein was administered at 20 µg per dose, and was also mixed with a 1:3 dilution of Imject alum adjuvant (containing 40 mg/ml aluminiuim hydroxide and 40 mg/ml magnesium hydroxide, from Pierce). The alum adjuvant was diluted in PBS. The total volume of each immunisation was 100 µl. Immunisation suspensions were mixed for 30 minutes, at room temperature, before administration. The proteins were prepared, and administered by the I.P. route, twice more with 7 days between each immunisation.

Q. Intraperitoneal Immunisations

The immunisation schedules for I.P. immunisations were modified from Alexander et al., (1994). The immunisation schedule, outlined in Figure 5, was followed for the I.P. immunisations of Mf1 mice with both Nan A and PdB proteins.

Forty MF1 mice were used in each immunisation experiment. Mice were tail bled, before each immunisation, to measure specific serum antibody titres throughout the experiment. The blood, collected from tail bleeds was centrifuged, for 10 minutes at 12470 x*g*, and the serum was removed and stored, at -80°C, for ELISA analysis. Twenty mice from each experiment were immunised with 20 µg of Nan A or PdB, which was mixed with a 1:3 dilution of alum. The total volume administered to each mouse was 100 µl. The inocula were mixed, at room temperature, for 30 minutes before immunisation. Twenty mice from each experiment were sham immunised, (called the control group from here) with a 1:3 dilution of alum, glycerol and PBS. Each mouse in the control group received a total volume of 100 µl per inoculum. The control suspension was mixed, at room temperature, for 30 minutes before administration. Each mouse was immunised twice more as outlined in Figure 5.

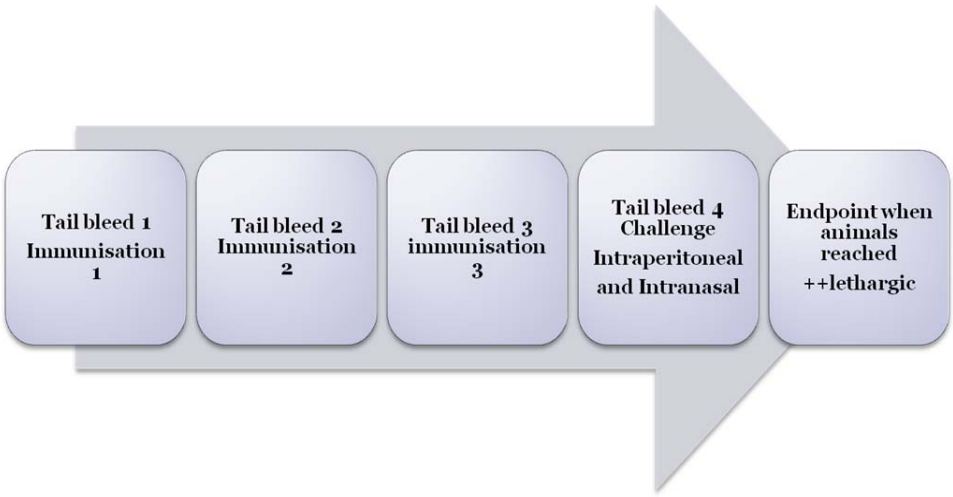


Figure 5- shows the immunisation schedule followed. This schedule was followed for immunisation of Mf1 mice with Nan A and PdB proteins. At time zero, mice were tail bled, and then immunised with either Nan A, PdB or control solutions. Seven days and fourteen days after the first immunisation, mice were tail bled and immunised again. Two weeks after the last immunisation, which was 28 days after the first immunisation, mice were tail bled once more and challenged with passaged serotype D39.

R. Subcutaneous Immunisations

Upon advice from NVI the immunisation route, and schedule, were modified. The time between each immunisation was changed, from seven days, to fourteen days. Nan A or PdB were administered in separate experiments, by subcutaneous immunisation, without alum adjuvant, at the time points indicated in Figure 6.

Twenty MF1 mice were tail bled and immunised, subcutaneously, with 20 µg of purified Nan A protein, diluted in 100 µl of PBS. In a different experiment twenty MF1 mice were immunised, subcutaneously, with 20 µg of purified PdB, diluted in 100 µl of PBS. Twenty mice from each experiment were control immunised, with the percentage of glycerol present in the purified protein mixed in PBS in total volume of 100 µl. The mice were tail bled and immunised as outlined

in Figure 6. Mice were then challenged, with virulent D39 pneumococci, as outlined below in section S.

In another experiment, twenty Balb/c mice were immunised subcutaneously, with 20 µg of purified PdB in 100 µl of PBS. Twenty mice were also control immunised with glycerol and PBS, in total volume of 100 µl. The mice were immunised and challenged according to the time points outlined in Figure 6. The mice were then challenged as outlined below in section S.

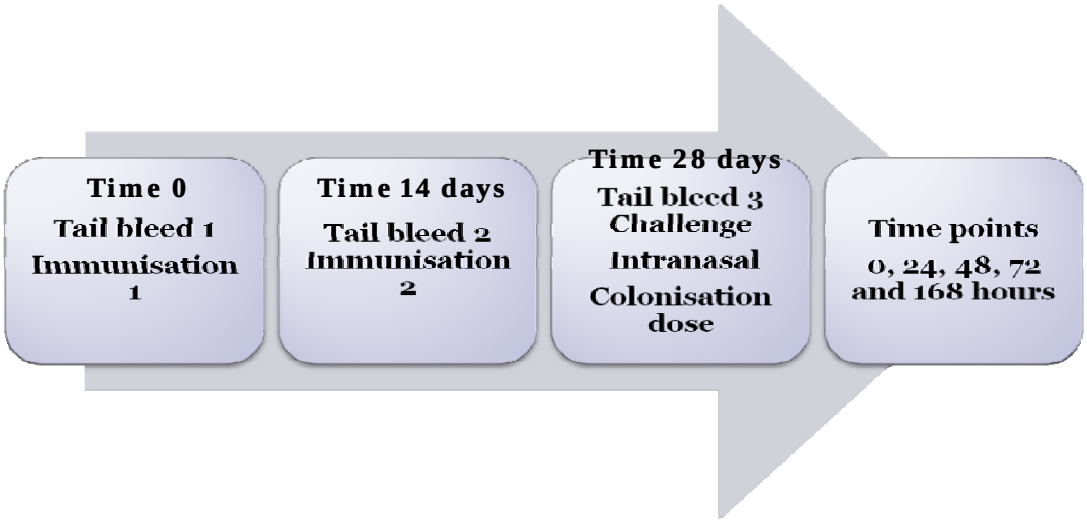


Figure 6 – shows the immunisation schedule carried out for subcutaneous immunisations with Nan A and PdB proteins. Mice were tail bled and immunised, with the required protein, at time zero. Fourteen days later mice were tail bled and immunised once more. Fourteen days after the last immunisation mice were challenged, with an acute dose or a colonisation dose of pneumococci.

S. Challenge

All immunisation routes, challenge routes and pneumococcal serotypes mice were challenged with are outlined in Table 8. The mice immunised, through the intraperitoneal route, were challenged by the intraperitoneal route in the first Nan A experiment. This route of challenge was chosen to assess whether the immunisations had elicited protective systemic immunity against peritonitis and sepsis. All further challenges of mice intraperitoneally immunised, with either Nan

A or PdB protein, were to test for protection against invasive pneumonia. Therefore the mice were challenged intranasally with D39 pneumococci.

The mice immunised by the subcutaneous route were challenged, with an acute dose, colonisation dose or an intravenous dose. A colonisation dose was chosen, as it was important to demonstrate any protection elicited by protein immunisations in the first step of *S. pneumoniae* infection. Without colonisation of the upper respiratory tract, the pneumococcus is unable to move down to the lower respiratory tract and cause pneumonia or sepsis. An intravenous dose was chosen to examine whether any protection was seen in mice immunised with the proteins during a sepsis model of infection. This model of infection mimics bacterieamia and septicaemia, which may follow pneumonia if the pneumococcal infection is uncontrolled.

In experiments where mice were pre-colonised with passaged virulent D39, mice were challenged 28 days after the first immunisation. Mice were then left for 21 days to ensure no disease signs were seen after colonisation. Finally, mice were challenged with an acute dose or an intravenous dose of D39. All other mice immunised, by the subcutaneous route, were challenged with a colonisation dose of virulent passaged A66, TiGR4 or 19F all in separate experiments.

All animals were monitored for disease signs after challenge. When any mouse reached ++lethargic it was anaesthetized, as described in section N, and culled by cervical dislocation.

Table 8 – shows the proteins used in immunisation experiments. It also shows the immunisation routes, mouse strains used, pneumococcal serotypes mice were challenged with and the dose used in each challenge.

Immunisation protein	Immunisation route	Mouse strain	Pneumococcal serotype	Route and dose of challenge
Nan A	Intraperitoneal	MF1	D39	Intraperitoneal $1x10^7$ CFU/ml in 100µl
Nan A	Intraperitoneal	MF1	D39	Acute Intranasal $1x10^7$ CFU/ml in 50µl
PdB	Intraperitoneal	MF1	D39	Acute Intranasal $1x10^7$ CFU/ml in 50µl
PdB	Subcutaneous	Balb/c	D39	Intranasal colonisation $1x10^7$ CFU/ml in 10µl
Nan A	Subcutaneous	MF1	D39	Intranasal colonisation $1x10^7$ CFU/ml in 10µl
PdB	Subcutaneous	MF1	D39	Intravenous $1x10^7$ CFU/ml in 100µl

Table 8 continued.

Immunisation protein	Immunisation route	Mouse strain	Pneumococcal serotype	Route and dose of challenge
PdB	Subcutaneous	MF1	D39	Intranasal colonisation $1x10^7$ CFU/ml in 10µl followed by acute intranasal dose $1x10^7$ CFU/ml in 50µl
PdB	Subcutaneous	MF1	A66	Intranasal colonisation $1x10^7$ CFU/ml in 10µl
PdB	Subcutaneous	MF1	TiGR4	Intranasal colonisation $1x10^7$ CFU/ml in 10µl
PdB	Subcutaneous	MF1	19F	Intranasal colonisation $1x10^7$ CFU/ml in 10µl

T. Tissue collection

After all acute intranasal challenges and intraperitoneal challenges, tissues were collected from each mouse for processing. Blood was collected by cardiac puncture and processed as described in section U. The lungs and nasopharynx tissue were also collected from each mouse and processed as outlined in section U.

After all colonisation challenges the blood, cervical lymph nodes (CLN), nasopharyngeal associated lymphoid tissue (NALT), the nasopharynx and lungs, were collected from each animal at different time points. These time points were 0, 24, 48, 72 hours and 7 or 14 days following challenge. For tissue collection mice were anaesthetized, with 2.5% (v/v) isoflorane in 1.6-1.8 L O_2/min, in an anaesthetic box. When the animals were fully anesthetized blood was taken, by cardiac puncture, and the animal was culled by cervical dislocation. Following this, the animal was dissected. The lungs, CLN, NALT and nasopharynx were removed. The lungs were stored in 10 ml PBS, the CLN were added to 1ml RPMI, the NALT was added to 1ml RPMI and the nasopharynx was collected and put into 5ml PBS. All tubes containing PBS, for the lungs and nasopharynx, were weighed before the tissue was added and again after the tissue was added to allow an accurate approximation of tissue weight. This allowed the colony forming units, per milligram of tissue, to be measured.

Nasal washes were performed after challenge with a colonisation dose. This was done for each challenge serotype (apart from A66), to assess the titre of secretory IgA in the nasopharynx. Firstly, the CLN were removed followed by the back of the nasopharynx associated with the back of the trachea. The nasal wash was then performed by inserting a tube up the trachea and washing the nasal cavity with 500 µl of PBS. The drops were then collected from the nose.

U. Tissue processing

The blood collected from cardiac punctures was centrifuged, for 10 minutes at 12470 x*g*, and then the serum was removed and stored, at -80°C, for further analysis.

After tissue collection the lungs and nasopharynx were weighed and homogenised (Ultra turrax T8 made by IKA, Germany). Following homogenisation 20 µl, from each tube, was serially diluted by ten -six times. Then, 60 µl of each dilution was dropped onto BAB plates, in three drops of 20 µl, the plates were then allowed to dry. Plates were incubated overnight, at 37°C, in a candle jar. The following day the colonies were counted. The blood collected from the cardiac punctures was also diluted (before centrifugation) and dropped on to plates in the same way.

CLN and NALT were homogenised by adding the tissue to a cell strainer (BD, Biosciences). The tissue was pushed through, using a syringe plunger, and then centrifuged, at 800 x*g,* for 10 minutes. Following this, the supernatant was removed and the pellet was resuspended in freezer mix containing 75% RPMI (Gibco), 15% FCS (Gibco) and 10% dimethyl sulphoxide ((DMSO) Sigma). The samples were then slowly frozen in alcohol, at -80°C for 24 hours, and then stored at -80°C for ELISPOT assays and FACS analysis.

Nasal washes were serially diluted by 10 and plated on BAB, containing 2 µg/ml of gentamicin. The plates were allowed to dry and were than incubated, at 37°C, overnight. The viable colonies were counted the following day.

V. ELISA

ELISA was performed as described in Morreno et al., (2010). Serum samples were analysed for specific, IgG and IgG2a, antibody content by indirect ELISA. ELISA was performed by coating maxisorp 96 well plates (Nunc), with 2 μg of the respective protein diluted in 100 μl of PBS per well. The plates were incubated overnight at 4°C. After, the plates were washed, three times, with PBS and 0.05% v/v Tween 20 (Sigma). The plates were then blocked with 5% milk powder (Fluka, Sigma), in PBS, and incubated overnight at 4°C. The next day the plates were washed, three times, with PBS and 0.05% v/v Tween. Following this, the serum samples were added to the plate. For sera collected from protein immunised mice, a starting dilution of 1:200 was added to the first well. For sera collected from control immunised mice, a starting dilution of 1:50 was added to the first well. The sera were then serially diluted by two, eleven times. The plates were then incubated for 2 hours at 37 °C. All serum samples, from each experiment, were assayed at the same time. All plates contained a PBS negative control. After incubation, the plates were washed with PBS and 0.05% v/v tween. The goat anti-mouse IgG conjugated to horse radish peroxidase (Invitrogen), or the goat anti mouse IgG2a conjugated to horse radish peroxidise (Invitrogen) secondary antibodies were added, at dilution of 1:5000 in PBS. The secondary antibody was added at a volume of 100 μl per well. The plates were incubated for 1.5 hours at 37°C. The plates were then washed with PBS and 0.05% v/v Tween, three times. To develop the ELISA, 100 μl of TMB solution (Invitrogen) was added to each well, and after 3 minutes 1M H_2SO_4 was used to stop the reaction. The absorbance of each plate was then read at 450nm (with an ELISA plate reader).

Nasal washes were also assayed for specific IgA titre by indirect ELISA. Plates were coated and incubated with PdB protein and washed as above. Following this, nasal washes were added to the plates neat and serially diluted by two, eleven times, and incubated for 2 hours at 37°C. The plates were washed as above. The goat anti mouse IgA conjugated to horseradish peroxidise (Sigma), secondary antibody, was added to the plates at a dilution of 1:5000 and incubated, at room temperature, for 1.5 hours. Plates were then washed and developed and read as described for the IgG and IgG2a indirect ELISA.

W. ELISPOT assays

ELISPOT assays were performed to establish whether there were any differences, between amounts of antibody producing cells, in the CLN of control mice and immunised mice. The CLN were removed from the mice as described in section T. This assay was modified from Richards et al., (2010) and allowed the detection of mucosal immunity elicited by immunisation. ELISPOT assays were performed for IgG producing antibody cells and IgA producing antibody cells.

A sterile multiscreen plate (Millipore) was opened and the membrane was activated, with 15 % ethanol in sterile PBS. The plate was then coated with unlabelled antibody (Southern Biotech), and incubated, at 37 °C, for 2 hours. Following this, the plate was washed, three times, with PBS under sterile conditions and blocked with R10 media (see appendix 1) for 1 hour. During this time CLN cells, collected earlier, were quickly defrosted in a 37 °C water bath and washed once in RPMI. The cells were centrifuged at 300 x*g* for 7 minutes. The supernatant was removed and the cells were resuspended in 300 µl of RPMI. Following this, 10 µl of CLN cells were removed and mixed with 10 µl of trypan blue, for counting

viable cells with a hemocytometer. Cells were counted under the microscope and the concentration of cells was adjusted to $2x10^6$ cells/ml. If there were not enough cells samples were pooled together, (usually 5 samples were pooled together to gain the desired amount of cells per well).The ELISPOT plate was then washed three times with PBS. Following this 50 µl of R10 media, containing 0.07% of 2-mercapetoethanol, was added to each well. Then, 50 µl of CLN cells was added to the plate, in duplicate, and incubated at 37°C overnight in a 5% CO_2 incubator. The following day the plate was washed with PBS, three times, and a secondary antibody of IgG (Southern Biotech) or IgA (Southern Biotech) was added, at a 1:1000 dilution, to the appropriate wells. The plate was incubated at room temperature for 2 hours. The plate was washed again, three times, with PBS and developed using an AP colour developer kit (Bio-rad). The plate was developed for up to 10 minutes, or until spots could be seen in the wells. The reaction was stopped by adding water to the wells. The plate was left on the bench to dry and stored at room temperature, until it could be read with an ELSIPOT reader. Plates were read with a Scancell ELISPOT reader (many thanks to Andrew Jackson of Nottingham University for use of this equipment).

X. Cholesterol removal from serum samples

Cholesterol was removed from serum samples to prevent it from inhibiting pneumolysin protein activity. Cholesterol removal solution (see appendix 1) was added to each sample, at 1:10 dilution, and then mixed well. The samples were incubated at room temperature for 5 minutes. Following this the samples were centrifuged, at 12470 x*g,* for 30 minutes. The supernatant was then removed and

the process was repeated once more. Serum samples were then stored at -20 °C for future analysis.

Y. Hemolysis inhibition assays

Hemolysis inhibition assays were performed to determine whether the antibodies generated by immunisation were functional antibodies. The assays showed whether the antibodies present in the sera, of mice immunised with PdB, had the functional ability to block the action of purified pneumolysin, by inhibiting the lysis of red blood cells.

Mouse sera, collected from PdB immunised and control immunised mice, had the cholesterol removed to prevent pneumolysin inhibition. The cholesterol was removed as described in section X. Following this, 20 µl of each sample was added to PBS and serially diluted, twelve times, in a 96 well plate. Purified pneumolysin was added to the plate, at the concentration needed to lyse 50% of the red blood cells. The plate was incubated, at 37°C, for 30 minutes. Following this, 50 µl of 4% sheep red blood cell solution was added to each well and incubated, for 30 minutes, at 37°C. One row of the 96 well plate contained the positive control, this was pneumolysin and red blood cells. The negative control contained only red blood cells and PBS. Hemolysis inhibition, at the dilution the sample was able to inhibit PLY, was then determined by eye.

Z. Statistical analysis

Statistical analysis was performed using the computer programme Graphpad Prism 5.5. Two tailed T tests and two way ANNOVA were used to analyse the

significance, significance was defined as * = $P \leq 0.05$ and ** $P \leq 0.01$. Error bars are represented as standard error of the mean (SEM).

III. Results

A. Confirmation of expression and purification of proteins by SDS PAGE and Western blotting

Neuraminidase purification was assessed by SDS PAGE. Nan A was purified and desalted, as shown in Figure 7. The molecular weight of purified Nan A was confirmed as 107kDa (Yesilkaya et al., 2006).

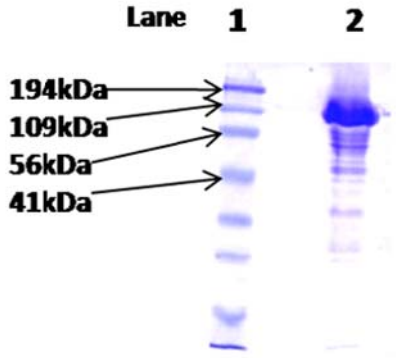


Figure 7 – Lane 1 shows the molecular weight marker, lane 2 shows purified neuraminidase A.

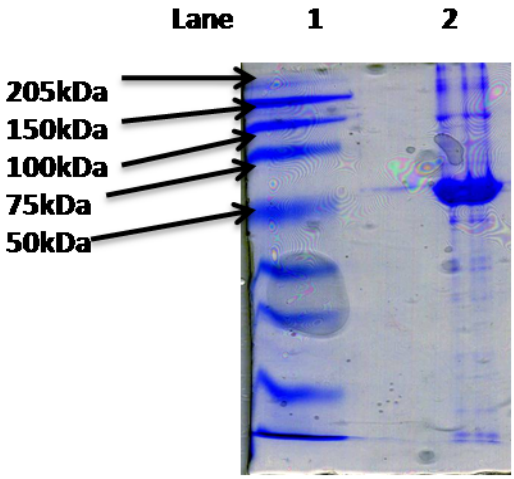


Figure 8 – shows the result of the SDS Page gel ran to verify the molecular weight of PdB protein. Lane 1 shows the molecular weight marker and lane 2 shows PdB protein purified by NVI.

The PdB protein was kindly provided by the Netherlands Vaccine Institute (NVI) for immunisation of mice. To verify the purity of the PdB protein an SDS PAGE gel was performed and stained with coomaisse blue. PdB protein was also purified for specific ELISA analysis, by ion exchange chromatography, from a vector kindly provided by Eliane Miyaji (Centro de Biotecnologia - Instituto Butantan, Brazil). The elutions (collected in separate 1ml tubes) from the purified PdB were run on an SDS Page gel to check for purity, and the results are shown in Figure 9. The molecular weight of PdB is 52 kDa, which is the same molecular weight as pneumolysin (Alexander et al., 1994).

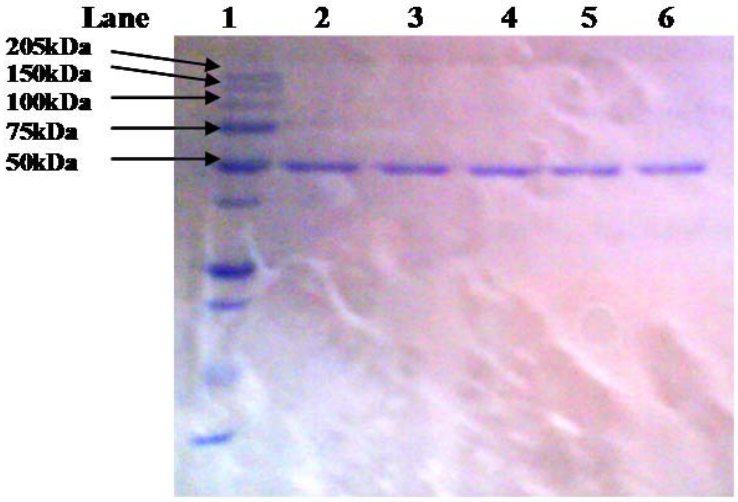


Figure 9 – shows an SDS PAGE gel containing the elutions from purified PdB proteins expressed from *E. coli.* Lane 1 shows the molecular weight marker, lanes 2 to 6 show different elutions of purified PdB protein.

The conformation of the PdB protein from NVI and the Nan A protein purified in house was then verified by western blot analysis. Figure 10 shows the results of the western blots. PdB has a clear defined band. However, Nan A has more than one band detectable on the western blot membrane; this may be due to

the secondary antibody binding to breakdown products of the proteins or may be non specific binding. The secondary antibody used to detect Nan A was a specific rabbit sera, which may have bound to non specific targets.

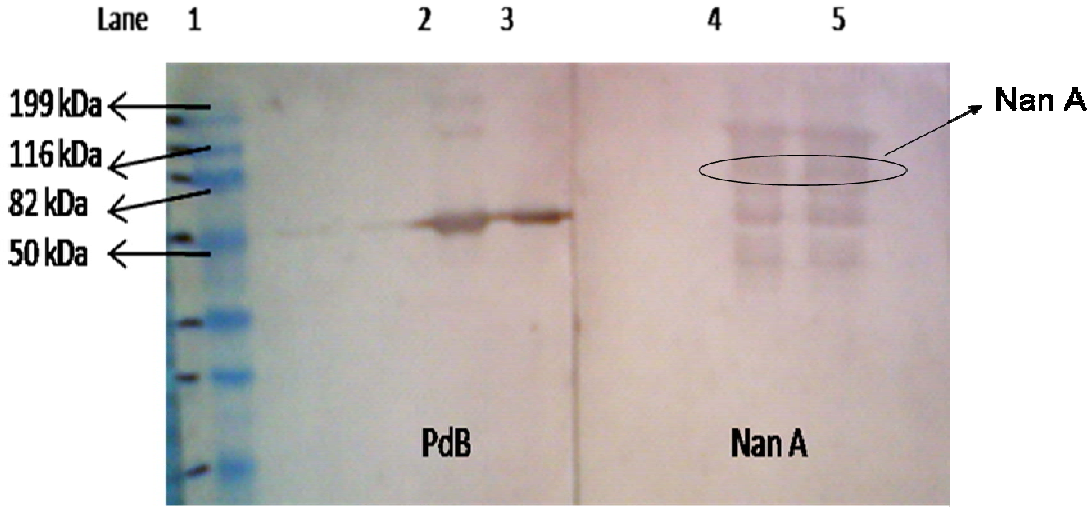


Figure 10 – Western blot shows the immunoreactivity of the purified proteins PdB and Nan A**.** Lane 1 shows the molecular weight marker, lanes 2 and 3 show the bands developed specific for purified PdB protein. Lanes 4 and 5 show bands developed specific for purified Nan A protein.

The concentration of purified proteins was measured by Bradford assay, with BSA as a standard, or were analysed with a nanodrop machine (Fisher Scientific). The activities of neuraminidases A and B were measured by substrate cleavage of 2-O-(p-Nitrophenyl)-α-D-N-acetylneuraminic acid. The activity of purified PLY and PdB proteins were measured by hemolysis assay. The hemolytic acitivity of the proteins was defined as; the point at which the proteins were able to lyse 50% of the red blood cells. This was then converted to hemolytic units, in one microgram of protein. Table 9 shows the concentrations of the different purified proteins and their respective activities and what each elution was used for

throughout the work. Protein concentration was determined by Bradford Assay or Nanodrop spectrophotometer. Bradford assay was used with a BSA standard curve of 100 ug/ml to 1.5 ug/ml. Hemolytic activity of PdB and PLY were determined by hemolysis assays. Activity of NanA was measured by adding the synthetic substrate PNP-Neu5Ac. The release of p-nitrophenol was recorded.

Table 9- proteins received from NVI and proteins purified by ion exchange chromatography, their concentration, respective activities and the experiments each protein was used for.

Purified Protein	**Protein Concentration mg/ml**	**Activity**	**Used in Experiment**
Neuraminidase A Batch 1	2.1	0.316 (concentration of substrate cleaved/min/mg protein)	Nan A immunisations
Neuraminidase A Batch 2	2.1	0.540 (concentration of substrate cleaved/min/mg protein)	Nan A ELISA plate coating
Purified Protein	**Protein Concentration mg/ml**	**Hemolytic activity**	
PdB (purified on arrival from NVI) Batch 1	1.2	1µg of protein = 3 Hemolytic units	D39 I.P., I.N. challenges, colonisations 1 and 2, I.V. challenge.
PdB (purified on arrival from NVI) Batch 2	0.7	1µg of protein = 3 Hemolytic units	D39 colonisations 3 and 4, TiGR4, A66 and 19F colonisations
PdB (purified in house) Batch 1	2.57	1µg of protein = 27.3 Hemolytic units	ELISA plate coating
PdB (purified in house) Batch 2	1.42	1µg of protein = 25.6 Hemolytic units	ELISA plate coating
PLY Batch 1	0.61	1µg of protein = 1061 Hemolytic units	Hemolytic Inhibition experiments

B. Adverse Reaction Test

The purified proteins were tested for toxicity to animals to ensure there were no adverse effects of administering them, repeatedly, over a three week period of time. The purified proteins Nan A, Nan B (batch 1) and PdB (from NVI, batch 1) were individually administered, intraperitoneally, to three MF1 mice. This was then repeated after seven days and again fourteen days later to demonstrate that the immunisation schedule that was to be followed later would not produce any adverse reactions in the mice. The animals were closely monitored for any signs of disease. Administration of the purified proteins caused no adverse effects to the animals during the 21 days of immunisation. This showed that the proteins contained no contaminants that were toxic to the mice, and there were no adverse effects of administering the proteins in succession.

C. Challenge Doses

Mice were challenged by differing routes with virulent D39 pneumococci. The doses given in each of the experiments are listed below in Table 10.

Table 10 – dose and route of pneumococci administration in each immunisation experiment**.**

Immunisation Protein	Immunisation Route	Mouse Strain	Pneumococcal Serotype	Route and actual dose of challenge
PdB	Intraperitoneal	MF1	D39	High Intranasal dose 1.4 x 10^7 CFU/ml in 50µl
PdB (boosted with one extra 40µg dose of protein)	Intraperitoneal	MF1	D39	High Intranasal dose 1.6 x 10^7 CFU/ml in 50µl
PdB	Subcutaneous	MF1	D39	High Intranasal dose 1.7 x 10^7 CFU/ml in 50µl
PdB	Subcutaneous	MF1	D39	Lower Acute Intranasal 1.9 x 10^6 CFU/ml in 50µl
PdB	Subcutaneous	MF1	D39	Intravenous 9.6 x 10^6 CFU/ml in 100µl
PdB	Subcutaneous	Balb/c	D39	Intranasal 1.8 x 10^7 CFU/ml in 10µl for colonisation
PdB	Subcutaneous	MF1	D39	Intranasal 3.2 x 10^7 CFU/ml in 10µl for colonisation
PdB	Subcutaneous	MF1	A66	Intranasal 6.3 x 10^7 CFU/ml in 10µl for colonisation
PdB	Subcutaneous	MF1	TiGR4	Intranasal 5 x 10^7 CFU/ml in 10µl for colonisation
PdB	Subcutaneous	MF1	19F	Intranasal 7 x 10^7 CFU/ml in 10µl for colonisation
Nan A	Intraperitoneal	MF1	D39	Intraperitoneal 2.5 x 10^7 CFU/ml in 100µl

Table 10 cont.

Immunisation Protein	Immunisation Route	Mouse Strain	Pneumococcal Serotype	Route and actual dose of challenge
Nan A	Intraperitoneal	MF1	D39	High Intranasal dose 1.7 x 10^7 CFU/ml in 50µl
Nan A	Subcutaneous	MF1	D39	High Intranasal dose 1.2 x 10^7 in 50µl

D. PdB Immunisations

1. Intraperitoneal immunisations

Twenty MF1 mice were immunised, intraperitoneally, with 20µg of PdB protein and alum adjuvant three times, seven days apart. Twenty mice were also immunised with PBS, glycerol and alum adjuvant as a control. The mice were split into two groups of twenty for challenge, with each group consisting of 10 PdB immunised mice and 10 control immunised mice. The first group of mice received three immunisations and were challenged, intranasally, with an acute dose of virulent D39 pneumococci. This was done to determine whether PdB immunised mice were protected from developing pneumonia following challenge.

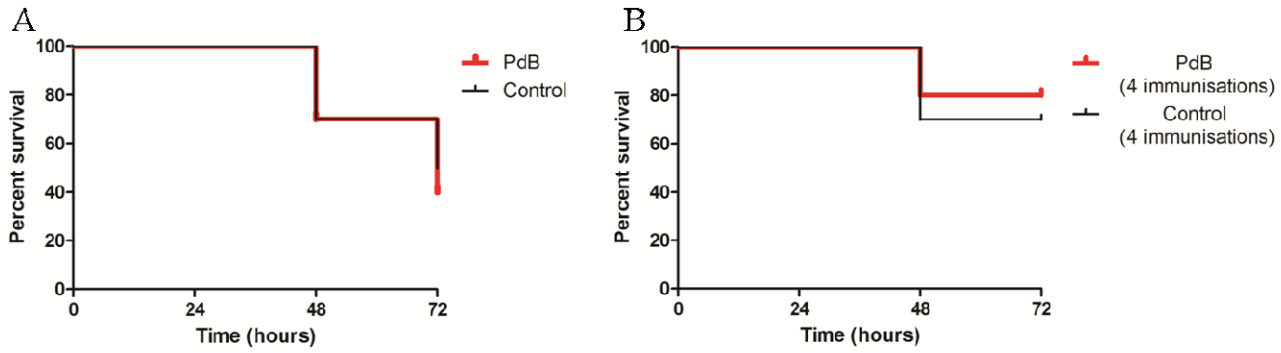


Figure **11** shows the survival time of mice in both immunisation groups after acute challenge with virulent D39. Although there was increase in the survival time of mice immunised with PdB (mean survival time of 62 hours), compared to the control group (mean survival time of 59.2 hours), this difference was not significant.

Following this the second group of mice were immunised once more with 40μg of PdB protein. This was to ensure the mice were given enough protein to be mounting a protective immune response. After 14 days, mice were tail bled and the serum samples were assayed by western blot, to detect the presence of any anti PdB antibodies. Mouse sera were used as the primary antibody to probe the blot membrane. Figure 12 shows the positive bands of anti-PdB antibodies present in the serum, collected from the tail bleeds of two mice, confirming that the PdB immunised mice were mounting a specific immune response to the protein. These mice were then challenged, intranasally, with an acute dose of D39. Figure 11, graph B, show the survival times of PdB immunised and control mice. There was no significant difference between the mean survival of PdB immunised mice (mean survival of 66 hours), and the control immunised mice (mean survival of 63 hours). Figure 13 shows the bacterial numbers present in the lungs of mice at the endpoint of the experiment. PdB immunised mice had no reduction in bacterial numbers recovered from the lungs, in comparison to control immunised mice. However, mice in both immunisation groups survived for a longer than expected after challenge. This is according to the survival time published by Canvin et al., 1995, who also used a high dose of D39 and MF1 mice. This may have been due to a decrease in virulence of the passaged stock of D39 bacteria used for challenge.

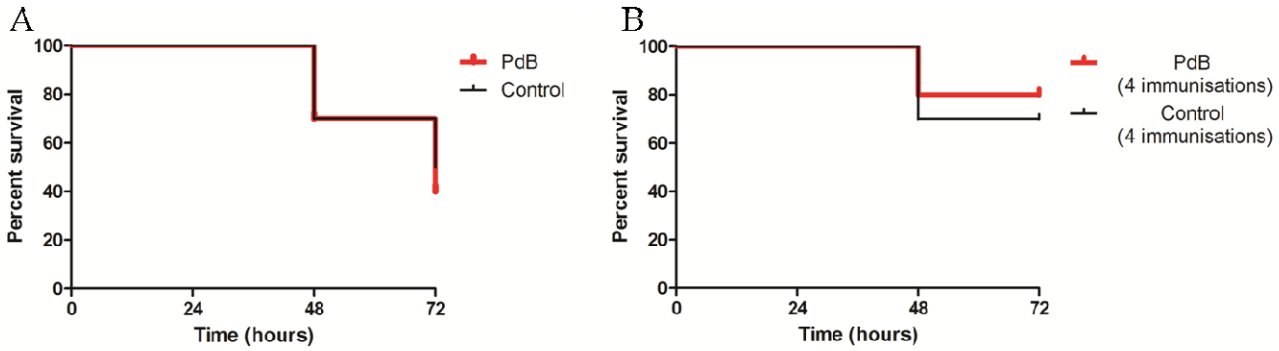


Figure 11- survival of mice intranasally infected with D39, after 3 immunisations with PdB or control (A). B – Shows mice immunised once more, with PdB (double dose of 40 µg) or the control substance, before challenge.

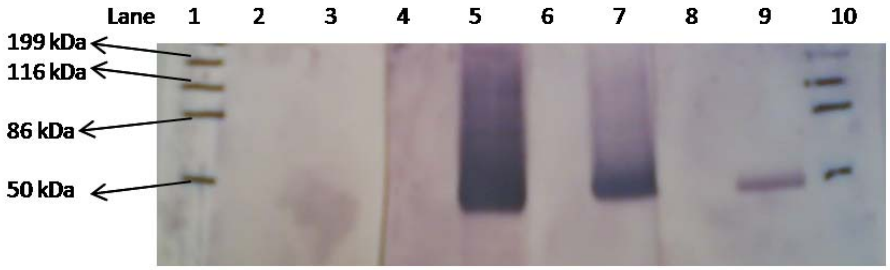


Figure 12- anti-PdB antibodies present in tail bleed sera of two mice. The sera was taken from the mice immunised four times with PdB or the control (graph B above). Lane 1 shows the molecular marker, lane 2 shows no positive bands of anti-PdB antibody in sera from a control immunised mouse. Lane 3 shows serum from a different control immunised mouse, lane 4 shows BSA a negative control for this assay. Lane 5 shows serum from a PdB immunised mouse, lane 6 shows BSA, lane 7 shows a different serum sample from a PdB immunised mice. Lane 8 shows BSA, and lane 9 shows PdB protein, which was used as a positive control in this assay.

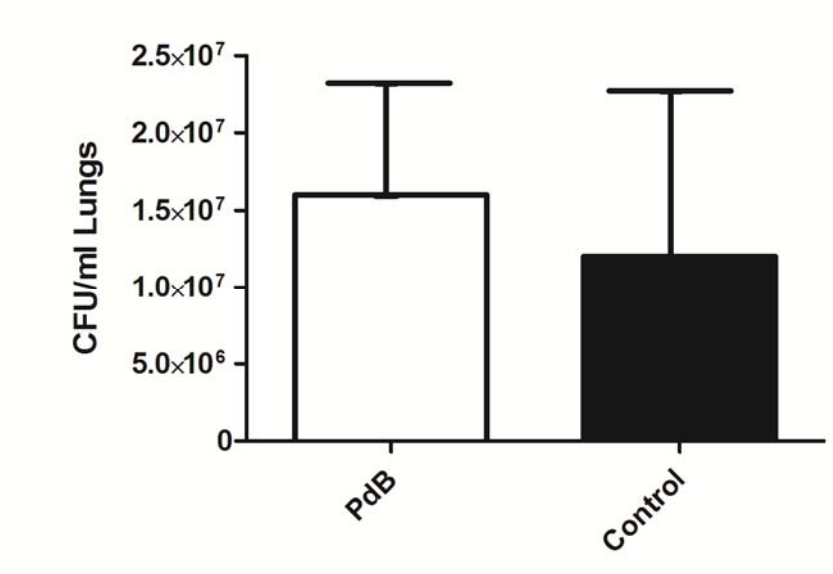


Figure 13- bacterial numbers present in the lungs of PdB and control immunised, at the endpoint of the experiment, after an acute intranasal challenge with D39. Error bars represent SEM and n=5 per group.

Serum samples were collected over the course of the immunisation period were assayed by ELISA for the presence of specific antibodies. Figure 14 shows the specific antibody titres of sera collected from tail bleeds of PdB and control immunised mice, over the course of the experiment. There was significantly more specific anti PdB IgG and IgG2a present in the sera collected from PdB immunised mice, compared to the amounts of antibody present in the sera from control immunised mice. This significant increase in specific anti- PdB antibody is present after the second immunisation. However there is only a small difference in antibody titre after the third immunisation, in both IgG and IgG2a titres. There is also a small increase in specific IgG and IgG2a, after intranasal challenge of mice, at the endpoint of the experiment (when mice reach ++ lethargic).

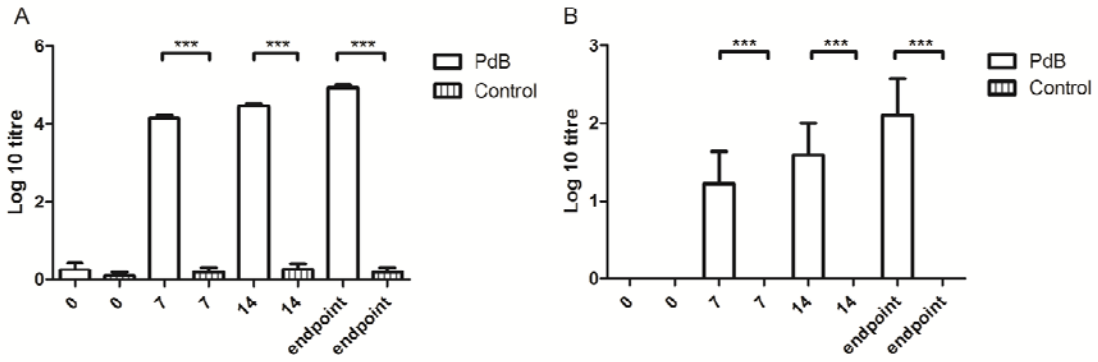


Figure 14- titre of IgG and IgG2a in sera of mice, immunised with PdB and control, collected from tail bleeds and cardiac punctures during the experiment. A - shows the IgG titre, where n=18 samples per time point. B - shows the IgG2a titre where n=14 samples per time point. Error bars represent SEM. Mice immunised with PdB have significant amounts ($p<0.01$) of specific IgG and IgG2a, at all time points, apart from t=0.

PdB immunised mice have a significant increase in specific antibodies present in their sera. However, the antibodies did not confer protection from infection following acute challenge. The antibodies recovered from cardiac puncture serum samples were tested for functional activity by hemolysis inhibition assays. Figure 15 shows an example of a hemolysis inhibition assay. The assay shows the wells containing serum from PdB immunised mice were able to inhibit the activity of PLY, by preventing red blood cell lysis (as shown in rows C, D and E of Figure 15). The serum samples from control immunised mice have no specific antibody and were unable to prevent red cell lysis (as shown in rows F, G and H of Figure 15). The titre of inhibition was decided by eye at the point where 50% of the red blood cells had been lysed by the PLY. Fig 15b shows the titre of inhibition of PLY protein from all samples collected from both PdB and control immunised mice. There was significantly more inhibition of PLY in the serum samples collected from PdB immunised mice, compared to serum samples from control immunised mice. Therefore the antibodies raised by PdB immunisation are functional, as well as being present in the sera in a high titre.

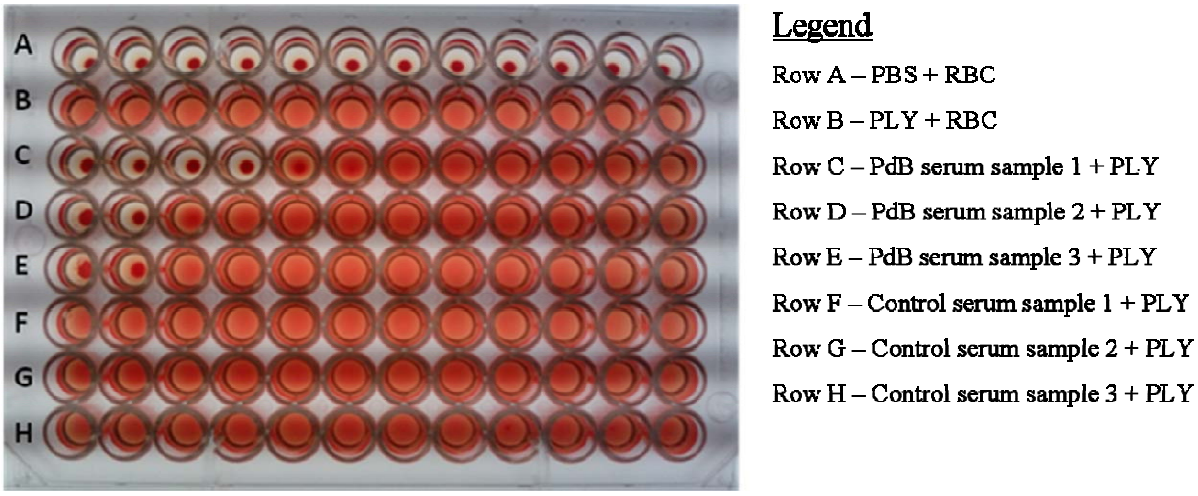


Figure 15- an example of a hemolysis inhibition assay. Row A shows the negative control, which contains red blood cells and PBS. Row B shows the positive control of red blood cells, PBS and PLY. Rows C, D and E show inhibition of PLY by serum samples from PdB immunised mice. All serum samples were depleted of cholesterol before assays. In rows F,G and H there is no inhibition of PLY by serum samples from control immunised mice.

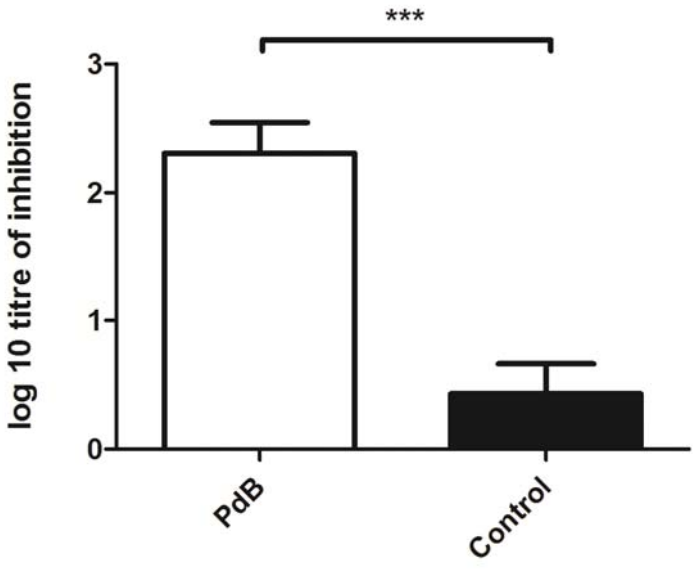


Figure 15b- shows the titre of inhibition of purified pneumolysin, by antibodies present in the serum of PdB and control immunised mice. Error bars represent SEM and n= 12 per group.

2. Subcutaneous immunisations

a) High dose challenge

No protection was seen following intraperitoneal immunisation as shown in Figure 11. Therefore, another immunisation schedule was set up, with advice from the Netherlands Vaccine Institute, to immunize mice subcutaneously. The PdB protein was administered three times, fourteen days apart and mice were challenged fourteen days after the last immunisation. The alum adjuvant was not included to the antigen preparation, as the protein was immunogenic alone.

Twenty MF1 mice were immunised subcutaneously, with 20µg of PdB protein, and twenty MF1 mice were also control immunised with PBS and glycerol. Mice were challenged in two groups of twenty as before, with each group containing ten PdB immunised mice and ten control immunised mice. Mice were tail bled and the sera were assayed for specific IgG and IgG2a anti PdB antibodies. Figure 16 shows the titre of specific anti PdB antibodies present in the sera of control and PdB immunised mice. PdB immunised mice had a significant increase in both specific IgG and IgG2a anti PdB antibodies present in their sera in comparison to control immunised mice.

The first sets of mice were challenged intranasally with a high dose of virulent D39 (50µl of $1x10^7$cfu/ml). The mean survival time of mice in the PdB immunised group (mean survival of 45 hours) was less than the survival time in the control group (mean survival of 55.2 hours), as shown in Figure 17. However, this difference was not significant. The numbers of pneumococci recovered from the lungs and blood of mice at the endpoint of the experiment (where mice became ++ lethargic) are shown in Figure 18. There was no significant difference in numbers of pneumococci present in the lungs and blood of mice immunised with PdB or the

control. This demonstrates that subcutaneous immunisation with PdB is not protective against acute pneumonia.

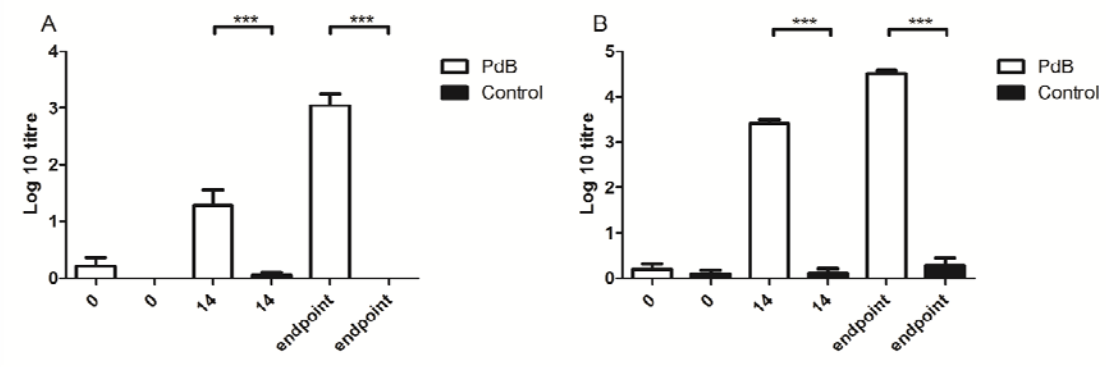


Figure 16- shows the specific anti- IgG and IgG2a present in sera collected from PdB and control immunised mice. A- shows the specific titre of anti- PdB IgG antibodies. B- shows the specific titre of anti-PdB IgG2a antibodies. Error bars represent SEM, n= 20 per group, per bar.

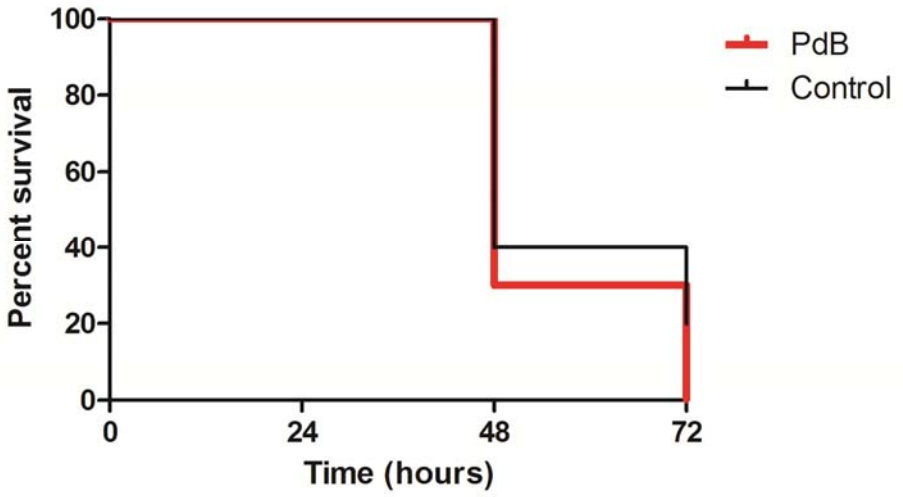


Figure 17- survival of mice challenged intranasally with a high dose of pneumococci. n=10 mice for each group.

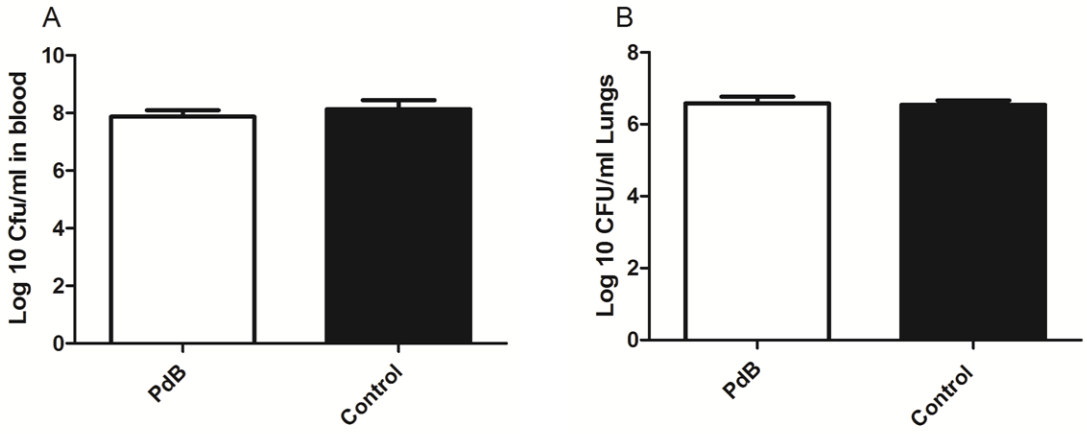


Figure 18- shows the pneumococcal numbers recovered from the lungs and blood of acute challenged mice. A- shows the log 10 cfu/ml of D39 pneumococci recovered from the blood of control and PdB immunised mice. B- shows the log 10 cfu/ml of D39 pneumococci recovered from the lungs of control and PdB immunised mice. Error bars represent SEM and n=8 per group.

b) Low dose challenge

The second group of mice subcutaneously immunised with PdB or the control were challenged intranasally with a lower amount of bacteria; the dose was $1x10^6$CFU/ml in 50µl. Following challenge there was no significant difference between the mean survival times of PdB immunised (mean survival 51.1 hours) and the control immunised group (mean survival 57.6 hours), as shown in of Figure 19. However, following challenge with a lower dose of pneumococci, a significant difference ($p<0.05$ (Mann Whitney U Test)) in the numbers of bacteria recovered from the nasopharynx was seen. PdB immunised mice had significantly less bacteria recovered from the nasopharynx, compared to the control group, as shown in Figure 20. There was no significant difference in numbers of pneumococci recovered from the lungs.

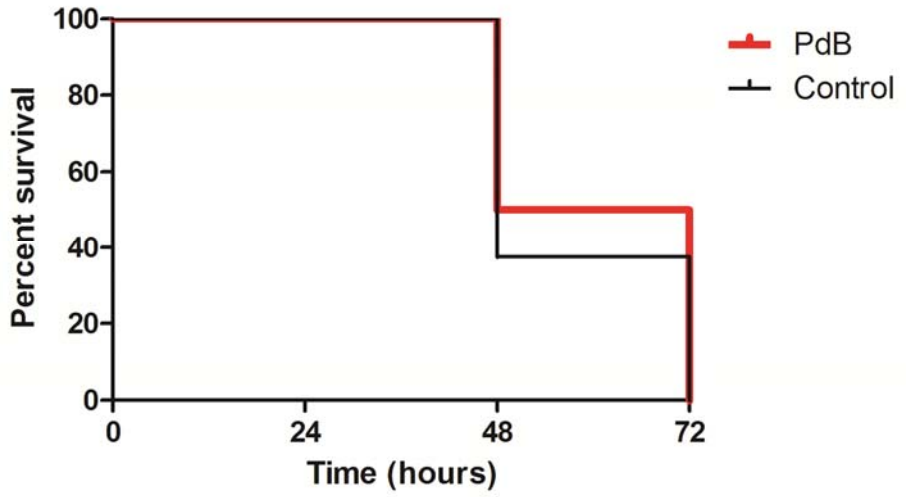


Figure 19- survival time of mice immunised with PdB or the control following challenge with a lower dose of pneumococci. n=10 mice per group.

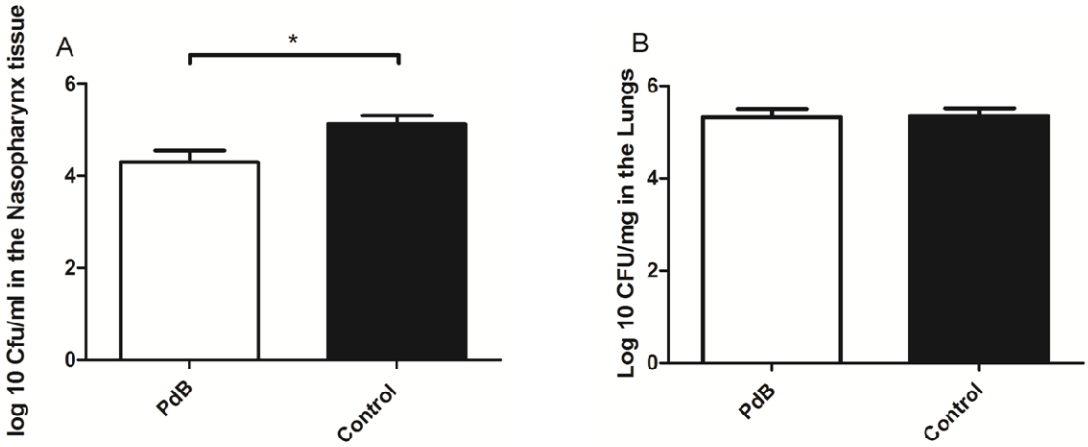


Figure 20- amount of pneumococci in the nasopharynx of both PdB and control immunised mice after intranasal challenge with a lower dose of bacteria (A). B- Shows the amount of pneumococci in the lungs of mice after intranasal challenge with a lower dose of bacteria. The error bars represent the standard error of the mean (n=10 per group).

c) Intravenous dose

Since specific anti PdB antibodies were present in the serum of mice, another group of mice were immunised and infected intravenously with a fatal dose of pneumococci, to determine whether any protection could be seen. Mice were immunised with PdB or the control, twice - fourteen days apart. Fourteen days later mice were challenged intravenously. Another naïve group of mice were challenged

as a control. Mice were culled when they reached ++ lethargic. Figure 21 shows the survival times of mice from all three treatment groups. There was a slight increase in survival time of PdB immunised mice, compared to the control immunised mice and naïve mice. However, at the end point of the experiment the PdB immunised mice had the same numbers of surviving mice as the naïve group of mice. Figure 22 shows the pneumococci recovered from the blood, lungs and nasopharynx of mice after intravenous challenge. There was no significant difference in pneumococcal numbers recovered from these samples in the PdB immunised group, the control immunised group or the naive group of mice. This shows that although immunisation with PdB may delay death from bacterieamia, it is unable to reduce bacterial numbers in the lungs, blood and nasopharynx. At the endpoint of the experiment, the same survival and bacterial numbers are seen, in the PdB immunised mice as the non immunised naïve mice. Therefore, these results show that immunisation with PdB is not protective against an intravenous dose of pneumococci.

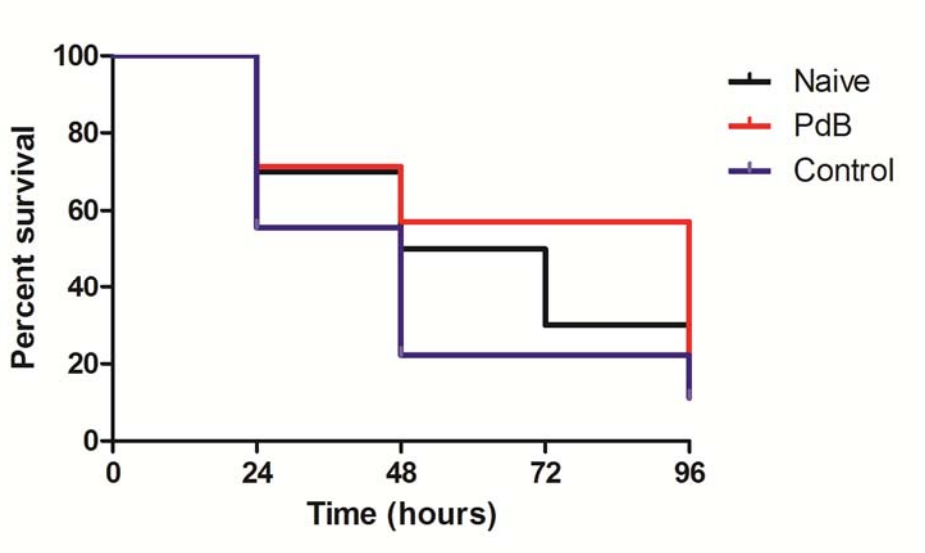


Figure 21- shows the survival of mice after intravenous infection with D39. The three groups are PdB immunised mice, control immunised mice and naïve mice. n=10 mice per group.

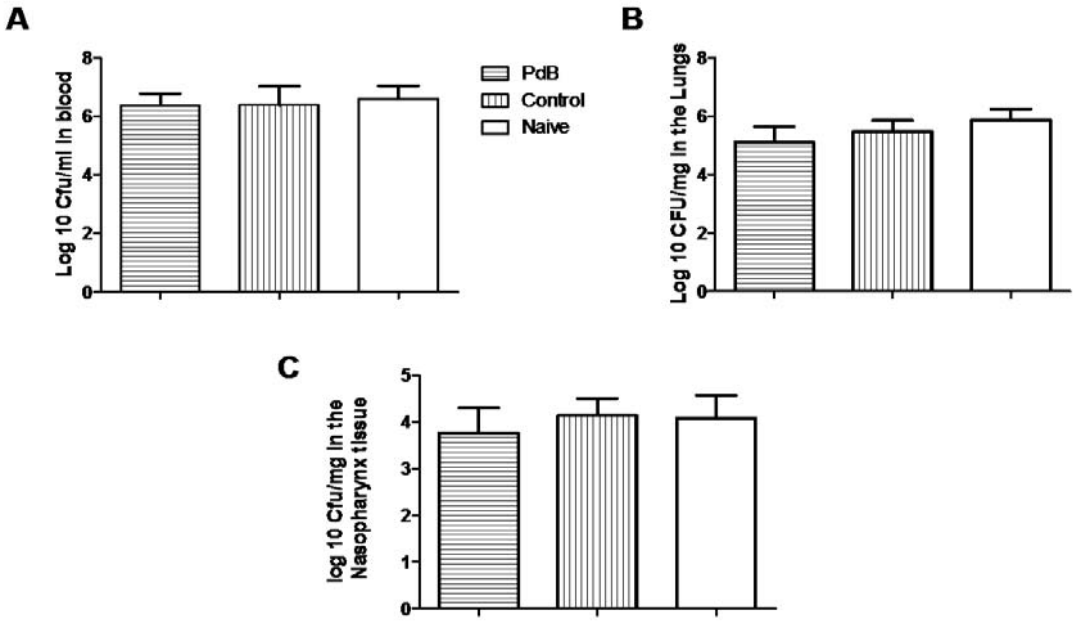


Figure 22- viable pneumococci present in the blood, lungs and nasopharynx of PdB, control or naïve mice challenged intravenously. A - shows the $\log_{10}$ Cfu/ml in the blood of mice, B - shows the $\log_{10}$ Cfu/ml in the lungs of mice and C - shows the $\log_{10}$ Cfu/ml of pneumococci in the nasopharynx of mice. Error bars represent the SEM. n=9 for PdB and control groups, n=8 for the naïve group.

E. Balb/c immunisation followed by colonisation

The previous results show that MF1 mice may be succumbing to infection too quickly to see a protective effect from the immunisations, therefore Balb/c mice were chosen to be immunised next. Balb/c mice are an inbred strain of mice, which are resistant to pneumococcal infection (Kadioglu and Andrew, 2005).

The immunisation schedule was set up as before and mice were immunised, subcutaneously, three times - fourteen days apart. Mice were tail bled before each immunisation and cardiac punctured at the endpoint. The serum was collected from these samples for ELISA analysis. Figure 23 shows the antibody titre of serum samples, from PdB and control immunised mice, collected over the course of the experiment. The mice immunised with PdB had a significant increase in the amount of specific anti-PdB IgG antibody after the first and second immunisations compared to the control group. Following immunisation, Balb/c mice were challenged intranasally in two groups of twenty (ten PdB immunised and ten control immunised mice). The mice were sacrificed at 24 and 48 hours after challenge. The results showed that none of the mice had any bacteria present in their lungs or in their blood. However, the PdB immunised mice had a significantly lower number of bacteria recovered from the nasopharynx, in comparison to control immunised mice, as shown in Figure 24. This significant difference in bacterial numbers was seen at both 24 hours ($p<0.005$) and 48 hours ($p<0.001$). The number of bacteria recovered from the PdB immunised group of mice does not increase from 24 to 48 hours. This is unlike the control group where the amount of bacteria continues to increase in the nasopharynges.

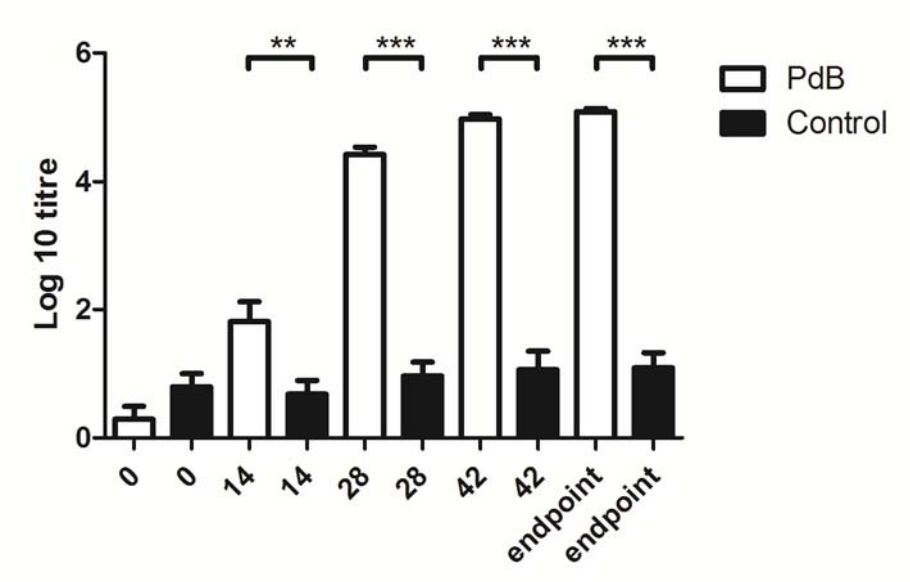


Figure 23- titre of anti-PdB IgG antibodies in serum collected from Balb/c mice immunised with PdB protein or the control. Error bars represent the SEM and n=20 at all time points, apart from 42days where n= 10 per group.

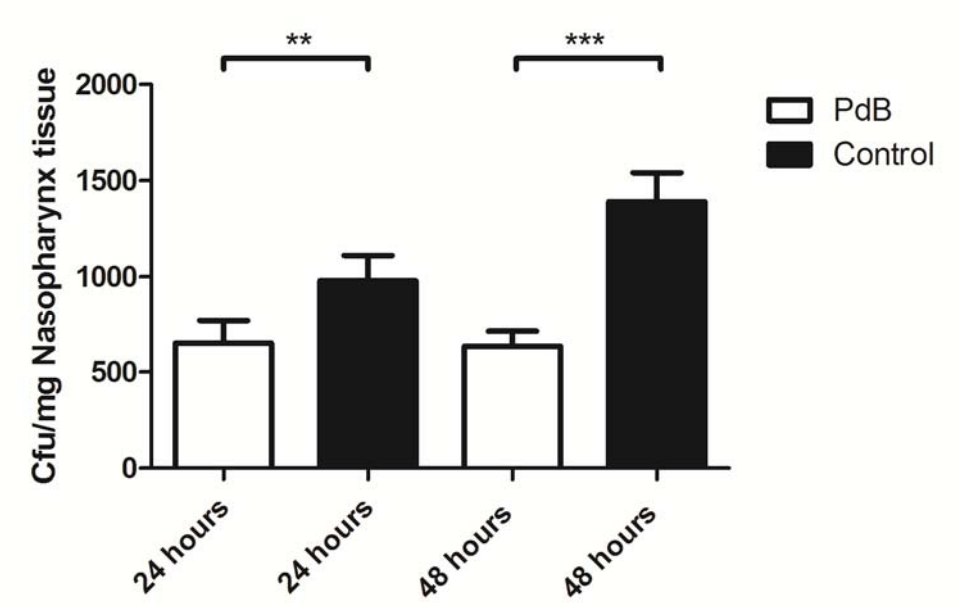


Figure 24- CFU/mg of pneumococci recovered from the nasopharynx tissue of Balb/c mice sacrificed at 24 hours and 48 hours after challenge. Each error bar represents the SEM. n=8 for the PdB group and n=10 for control group at each time point.

The functional ability of antibodies elicited by immunisation with PdB protein in BALB/c mice was tested by hemolysis inhibition assays. Figure 25 shows the antibodies present in the serum samples of PdB immunised mice had a significantly higher titre of inhibition of PLY activity compared to the control

group. Although the titre of functional antibody activity is low in these samples there is a specific antibody response which is likely to relate to a decrease in the bacterial numbers present in the nasopharynx of PdB immunised mice. Therefore, immunisation with PdB may elicit a site specific reaction which may be protective against colonisation of the nasopharynx.

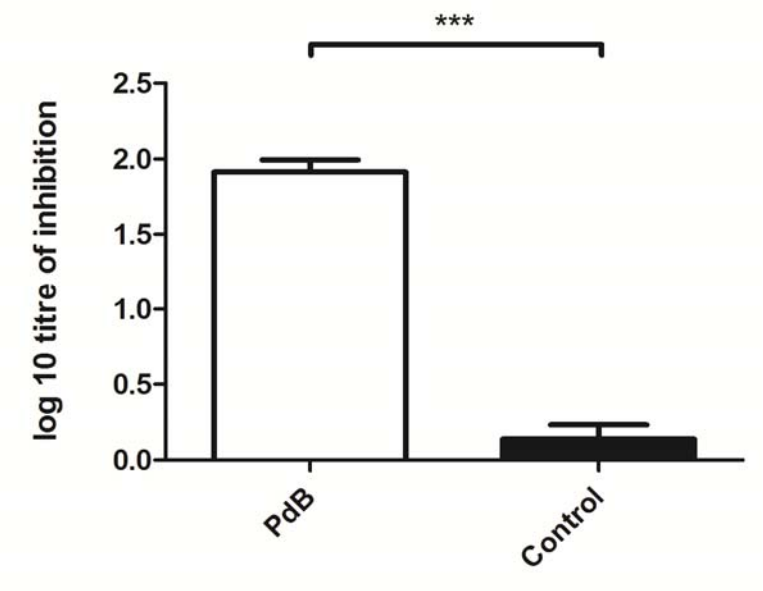


Figure 25- hemolysis inhibition of PLY protein by sera collected from the cardiac punctures of Balb/c mice subcutaneously immunised with PdB and control. Error bars represent SEM and n= 10 per group.

F. MF1 Immunisation followed by colonisation

The numbers of pneumococci recovered from the nasopharynx of Balb/c mice following immunisation with PdB protein were decreased in comparison to the control immunised mice. Therefore, further study was done to discover if this difference could be seen in MF1 mice. MF1 mice were used in these experiments, as a colonisation model is well established in this strain. MF1 mice are also outbred and are able to mimic the genetic variation present in the human population. The immunisation schedule was altered, since the previous results showed only a small

increase in specific antibody titre after the third immunisation. The immunisation schedule was set out so that mice were immunised twice, fourteen days apart, and challenged fourteen days after the last immunisation. Colonisation of the nasopharynx is asymptomatic; therefore mice were sacrificed at different time points after challenge. Four different pneumococcal serotypes were used for colonisation, in separate experiments, to examine whether cross serotype protection was elicited by immunisation with PdB.

a) D39 Immunisation followed by colonisation

Before immunisation with PdB twenty four MF1 mice were challenged with a colonisation dose of D39 pneumococci. A colonisation dose is the administration of 10µl of $1x10^7$cfu/ml intranasally (Richards et al., 2010). Mice were sacrificed at time 0, 24, 48, 72, 168 and 336 hours after challenge. No pneumococci were present in the lungs or blood of colonised mice. Pneumococci were present only in the nasopharynx of mice challenged with a colonising dose. Figure 26 shows the numbers of pneumococci recovered from the nasopharynx of MF1 mice following colonisation with D39. Pneumococci were carried in the nasopharynx for longer than fourteen days, as shown in Figure 26.

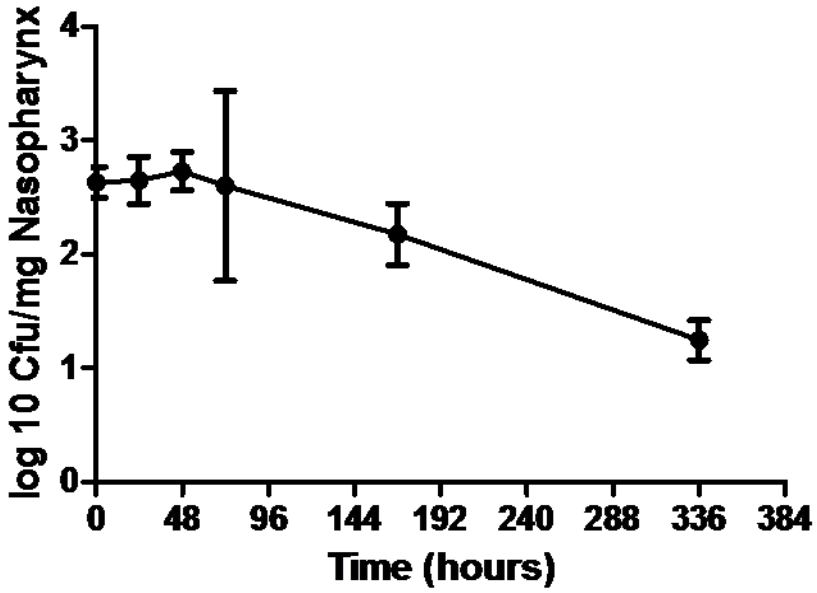


Figure 26- shows the carriage of D39 pneumococci in the nasopharynx of mice challenged with a colonising dose of virulent D39. Error bars represent the SEM and n=4 mice per time point.

The effect of PdB immunisation upon carriage of D39 in the nasopharynx was investigated. Fifty MF1 mice were subcutaneously immunised with either PdB protein (batch 1 NVI) or the PBS control twice, fourteen days apart. Mice were tail bled before each immunisation and serum samples were collected and assayed by ELISA for the presence of specific antibodies. Figure 27 shows the antibody profile of both PdB and control immunised mice. There are significantly more anti-PdB IgG and IgG2a antibodies present in PdB immunised mice, at all time points, when compared to the control immunised mice. Since earlier results showed only a slight increase in antibody titre after the third immunisation, two immunisations were deemed to be enough for the mice to mount a specific immune response.

Fourteen days after the second immunisation mice were divided into two groups of fifty, and intranasally challenged with a colonisation dose of virulent D39

pneumococci. This protocol was repeated for a second time to give results from two separate challenge experiments.

Mice were sacrificed at time points 0, 24, 48, 72 and 168 hours after challenge. The nasopharynx, blood, lungs, NALT and CLN were removed and processed. The lungs and blood contained no pneumococci, confirming the bacteria did not move to the lungs and cause infection. The numbers of pneumococci present in the nasopharynx of both PdB immunised and control immunised mice, over seven days, is shown in Figure 28 (this is the result of two separate challenge experiments). The figure shows that PdB immunised mice had consistently less D39 present in the nasopharynx at all time points; however there were significantly less D39 present at 72 ($p<0.01$) and 168 ($p<0.05$) hours after challenge.

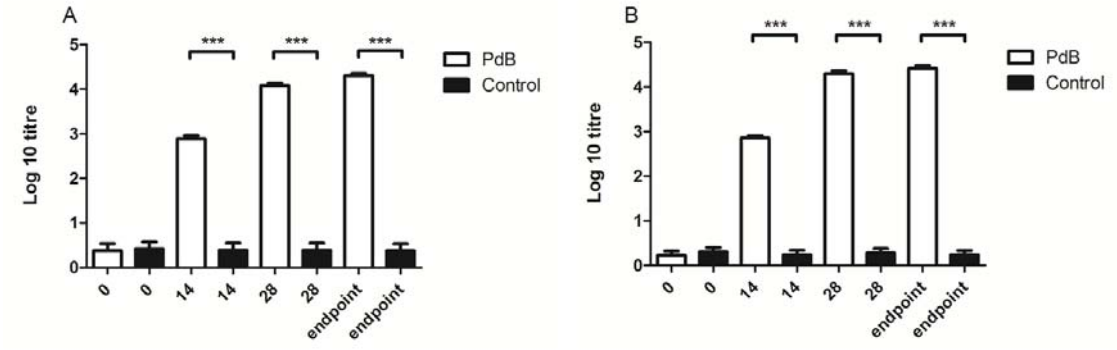


Figure 27- titre of specific antibodies present in sera collected from subcutaneously immunised mice subsequently colonised with D39. A - specific anti-PdB IgG titre. B - specific anti-PdB IgG2a titre. Error bars represent SEM. For graph A - n=45 and for graph B - n=39 per group, per time point**.**

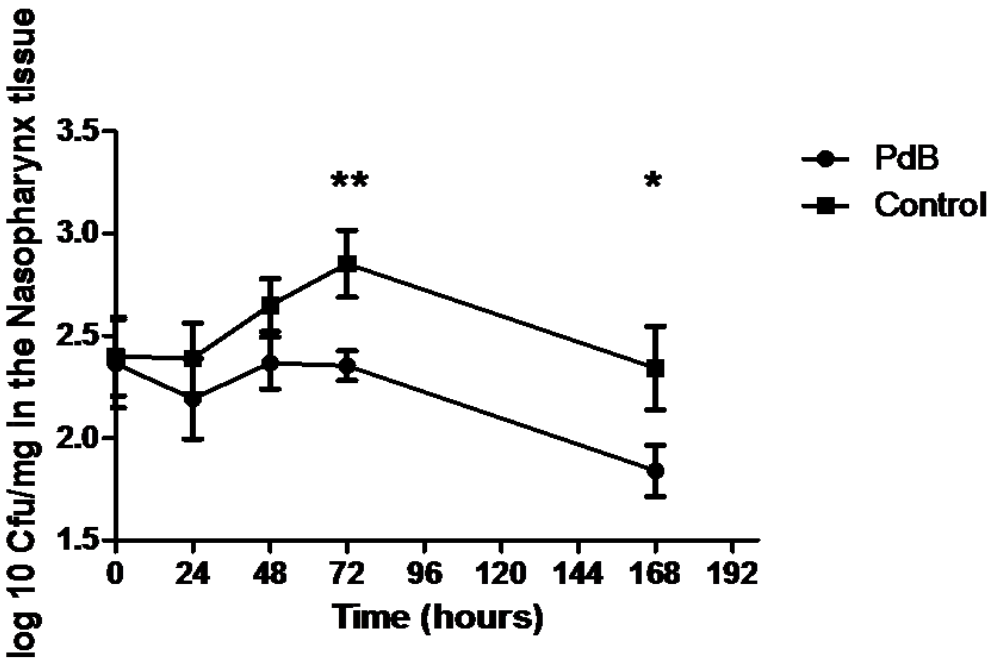


Figure 28- combined results of colonisation experiment 1 and 2 (two different colonisation challenges). The figure shows the concentration of D39 recovered from the nasopharynx of PdB immunised mice and control immunised mice over 7 days. n=7 at time 0 and n=10 at all other time points, error bars represent the SEM.

The anti PdB antibodies were assayed for activity by hemolysis inhibition assays. Figure 29 shows the titre of inhibition of PLY by sera from both control and PdB immunised mice. Therefore, antibodies elicited by immunisation are functional, and are able to neutralise purified PLY protein.

Through ELISPOT analysis the numbers of antibody producing cells present in the CLN were examined. Figure 30 shows the numbers of IgG and IgA producing B cells present in the CLN of both PdB and control immunised mice. There was a significant increase in IgG producing B cells, in PdB immunised mice, seven days after infection. Although not significant, there is also an increase in the number of IgA producing B cells, seven days after colonisation. An increase in B cells may contribute to a decrease in pneumococcal numbers present in the nasopharynx of PdB immunised mice. This decrease in pneumococcal numbers

recovered from the nasopharynx of PdB immunised mice, correlates with an increase in antibody producing B cells present in the CLN.

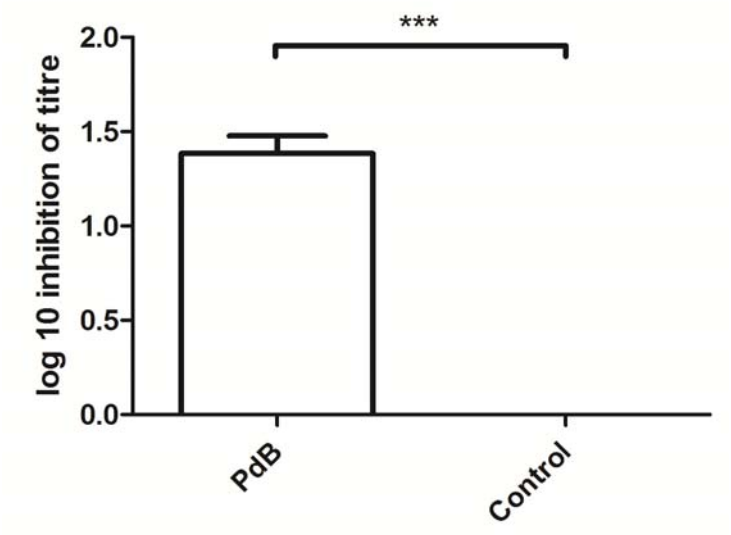


Figure 29- shows hemolysis inhibition of PLY protein by sera collected from the cardiac punctures of MF1 mice subcutaneously immunised with PdB and control. Error bars represent SEM. n=25 for PdB immunised mice and n=14 for control immunised mice.

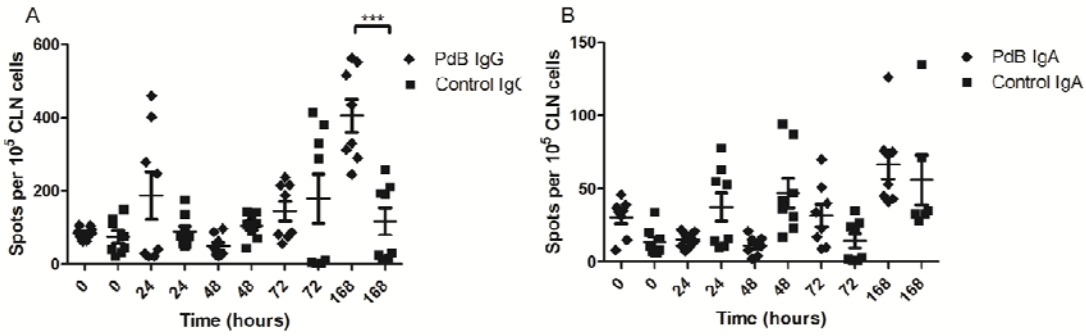


Figure 30- shows the ELISPOT analysis of cells from the CLN of mice immunised with PdB or control and subsequently colonised. One spot correlates to one antibody producing B cell. A- Shows the numbers of IgG producing B cells present. B- Shows the numbers of IgA producing B cells present. Error bars represent SEM. Each dot represents 5 CLN samples pooled together, n=8 per group per time point.

b) Immunisation and colonisation of mice followed by Intravenous challenge

Colonisation of MF1 mice with D39 has previously been shown to reduce the numbers of bacteria present in the nasopharynx after another colonisation with the same serotype (Richards et al., 2010). Therefore immunisation and colonisation were combined to discover if any additive protection could be seen after an intravenous dose of D39.

Mice were subcutaneously immunised with PdB (batch 1 NVI) or control twice, fourteen days apart. Fourteen days later mice were colonised with D39. Twenty one days later mice were challenged intravenously with D39. PdB and control immunised mice (which had not been pre-colonised) were intravenously challenged, as well as a group of naïve mice. Mice were sacrificed when they became ++ lethargic.

Figure 31 shows the percentage survival of mice from all five treatment groups. The figure shows that colonised mice had the highest number of surviving mice at the endpoint of the experiment. Although this difference is not statistically significant, colonisation and immunisation combined increase the survival of mice after intravenous challenge by 20%, when compared to colonised control immunised mice. As shown earlier mice immunised with PdB alone survive for longer than the naïve and control immunised mice. However, this difference only appears to prolong the life of mice as, at the endpoint of the experiment, the same numbers of PdB immunised mice survive as the naïve group of mice. Figure 32 shows the numbers of pneumococci recovered from the blood, lungs and nasopharynx of mice after intravenous challenge. There is a decrease in pneumococci recovered from the lungs and blood in the PdB immunised and colonised group of mice compared to all other treated groups of mice. Colonisation also decreases the amount of bacteria present in the nasopharynx. However, there seems to be no additive protection from immunisation as the control immunised group also have a decrease in D39 in the nasopharynx.

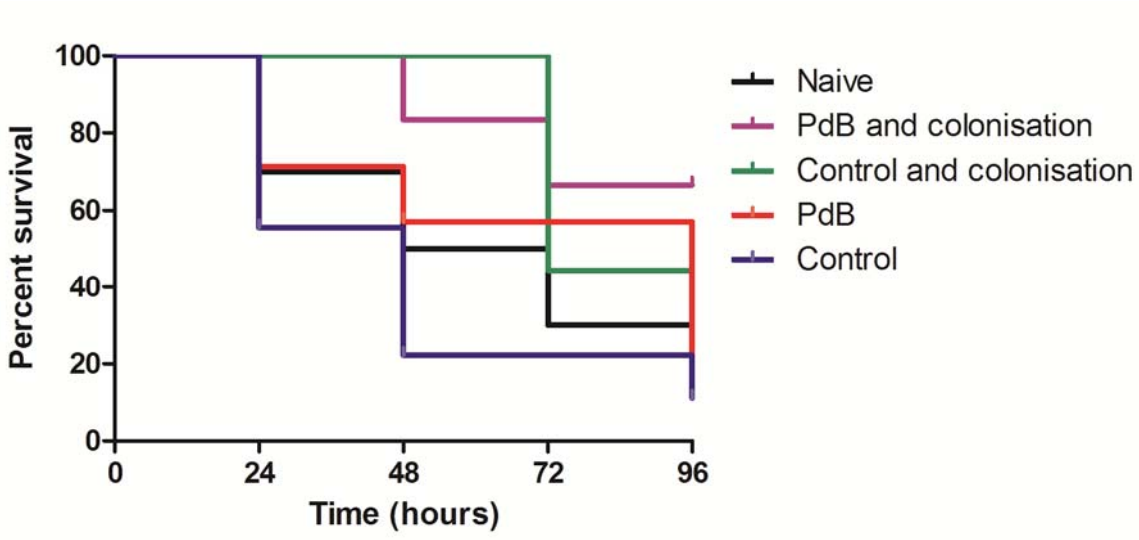


Figure 31- shows the survival percentage of five different groups of mice following intravenous challenge. The five groups were PdB immunised, control immunised, PdB immunised and colonised, control immunised and colonised and naïve mice. Error bars represent SEM and n=10 mice per group.

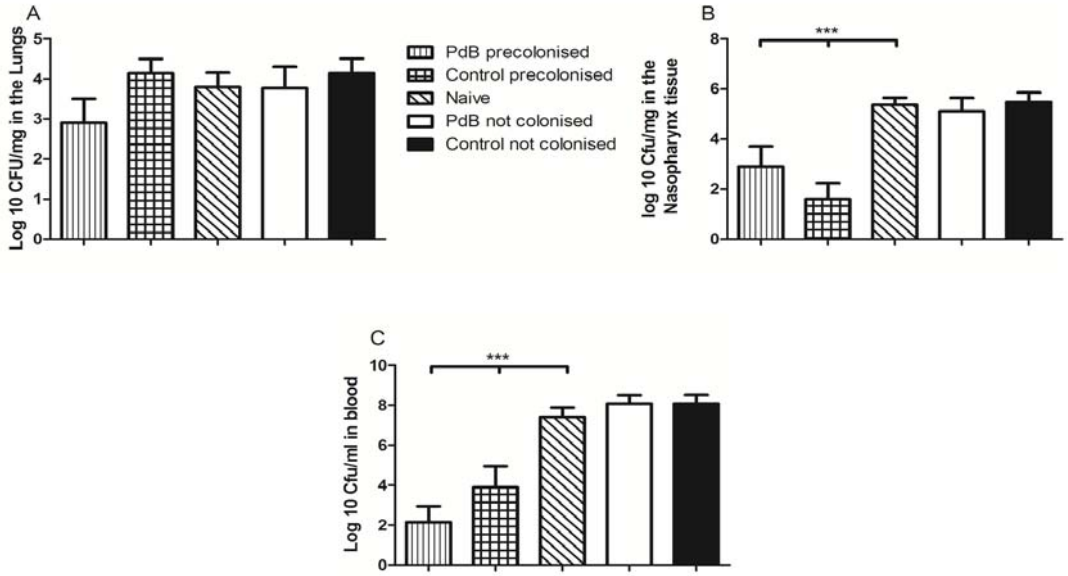


Figure 32- shows the numbers of D39 recovered from lungs, blood and nasopharynx of five differently treated groups of mice. The groups were PdB or control immunised mice followed by colonisation and/or intravenous challenge as well as a naïve group of mice that previously had no other treatment. A – Shows the numbers of D39 recovered from the lungs of mice, where n=8 per group. B- shows the numbers of D39 recovered from the blood of mice. n=13 per group apart from control not colonised where n=5 C- shows the numbers of D39 recovered from the nasopharynx of mice, n=8 per group. Error bars represent the SEM.

c) Immunisation and colonisation of mice followed by high dose intranasal challenge

The previous results show that a combination of immunisation and colonisation is able to reduce the numbers of bacteria present in the nasopharynx, lungs and blood. Therefore immunisation and colonisation were combined once more to discover if there was any additive protective effect after an acute challenge.

Mice were subcutaneously immunised with PdB (batch 1 NVI) or control twice, fourteen days apart. Fourteen days later mice were colonised intranasally with D39. Twenty one days later mice were challenged intranasally with an acute dose of D39. Mice that had only been immunised with PdB or control (no colonisation) were also intranasally challenged as well as a group of naïve mice. Mice were sacrificed when they became ++ lethargic. Figure 33 shows the survival of mice from all five treatment groups after acute challenge. Mice that were colonised with D39 survived for longer and at the endpoint of the experiment had a higher percentage survival rate. However, immunisation with PdB combined with colonisation gives no additive protection to mice after acute challenge. Figure 34 shows the numbers of pneumococci recovered from the lungs, blood and nasopharynx of all mice from the different treatment groups. Colonisation of mice decreases the numbers of pneumococci recovered from the lungs, blood and nasopharynx. Immunisation with PdB combined with colonisation decreases the numbers of pneumococci present in the nasopharynx although this difference is not significantly different from the colonised control immunised mice.

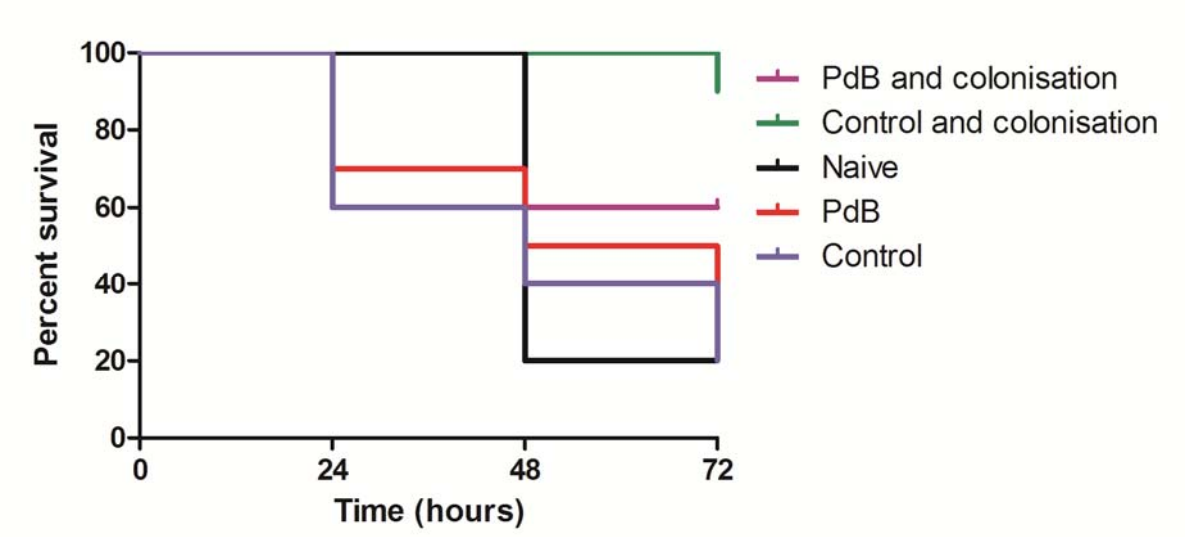


Figure 33-shows the survival percentage of five different groups of mice following intranasal challenge with a high dose of pneumococci. The groups were; PdB immunised, control immunised, PdB immunised and colonised, control immunised and colonised, and naïve mice that previously had no other treatment. Error bars represent SEM and n=10 per group except for the naïve group where n=5.

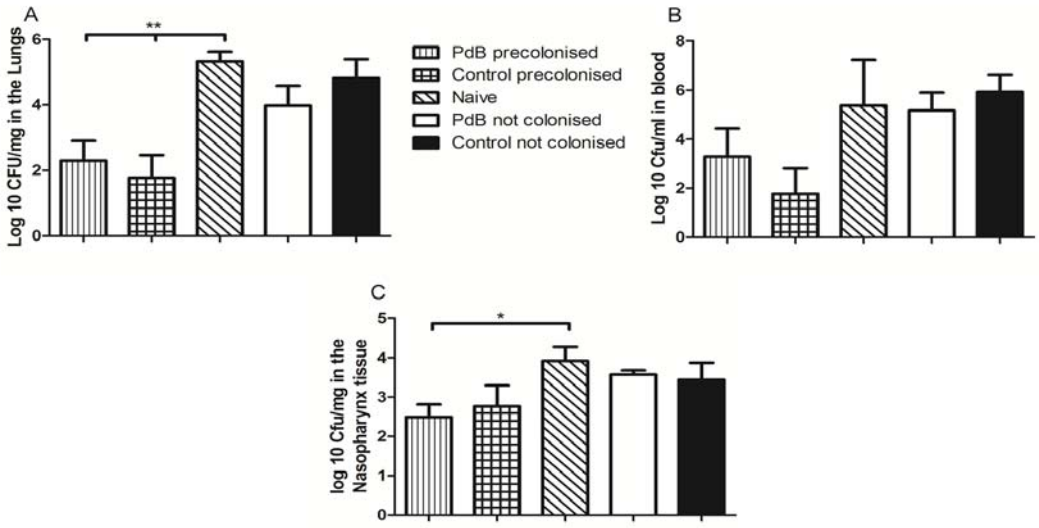


Figure 34- shows the amounts of pneumococci present in the lungs, blood and nasopharynx of mice from the five different treatment groups. A - Shows the amount of D39 present in the lungs. B - Shows the amount of D39 present in the blood. C - Shows the amount of D39 present in the nasopharynx. Error bars represent SEM and n=10 per group except for the naïve group where n=5.

d) A66, 19F, and TiGR4 colonisation

Previous results show that immunisation of mice with PdB is able to decrease colonisation of the nasopharynx with D39. Following this, it was investigated whether immunisation with PdB conferred protection from colonisation with other pneumococcal serotypes.

To show that A66 (serotype 3) was able to be carried in the nasopharynx; twenty four MF1 mice were challenged with a colonisation dose of pneumococci. Figure 35 shows the numbers of A66 recovered from the nasopharynx of mice challenged with A66 at different time points after infection. There were pneumococci present in the nasopharynx after fourteen days. This result shows that A66 is able to be carried in the nasopharynx without being cleared. Therefore, A66 could be used for evaluating whether immunisation with PdB is cross serotype protective.

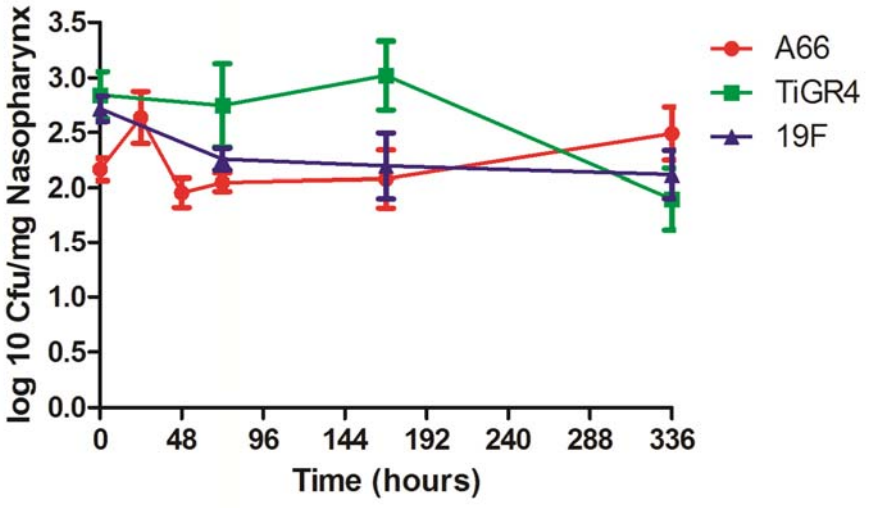


Figure 35- the amount of A66, TiGR4 and 19F present in the nasopharynx over a fourteen day time period. Error bars represent SEM and n=4 per time point.

The immunisation schedule was set up as described for D39 colonisation experiments. MF1 mice were subcutaneously immunised with two doses of either PdB (batch 2 NVI) or control over fourteen days. Fourteen days later mice were challenged with a colonisation dose of A66, TiGR4 or 19F. As in the D39 colonisation experiments mice were sacrificed at several different time points after challenge. Figure 36 shows the amounts of pneumococci recovered from the nasopharynx at time points 0, 24, 48, 72 168 and 336 hours after challenge. Unlike the D39 colonisation experiment there is no difference in the amounts of pneumococci recovered from the nasopharynx of PdB immunised mice when compared to the control immunised group, following colonisation with A66, TiGR4 or 19F.

No serum samples were assayed for antibody content, since ELISA analysis had previously been performed for mice that had been immunised twice. Previous results showed that specific antibodies had been elicited by PdB immunisation. However, at time points following colonisation of the nasopharynx with TiGR4 and 19F, nasal washes were performed to show if any secreted IgA was present. Secreted IgA is important in preventing colonisation of the nasopharynx. Any good vaccine developed against nasopharyngeal colonisation should be able to elicit secretory IgA. Figure 37 shows the titre of IgA elicited by immunisation at time points after colonisation with TiGR4 and 19F. There are very low levels of secretory IgA. This may be one reason that the pneumococci are unable to be cleared from the nasopharynx.

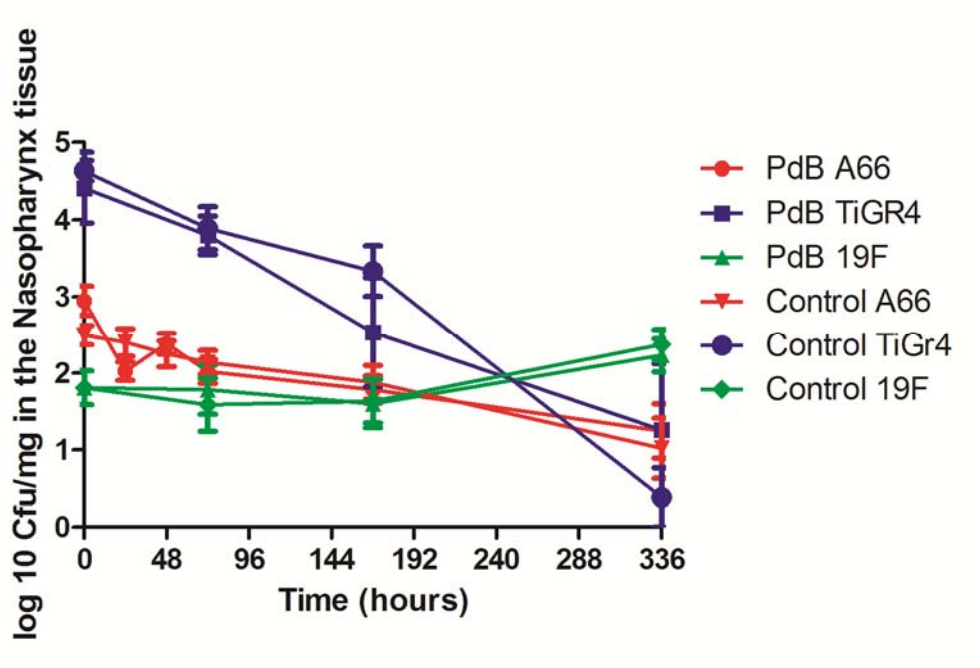


Figure 36- shows the numbers of A66 recovered from the nasopharynx of mice immunised with PdB or control at 0, 24, 48, 72, 168 and 336 hours following colonisation. Error bars represent SEM and n=5 per group, per time point.

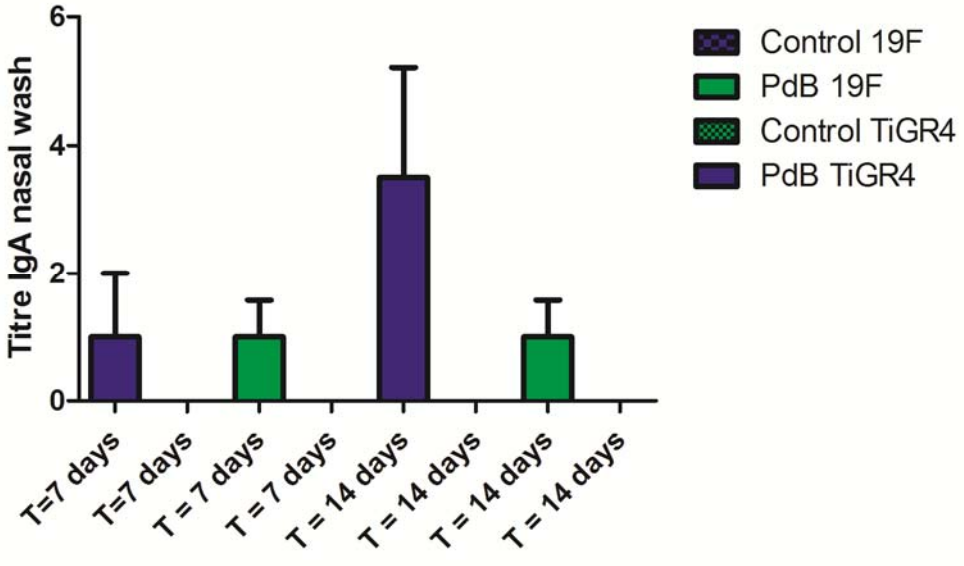


Figure 37- shows the titre of IgA present in nasal washes of PdB and control immunised mice after colonisation with TiGR4 and 19F. Error bars represent SEM and n=4 per group per time point.

ELISPOT analysis of the CLN from both PdB and control immunised mice, was performed to determine the numbers of IgG and IgA producing B cells present following colonisation with each pneumococcal serotype. Figure 38, Figure 39 and

Figure 40 show the numbers of IgG and IgA producing B cells from the CLN of PdB and control immunised mice. Although there are significant differences in the numbers of IgA and IgG producing B cells (plasma cells) present at different time points after challenge, these differences in the numbers of B cells do not correlate with any reduction of pneumococci in the nasopharynx. Therefore, immunisation with PdB does not protect mice from colonisation with the pneumococcal serotypes A66, TiGR4 or 19F.

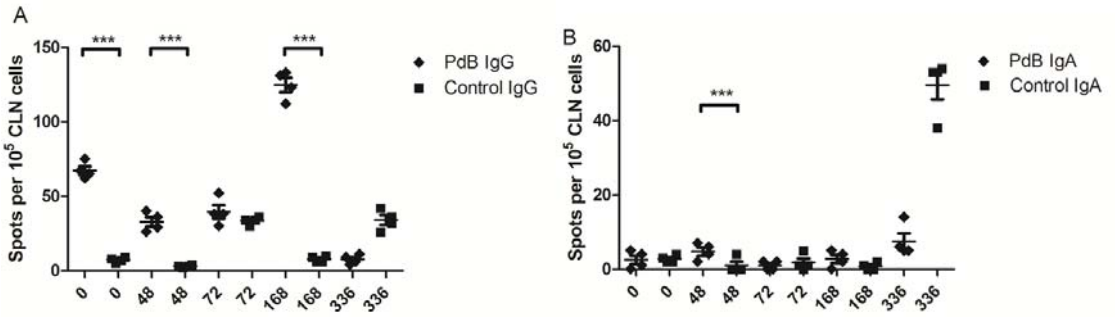


Figure 38- shows the numbers of IgG and IgA producing B cells present in the CLN of both control and PdB immunised mice. A - Shows the numbers of IgG producing B cells. B - Shows the numbers of IgA producing B cells. Error bars represent SEM and n=4 per group per time point.

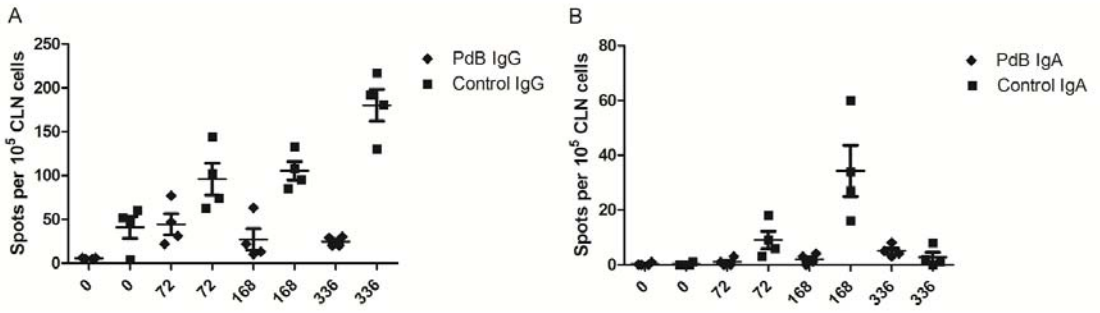


Figure 39- shows the numbers of IgG and IgA producing B cells present in the CLN of PdB and control immunised mice subsequently challenged intranasally with a colonisation dose of TiGR4. A - Shows the numbers of IgG producing B cells. B- Shows the numbers of IgA producing B cells. Error bars represent SEM and n=4 per group per time point.

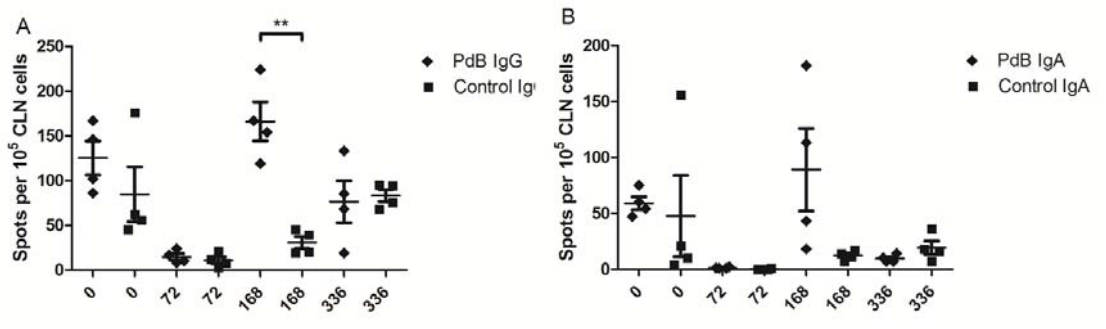


Figure 40- shows the numbers of IgG and IgA producing B cells present in the PdB immunised and control mice following colonisation with TiGR4. A - Shows the number of IgG producing B cells present in the CLN of control and PdB immunised mice. B - Shows the number of IgA producing B cells in the CLN of control and PdB immunised mice. Error bars represent SEM and n=4 per group per time point.

e) D39 colonisation repeated

D39 colonisation experiments were repeated, as previous results showed protection from colonisation. However, no protection was seen in the three other pneumococcal serotypes tested. The immunisation schedule was repeated as before with batch 2 of the PdB protein provided by NVI.

Mice were subcutaneously immunised with PdB or control twice, fourteen days apart, and then challenged with a colonising dose of D39 pneumococci. The numbers of pneumococci recovered from the nasopharynx of mice are shown in

Figure **41**. Surprisingly, there were no differences in the numbers of pneumococci recovered from the nasopharynx of PdB immunised mice, at any time point, in comparison to the control immunised group. This result is in contradiction

to the previous colonisation experiments with D39, where a decrease in the numbers recovered from the nasopharynx was seen in PdB immunised mice.

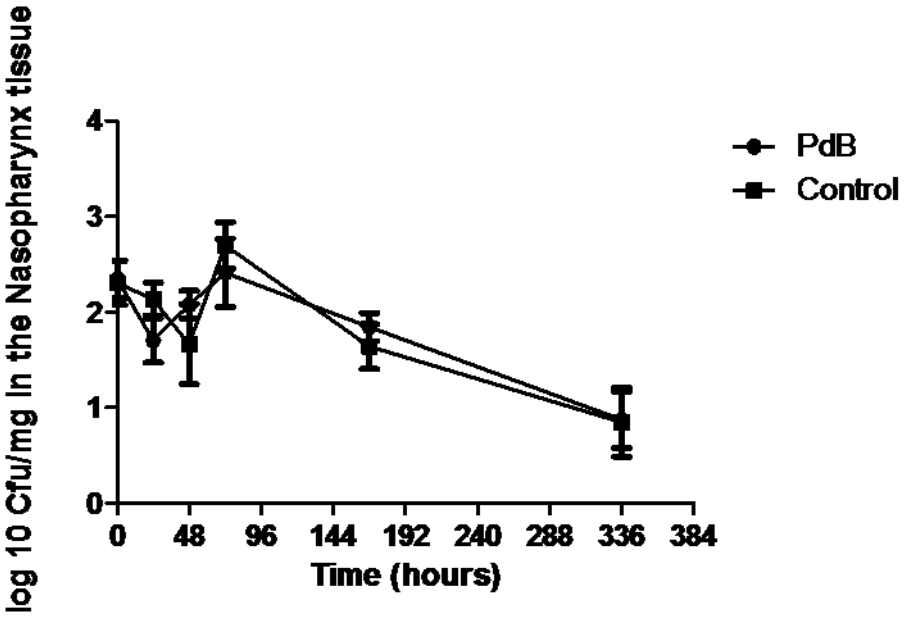


Figure 41- shows the numbers of pneumococci recovered from the nasopharynx of mice immunised with PdB or control following challenge with a colonisation dose of D39. This is the result of two separate challenges. Error bars represent SEM and n=8 per group at t=0, n=5 per group at t=24 and 48, n=10 per group at t=72, 168 and 336.

However, there is a mucosal response elicited in the nasopharynx. Figure 42 shows the secretory IgA recovered from nasal washes of both PdB and control immunised mice. PdB immunised mice showed a significant increase in secretory IgA production, at 168 and 336 hours following colonisation with D39. The titre of secretory IgA was low, however this was also seen in other mice colonised with different serotypes. The titre of secretory IgA elicited may not be enough for protection against colonisation in this case.

The numbers of IgG and IgA producing B cells present in the CLN's of PdB and control immunised mice were measured by ELISPOT analysis. PdB immunised mice had a significant increase in the numbers of IgG producing B cells present in

the CLN, in comparison to the control immunised group (as shown in Figure 43). However, this difference in IgG producing B cells does not correlate with a reduction of pneumococci recovered from the nasopharynx of mice. There was no significant difference in the numbers of IgA producing B cells present in the CLN of PdB immunised mice when compared to the control group. This result is unlike the previous D39 colonisation challenge experiments, where there was a significant increase in IgG producing B cells as well as an increase in IgA producing B cells, compared to the control group. This may explain why there is no reduction in bacterial numbers in these particular mice immunised with PdB.

These experiments, using PdB immunisation followed by the same colonisation dose of D39, should have shown some protection in the nasopharynx. However there is a difference in protection between the first set of D39 colonisation experiments and the second set. The only difference was the PdB protein used for immunisation of the mice. The first set of mice were immunised with PdB batch 1 and during the second set of experiments mice were immunised with PdB batch 2.

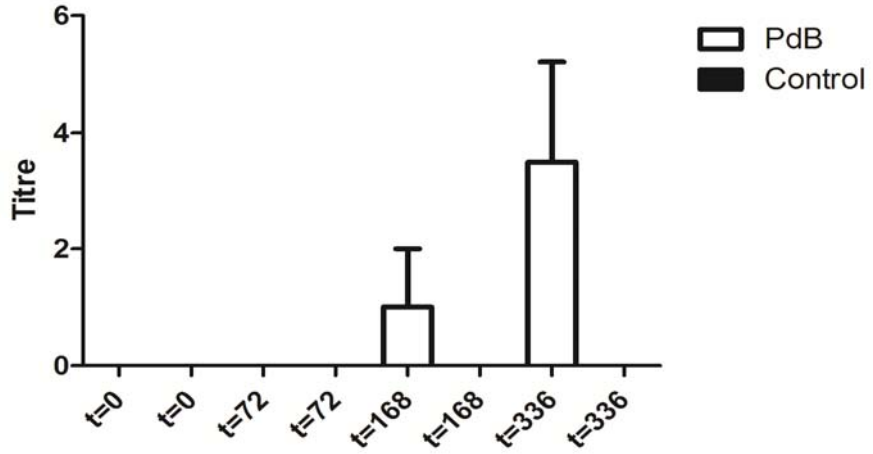


Figure 42- shows the titre of secretory IgA at recovered from nasal washes of mice, at different time points after colonisation with D39. Error bars represent SEM and n=5 per group per time point.

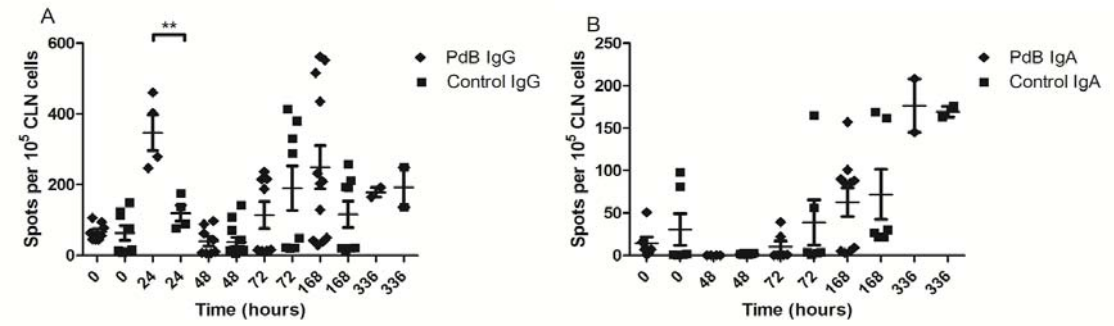


Figure 43- shows the numbers of IgG and IgA producing B cells present in the CLN of PdB and control immunised mice. A - Shows the IgG producing B cells present at different time points following challenge. B - shows the IgA producing B cells present at different time points after challenge. Error bars represent SEM. Each data point represents 5 CLN samples pooled together. For the IgG graph n=8 per group, per time point except for 24 hours where n=4, and 336 hours where n=2. For the IgA graph n=6 per group, per time point except for 336 hours where n=2.

2. PdB batch comparison and its importance in decreasing nasopharyngeal colonisation with D39

Immunisation with PdB was unable to protect mice from colonisation in the second set of experiments carried out. Therefore, samples collected from earlier colonisation challenges (colonisations 1 and 2) were compared with the samples collected in the latest experiments (colonisations 3 and 4).

Figure 44 shows the titre of IgG in the serum of mice immunised with PdB and control from colonisation experiments 1 and 2, in comparison to the titre of IgG elicited in the serum of mice from experiments 3 and 4 combined. In all colonisation experiments, the control group had significantly less specific anti- PdB IgG present in the serum samples, compared to the PdB immunised group. This is in concurrence with all ELISA analysis performed throughout all of the colonisation experiments. However, there is a significant difference ($p<0.0001$) between the titre of IgG elicited in the first two colonisation experiments, compared

to experiments 3 and 4. These mice were immunised subcutaneously at the same time points, and therefore the difference in titre of IgG should not be significantly different.

Figure 45 shows the inhibition of purified PLY by serum samples, collected from all colonisation experiments. Mice immunised with the first batch of PdB protein had significantly more functional anti PdB antibodies ($p<0.0001$) present in the sera, in comparison to mice immunised with the second batch of PdB protein.

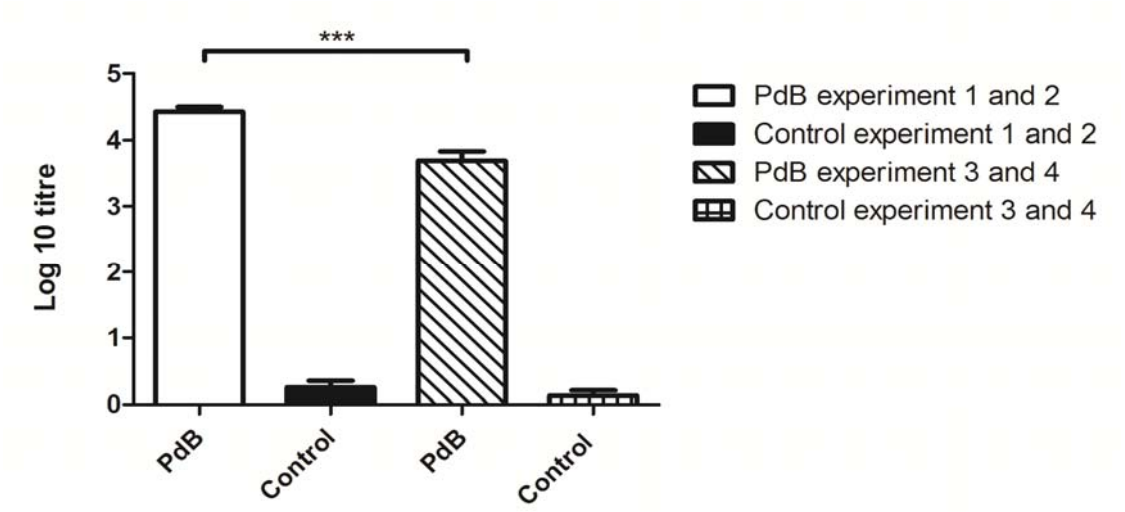


Figure 44- shows the titre of IgG in the sera of mice immunised with PdB batch one (colonisation experiment 1 and 2), and the titre of IgG in the sera of mice immunised with PdB batch two (colonisation experiments 3 and 4). Error bars represent SEM and n=41 per group.

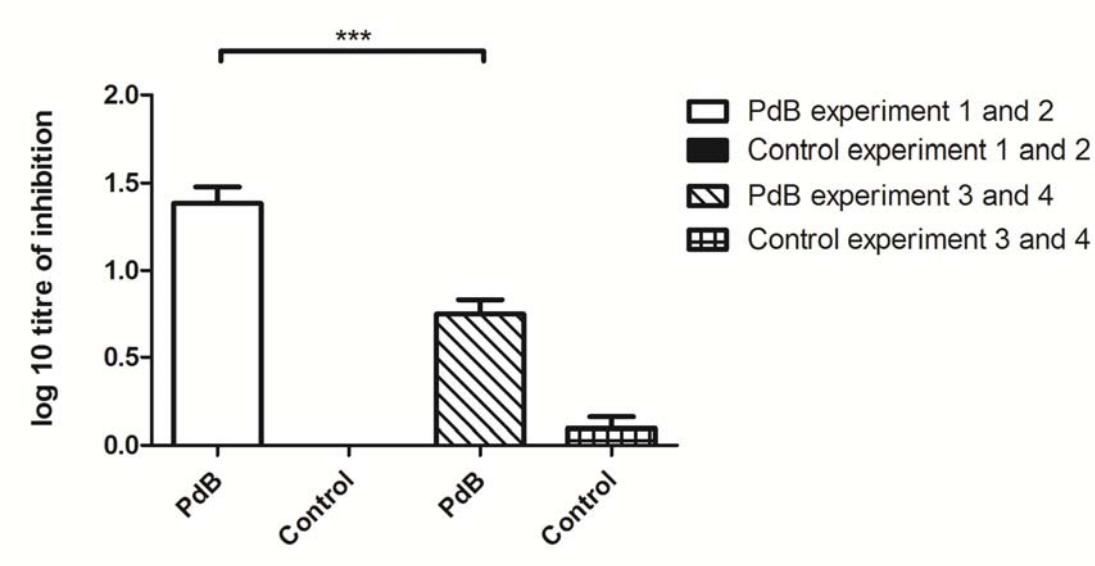


Figure 45- shows the inhibition of PLY by serum samples collected from the first (colonisation 1 and 2) and second (colonisations 3 and 4) colonisation experiments. Error bars represent the SEM and n=14 per experiment for the control samples, and n=25 per experiment for PdB samples.

Another difference between the two PdB protein batches used for immunisation can be seen in the ELISPOT assay results. Figure 46 shows the numbers of IgG producing B cells present in the CLN of PdB and control immunised mice, from colonisation experiments using PdB batch 1 and PdB batch 2. There is a significant difference in the numbers of IgG producing B cells present in the mice immunised with PdB batch 1 at time points 0, 72 and 168 hours after challenge when compared to the mice immunised with PdB batch 2. The numbers of IgG producing B cells present in mice immunised with PdB batch 2, are consistently lower than that of mice immunised with PdB batch 1. There is also a difference in the numbers of IgA producing B cells present in the mice immunised with PdB batch 1, when compared to PdB batch 2, as shown in Figure 47. There is a significant difference in IgA producing B cell numbers at time 0 in mice immunised with PdB batch 1, compared to mice immunised with PdB batch 2. Although it is not significant, there is a trend of increased numbers of IgA

producing B cells at all other time points in mice immunised with PdB batch 1, when compared to PdB batch 2.

These results taken together may explain the difference in results gained from D39 colonisation experiments. These results showing lower titres of IgG, as well as lower numbers of antibody producing B cells present in the CLN of mice immunised with PdB batch 2, may explain why these mice were unable to reduce the numbers of pneumococci in the nasopharynx. Mice that were challenged earlier with serotype 3, 4 and 19 were also immunised with PdB batch 2. Therefore, these mice may have mounted an inadequate immune response which in turn was unable to decrease the pneumococcal numbers recovered from the nasopharynx. Hence, it remains to be seen whether immunisation with PdB is able to elicit cross serotype protection.

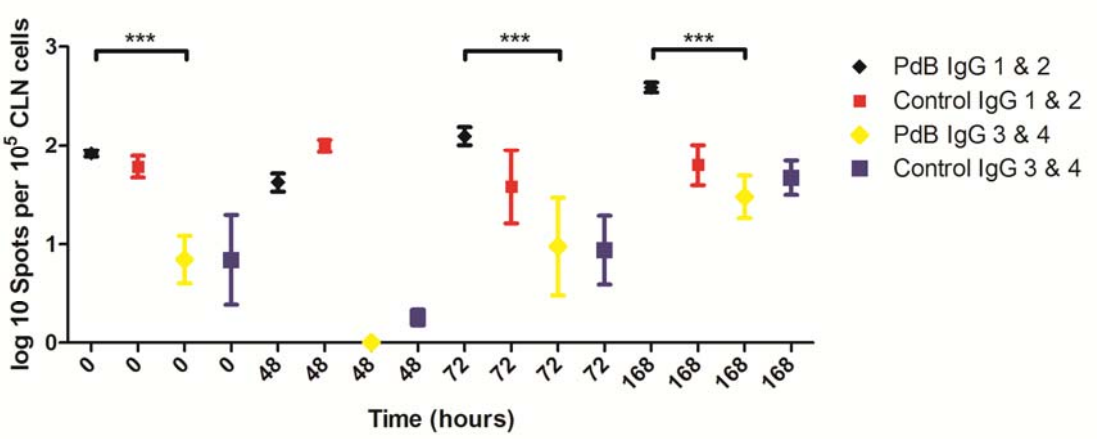


Figure 46- shows a comparison of the numbers of IgG producing B cells present in the CLN of PdB and control immunised mice (from mice immunised with different PdB batches). PdB IgG 1 & 2 were immunised with PdB batch 1. Control IgG 1 & 2 were immunised and challenged at the same time points. PdB IgG 3 & 4 were immunised with PdB batch 2. Control IgG 3 & 4 were immunised and challenged at the same time points. Error bars represent SEM. Five CLN samples were pooled together for an adequate number of cells to be present for the assay. n=8 per group, per time point.

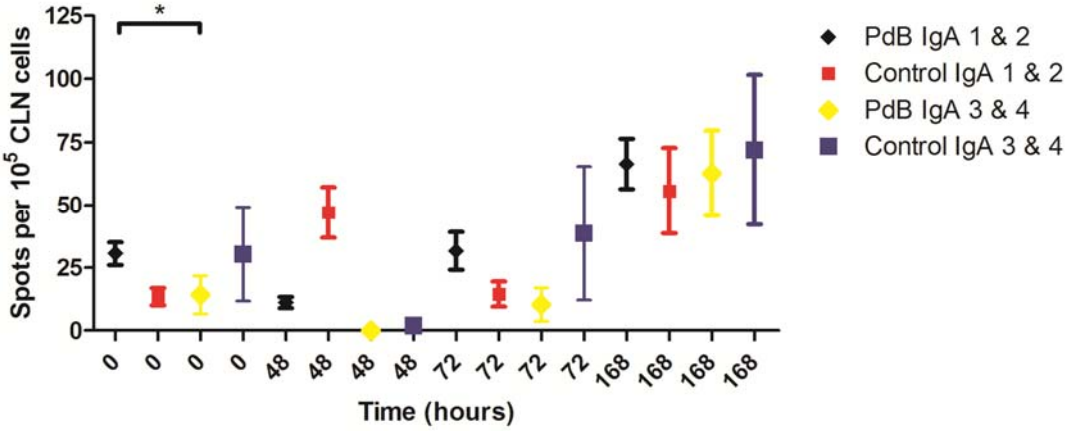


Figure 47- shows a comparison in the numbers of IgA producing B cells present in the CLN of PdB and control immunised mice (from mice immunised with different PdB batches). PdB IgA 1 & 2 were immunised with PdB batch 1. Control IgA 1 & 2 were immunised and challenged at the same time points. PdB IgA 3 & 4 were immunised with PdB batch 2. Control IgA 3 & 4 were immunised and challenged at the same time points.. Error bars represent SEM. Five CLN samples were pooled together for an adequate number of cells to be present for the assay. n=8 per group, per time point.

G. Neuraminidase A immunisations

1. Intraperitoneal immunisations

Twenty MF1 mice were immunised with purified Nan A protein and alum adjuvant three times, 7 days apart. A control group of twenty mice were also immunised with PBS, glycerol and alum adjuvant at the same time points. Mice were tail bled before each immunisation and blood was collected by cardiac puncture at the endpoint of the experiment. Sera collected were analysed for specific anti-Nan A IgG antibodies. Figure 48 shows the ELISA results from sera collected over the course of the experiment. The ELISA results show that mice immunised with Nan A had a significant increased titre of serum anti –Nan A IgG antibodies, when compared to the control immunised group. This difference in titre is seen from the second immunisation, where, a booster dose of the protein increases the serum antibody titre significantly.

To establish whether immunisation with Nan A gave mice protection against systemic infection induced by peritonitis animals were intraperitoneally challenged with virulent passaged D39 pneumococci. Ten mice which were intraperitoneally immunised with Nan A and ten control immunised mice were intraperitoneally challenged twenty one days after the first immunisation. Mice were anaesthetised (with 2.5 % v/v isoflurane in 1.6 – 1.8L of O_2/min) and exsanguinated by cervical dislocation when they reached ++ lethargic. The survival times of mice following challenge from both the Nan A and control immunised groups are shown in Figure 49. There was no significant difference in the mean survival time of mice immunised with Nan A (23.6 hours) and the control immunised mice (25.4 hours). Therefore, immunisation with Nan A gave mice no protection against peritonitis induced systemic infection with D39.

To establish whether immunisation with Nan A protected mice from an acute pneumococcal lung infection mice were intranasally challenged with virulent D39. Ten Nan A immunised mice and ten control immunised mice were challenged intranasally twenty one days after the first immunisation. The mean survival times of mice from the Nan A and control immunised groups are shown in Figure 49. The results show that although Nan A immunised mice had a slight increase in mean survival time (75.8 hours) when compared to the control immunised group (67.1 hours), this difference is not significant. Although there are specific anti Nan A IgG antibodies present in the sera of Nan A immunised mice, the antibodies are unable to protect mice from an acute pneumococcal lung infection.

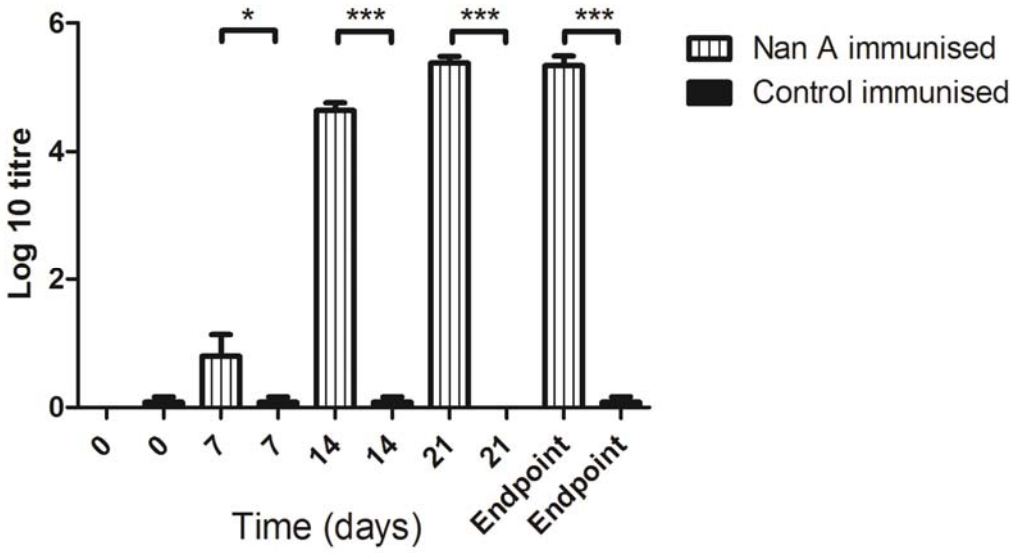


Figure 48 – shows the titres of specific anti – Nan A IgG antibodies present in the sera of Nan A and control immunised mice. These are the results from mice challenged intraperitoneally and intranasally. Each serum sample was assayed individually and n= 20 per time point, per group. Nan A immunised mice have significantly more anti - Nan A IgG $p>0.001$ at all time points apart from time 0. Error bars represent the SEM.

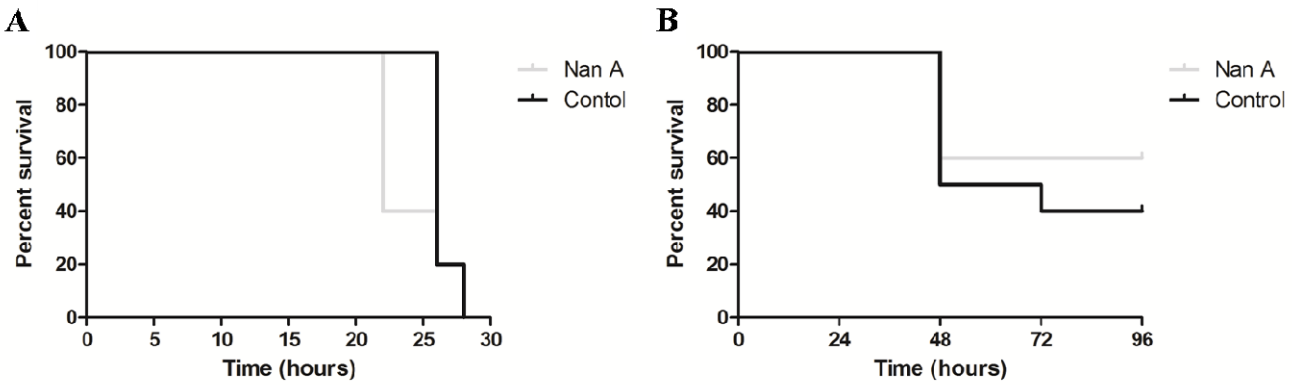


Figure 49- The survival time in hours of I.P. and I.N. challenged mice after three immunisations. A - Shows the survival times of mice challenged intraperitoneally with D39. B – Shows the survival times of mice intranasally challenged with D39. Each data point represents one mouse and n=10 mice per group. The mean survival time is shown with a horizontal line; the error bars represent the SEM**.**

2. Subcutaneous immunisations

Mice immunised with Nan A administered, by the intraperitoneal route, showed no protection from systemic infection or acute lung infection after challenge with virulent D39 pneumococci. For this reason another immunisation schedule was set up, with advice from the Netherlands Vaccine Institute (NVI), to immunise the mice subcutaneously. The time scale of the immunisation schedule was also altered to give 14 days between each immunisation, instead of 7 days. This was to ensure that adequate time was given for the mice to mount an immune response, following immunisation with Nan A, before challenge. The alum adjuvant was not included in the preparation as the protein was immunogenic alone.

Twenty MF1 mice were subcutaneously immunised with Nan A and twenty MF1 mice were immunised with the control. Mice were tail bled before each immunisation, and at the endpoint of the experiment blood was collected by cardiac puncture. The serum samples collected were then analysed for specific anti-Nan A antibodies. This was to ensure that the change in the route of protein administration

and the removal of the alum adjuvant did not alter the immune response to the protein, when compared to the intraperitoneal immunisation route. Figure 50 shows the anti-Nan A antibodies detected in the sera from Nan A and control immunised mice. A significant amount of specific anti Nan A IgG was detected at all time points apart from time 0. This showed removing the alum adjuvant and changing the route of protein administration did not change the significant amounts of anti Nan A antibody produced by the mice, when compared to intraperitoneal immunisation.

Fourteen days after the last immunisation mice were intranasally challenged with virulent D39 pneumococci. Mice were culled when they became ++ lethargic. Figure 51 shows the survival times of Nan A and control immunised mice. Although there is an increase in the survival time of Nan A immunised mice (61.2 hours), when compared to control immunised mice (56.9 hours), this difference is not significant. Figure 52 shows the numbers of pneumococci recovered from the lungs, blood and nasopharynx of both Nan A and control immunised mice. There was no decrease in the numbers of pneumococci recovered from the lungs ($p=0.2$) or blood ($p=0.7$) of the Nan A immunised mice, compared to the control immunised mice. Therefore immunisation with Nan A was unable to protect mice from acute challenge with virulent D39.

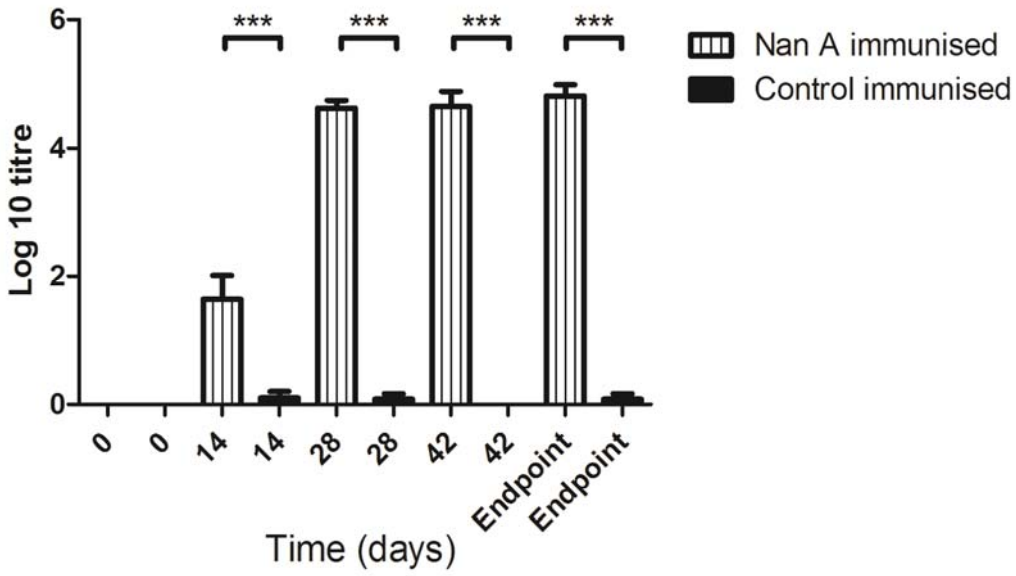


Figure 50- shows the titre of specific anti-Nan A antibodies detected in sera collected from mice over the time course of the experiment. Mice immunised with Nan A show significantly more anti-Nan A antibodies in the sera at all time points, compared to little or no anti-Nan A specific antibodies in control mouse sera. Error bars represent the SEM, n=19 per time point for Nan A and n=20 per time point for control, except from 42 days where n=10 for both groups.

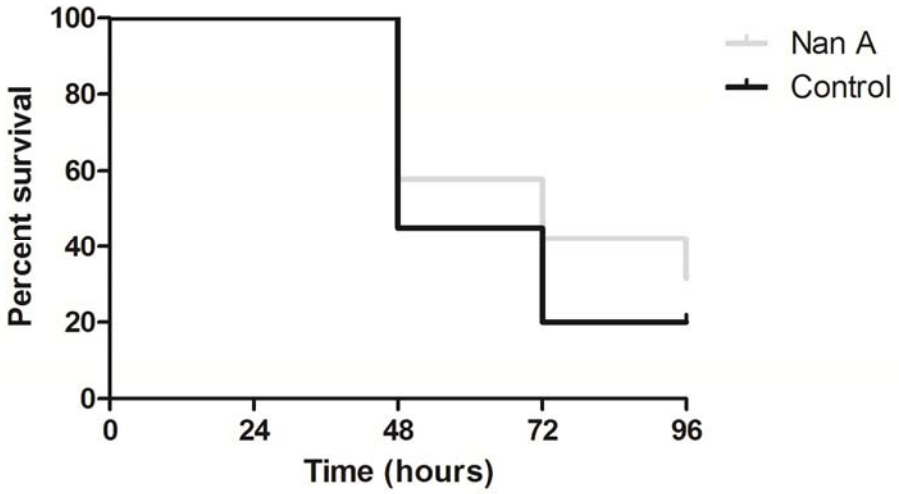


Figure 51 - Shows the survival time of mice immunised with Nan A, by the subcutaneous route, which were subsequently challenged with an acute dose of pneumococci. n=19 for the Nan A group and n=20 for the control immunised group. Each data point represents one mouse; the horizontal line shows the mean value and the error bars represent the SEM.

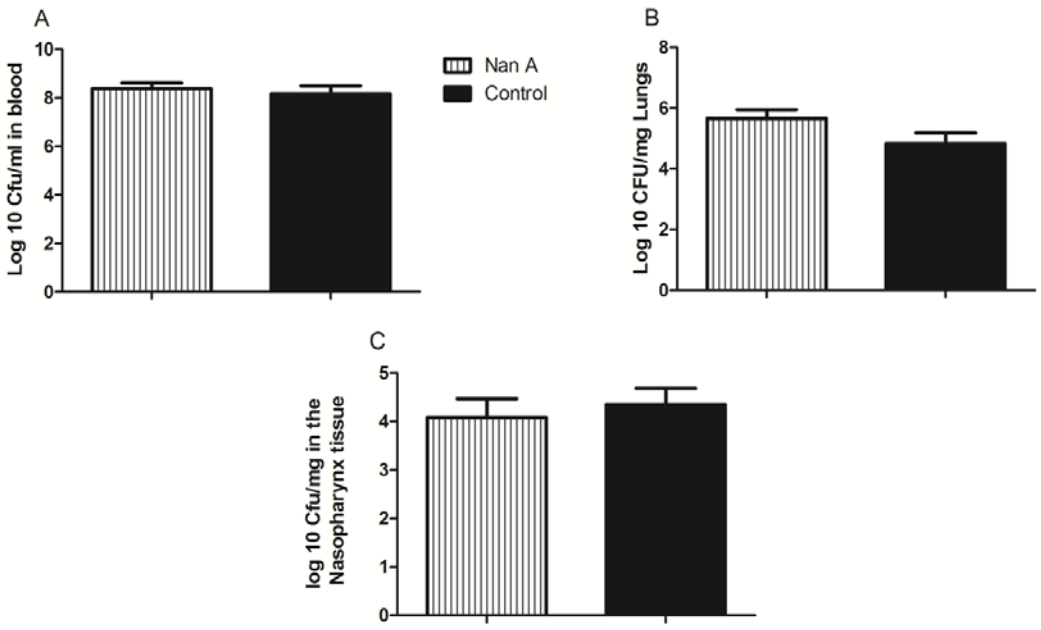


Figure 52- shows numbers of bacteria recovered from blood, lungs and the nasopharynx of Nan A and control immunised mice following an acute dose of D39. A- Shows the log 10 cfu/ml of bacteria recovered from blood, B- shows log 10 cfu/ml of bacteria recovered from lungs, C- shows log 10 cfu/ml of bacteria recovered from nasopharynx. n=10 per group. Error bars represent the SEM.

3. Summary of Nan A immunisations

Immunisation of MF1 mice with Nan A protein was unable to elicit protection from acute challenge or peritonitis induced systemic infection with D39 pneumococci. ELISA data showed that there were significant increases in specific anti IgG antibodies present in the sera of Nan A immunised mice, when compared to the control group. However, this increase in specific antibody was unable to protect the mice from subsequent systemic infection after intraperitoneal challenge. The antibodies are also unable to protect mice from lung infection following an acute dose of pneumococci. There is a small decrease in bacteria in the nasopharynx of Nan A immunised mice after acute intranasal challenge, however, this decrease is not significant. The decrease in pneumococci was unable to prevent colonisation and subsequent lung infection. Since there were no significant decreases in bacterial numbers in the lungs, blood or nasopharynx of Nan A immunised mice, the use of Nan A as an antigen was not continued in this work.

Nan A has been shown to be essential for colonisation of the nasopharynx. The effect that Nan A immunisation has upon nasopharyngeal colonisation was not tested in this work. Therefore, in the future it would be important to establish whether immunisation with Nan A, is able to decrease or prevent nasopharyngeal colonisation.

IV. Discussion

A. Immunisations with PdB

PLY has long since been considered a worthwhile vaccine candidate due to its sequence conservation and its presence in almost all pneumococcal serotypes tested. PLY is an essential virulence factor in colonisation, lung infection and sepsis. However, since PLY has hemolytic activity, as well as many other immuno-modulatory effects upon host cells, other pneumolysin toxoids have been constructed for use in immunisation studies. PdB is one of these proteins, and is a genetically detoxified derivative of pneumolysin. PdB has been used previously in other immunisation studies and was able to elicit protection against *Streptococcus pneumoniae* infection. Therefore PdB has been considered a vaccine candidate for further study.

During the course of this work, the protective effect of PdB has been tested in a variety of well established pneumococcal mouse models. The results presented here contribute to understanding the effect that PdB immunisation, has upon pneumococcal infections, and whether it is a worthwhile protein for future development.

1. Intraperitoneal immunisations with PdB did not protect mice from infection with a high intranasal dose of pneumococci

In the first of the PdB immunisation challenge experiments, mice were immunised intraperitoneally and challenged intranasally with a high dose of D39 pneumococci. The results showed that mice immunised with PdB had no significant difference in survival times, in comparison to control immunised mice. There was also no difference in the numbers of pneumococci recovered from the lungs of PdB

immunised mice compared to the control immunised mice. Therefore, PdB immunisation was unable to confer any protection against invasive pneumonia.

PdB was immunogenic and was able to elicit specific anti-PdB antibodies, determined by ELISA. Despite the high titre of specific antibodies present in the sera of PdB immunised mice, these antibodies were unable to protect mice from invasive pneumonia.

Since mice were not protected following three booster immunisations, mice were immunised once more with PdB before acute challenge. However, the additional immunisation was unable to protect mice against challenge from invasive pneumonia. ELISA results showed that the extra immunisation with PdB only slightly increased the amount of anti-PdB antibodies present in the sera of mice. Therefore, three immunisations with PdB were deemed sufficient to elicit a significant amount of antibody in further experiments.

The results presented here are in contrast to the work presented by Alexander et al., (1994). In the study by Alexander et al., (1994), 6-8 week old, female, MF1 mice were immunised with purified recombinant PdB protein. The PdB protein was purified from E.Coli containing a plasmid derived from serotype 2 pneumococci. Mice were subsequently challenged intranasally with D39 at a dose of $1x10^6$ CFU in 50µl. The authors showed that 72% of PdB immunised mice survived, in comparison to 6% of the control immunised following acute challenge.

However, Alexander et al., (1994), showed that the same antibody isotypes, as the work presented here, were present in the sera of mice immunised with PdB. Both IgG1 and IgG2a isotypes were elicited by immunisation of mice in the work presented here. ELISA results showed that specific IgG1 was present at the highest

titre in the sera of immunised mice. Specific IgG2a was also present, but at a lower titre, in the sera of immunised mice. The titres of antibodies were significantly higher than the control group, after two immunisations. These antibodies were also functional as they were able to block PLY activity *in vitro*. The titre of haemolytic inhibition was small but significant when compared to the control group.

Both IgG and IgG2a were present in the serum from mice immunised with PdB. IgG antibodies are a measure of the secondary response following immunisation. IgG2a has been shown to fix complement, on bacterial cell surfaces, at a higher level than any other antibody isotype (Oishi et al., 1992). The ability of IgG2a to fix complement subsequently aids in the opsonophagocytosis of pneumococci, therefore, it was important to test for levels of IgG2a following immunisation.

Alexander et al., (1994), showed that the antibodies elicited, following immunisation with PdB, were able to afford protection from 9 different serotypes of pneumococci. Pneumolysin has been shown to have low genetic variation (3.3%) in pneumococcal serotypes tested to date (Marriott et al., 2008). Therefore, immunisation was able to protect against infection with many different pneumococcal serotypes. PdB immunised mice were also protected from intraperitoneal challenge against nine different serotypes of pneumococci. However, these serotypes did not include serotype 2, D39.

Immunisation with PdB was also shown to be protective in an inbred mouse model (Ogunniyi et al., 2001). In the study by Ogunniyi et al. (2001), five to six week old, female Balb/c mice, were immunised with 10µg of PdB protein, three times, fourteen days apart. Fourteen days after the last immunisation mice were

challenged intraperitoneally with D39 at a dose of $1x10^5$ CFU. The results showed that immunisation with PdB protected mice from intraperitoneal challenge and increased survival time by 5 days compared to the control group (Ogunniyi et al., 2001).

In the work presented here intraperitoneal challenge was not performed as another model of systemic infection was used. This model was a well established bacteraemia model of infection induced by intravenous challenge with pneumococci.

2. Subcutaneous immunisations with PdB did protect mice from infection with a high intranasal dose of pneumococci

Intraperitoneal immunisations with PdB were unable to protect mice from invasive pneumonia. Therefore, the route of administration of PdB protein was altered to the subcutaneous route. The alum adjuvant was not included in the preparation, which allowed the investigation of the immunogenicity of PdB when used alone.

Following subcutaneous immunisation, mice were challenged intranasally with high dose of virulent D39. Following challenge, PdB immunised mice showed no difference in survival times, compared to the control immunised group. There was also no difference in the numbers of pneumococci recovered from the lungs, nasopharynges and blood of PdB immunised mice, compared to the control group. However, mice in both groups survived for longer than expected, as on average mice infected with a high dose of pneumococci to cause invasive pneumonia, succumb to infection within forty eight to seventy two hours (Canvin et al., 1995). This may have been due to the pneumococcal stock becoming less virulent.

This result is in contrast to the work performed by Briles et al., (2003), where mice subcutaneously immunised with PdB showed a significant decrease in pneumococci recovered from the lungs. Six to eight week old CBA (inbred mice) were immunised with 20μg of PdB protein, three times, fourteen days apart. Following this mice were challenged intranasally with capsular serotype 19 at a dose of $1x10^6$ CFU. With this dose of pneumococci mice survived at least until day five post infection, which in turn allowed the numbers of pneumococci present in the lungs to be counted. The results showed that mice immunised with PdB had a significant decrease in the numbers of pneumococci recovered from the lungs. In the model used in the work presented here, mice were given a terminal dose that caused the animals to succumb to infection within 48 to 72 hours therefore survival times were able to be determined, but lung colonisation was not.

3. Subcutaneous immunisations with PdB reduced nasopharyngeal carriage following challenge with a lower dose of pneumococci

PdB and control immunised mice showed no protection against a standard dose of D39 pneumococci. Therefore, the dose of challenge was lowered to a 10^6 CFU/ml instead of the usual 10^7 CFU/ml. Many immunisation studies have previously used lower doses of pneumococci for challenge.

Following challenge, PdB immunised mice showed no difference in survival times compared to the control immunised group. There was also no difference in the numbers of pneumococci recovered from the lungs of PdB immunised mice, in comparison to control immunised mice. This result differs from that of Briles et al., (2003), where immunisation with PdB was able to decrease the numbers of pneumococci recovered from the lungs. However, in the study by Briles et al.,

(2003), the mice used were, six to eight week old, inbred (CBA/N). The mice were challenged with serotype 19 at a dose of $1x10^6$ CFU. This difference may have been due to PLY gene variation between challenge serotypes. However, the difference in PLY's genetic variation has been shown to be low in different pneumococcal serotypes (Lock et al., 1992).

This difference in lung colonisation may be explained by differing amounts of PLY production in different serotypes of pneumococci. However, in a study by Benton et al. 1997, pneumococcal serotypes which produced less PLY, *in vitro*, were as virulent as pneumococcal serotypes producing higher amounts of PLY, *in vivo*. This may be explained by the many immmuno-modulatory effects of PLY at sublytic concentrations.

Although there were no significant differences between the numbers of pneumococci recovered from the lungs and blood of PdB immunised mice, there were significant differences in numbers of pneumococci recovered from the nasopharynges of PdB immunised mice. Immunisation with PdB significantly decreased the numbers of pneumococci recovered from the nasopharynges, compared to the control immunised group ($p<0.05$). Even though immunisation with PdB was able to significantly decrease the numbers of pneumococci recovered from the nasopharynges, it was unable to prevent colonisation. These results showed that subcutaneous immunisation with PdB may have elicited a site specific protective response. Therefore, the effect that subcuteanous immunisation with PdB had on pneumococcal colonisation of the nasopharynges was investigated.

4. Subcutaneous immunisation with PdB was able to reduce nasopharyngeal carriage in inbred Balb/c mice following challenge with a lower dose of pneumococci

The effect that immunisation with PdB had on colonisation was first tested using Balb/c mice. Balb/c mice are an inbred strain that are resistant to invasive pneumococcal infection and have been used in many pneumococcal immunisation studies. Balb/c mice have a Th2 phenotype which is driven by the production of IL-4 (Sacks and Noben-Trauth, 2002). IL-4 inhibits the Th1 type response. This phenotype prevents an inflammatory Th1 response. When seven week old, male, Balb/c mice were challenged with serotype 3 or serotype 14 pneumococci at a dose of 10^7 CFU, the results showed that no damage was caused to the lung tissues. However, there was still a large influx of lymphocytes to the site of infection (Mizrachi-Nebenzahl et al., 2003). Conversely, MF1 mice have a tendency towards a Th1 phenotype when challenged with pneumococci. This phenotype causes an inflammatory reaction which leads to the activation and influx of neutrophils to the lungs resulting in tissue damage (Kadioglu et al., 2000).

In this study mice were immunised subcutaneously and subsequently challenged intranasally with a colonisation dose (10^7 Cfu/ml administered in 10µl) of serotype 2, D39 pneumococci. Balb/c mice were then sacrificed at both 24 and 48 hours after first infection.

The results showed that mice immunised with PdB had a decrease in the number of pneumococci recovered from the nasopharynges, at both 24 and 48 hours after infection. ELISA results showed that these mice had significantly high titres of anti-PdB antibodies present in their sera, when compared to the control immunised group. These antibodies increased significantly after the second immunisation with

PdB. The antibodies elicited were able to neutralise recombinant PLY *in vitro*. Although the titre of PLY inhibition was low, the neutralising effect of the antibodies elicited by PdB immunisation could be detected.

Similarly, another immunisation study using PdT as the antigen, showed protection against nasopharyngeal colonisation (Basset et al., 2007). Mice were immunised with PdT, in combination with two more pneumococcal proteins PspA and PspC. Inbred C57BL/6J mice, which are susceptible to pneumococcal infection, were immunised intranasally with the purified proteins. Mice were then challenged with a colonisation dose of serotype 6B. Mice immunised with all three proteins had a significant decrease in pneumococci recovered from the nasopharynges (Basset et al., 2007). The study by Basset et al., (2007) also showed that protection from colonisation was CD4+ T cell dependent. This was shown by administration of an anti CD4+ T cell antibody before challenge, to both PdB immunised and control mice. The results showed that mice with depleted CD4+ T cells lost the protection that was seen previously, since similar amounts of pneumococci were recovered from the nasopharynges, as in the control immunised group.

The work by Basset et al., (2007) is in agreement with the work presented here. A decrease in the numbers of pneumococci recovered from the nasopharynges was seen in the PdB immunised mice up to 48 hours after infection. Therefore, the effect of PdB immunisation on colonisation of the nasopharynges was investigated here for up to fourteen days following colonisation.

5. Immunisations with PdB decreased the numbers of pneumococci in the nasopharynges of MF1 mice following challenge with a colonising dose of D39

To investigate the effect of PdB immunisation upon nasopharyngeal colonisation for a longer period of time, outbred MF1 mice were used. This was done as the colonisation/carriage model in MF1 mice was previously established (Richards et al., 2010). MF1 mice were subcutaneously immunised with PdB and subsequently challenged intranasally with a colonisation dose of D39 pneumococci. A colonisation dose of pneumococci is a high dose (1 x 10^7 Cfu/ml) in 10µl volume, allowing the pneumococci to stay in the upper respiratory tract. Mice were sacrificed at 0, 24, 48, 72 and 168 hours after colonisation and tissue samples were recovered for pneumococcal colony counts.

Colonisation of the nasopharynges was achieved, as the results showed that no pneumococci were present, in the lungs or blood of mice at any time after challenge. Mice immunised with PdB showed a significant drop in the numbers of pneumococci, recovered from the nasopharynges at both 72 and 168 hours after challenge. Although not significant, there was also a decrease in the numbers of pneumococci recovered from the nasopharynges of PdB immunised mice, at both 24 and 48 hours compared to the control group.

ELISA results showed that PdB immunised mice had a significant increase in specific anti-PdB antibodies present in their sera. A significant increase in both IgG1 and IgG2a isotypes was found, in the sera of PdB immunised mice. Therefore, a decrease in the number of pneumococci recovered from the nasopharynges of immunised mice would appear to involve antibodies.

The antibodies were also able to neutralise recombinant PLY. Hemolysis inhibition assays showed that when the antibodies were incubated with PLY and red blood cells, they were able to inhibit the pore forming action of PLY. Although the titre of inhibition was low, there was a significant difference in hemolysis inhibition, when compared to the control immunised group. Therefore, the antibodies produced by the mice in response to PdB immunisation were functional.

In the work presented here immunisations with PdB protein failed to protect mice against invasive pneumonia, but were able to protect against colonisation. Therefore, it would appear that a site specific response was elicited by subcutaneous immunisation with PdB. The basis of this immune response was investigated further.

The presence of B cells were analysed by ELISPOT analysis. Cervical lymph nodes collected from mice, after challenge, were used to identify if immunisation with PdB elicited any increase in antibody producing B cells. Results of the ELISPOT analysis showed that there was a significant increase, in the number of IgG antibody producing B cells at 168 hours, in PdB immunised mice compared to the control. Although not significant, there was also an increase in the number of IgA producing B cells present in PdB immunised mice, at both 72 and 168 hours after infection. These increases in B cells correlated with a drop in the pneumococcal numbers recovered from the nasopharynges.

It was important to measure both levels of IgG and IgA producing B cells, as the antibodies made by these cells have differing functions. IgA, more specifically secretory IgA, is a dimeric antibody produced to work at the mucosal surface (Quan et al., 1997). IgA typically has lower affinity epitopes than IgG

antibodies with the same specificity (Fukuyama et al., 2010). The IgA antibodies produced by B cells present in the CLN's of mice may not be secretory antibodies. However, nasal washes performed following immunisation and challenge (with a colonising dose of D39), showed that there were secretory IgA antibodies present, at both seven and fourteen days, following first infection.

Secretory IgA has been shown to decrease the numbers of pneumococci present in the nasopharyges following immunisation with another pneumococcal protein; PspA (Fukuyama et al., 2010). Six to eight week old C57BL/6 and C57BL/6 IgA knock out mice, were immunised intranasally with 1µg of PspA and adjuvant, four times, seven days apart. The results showed that mice without the ability to produce functional IgA were unable to reduce of pneumococci from the nasopharynges, following colonisation. Whereas, the wild type mice, with functional secretory IgA ability, were able to decrease the numbers of pneumococci recovered from the nasopharynges (Fukuyama et al., 2010).

IgG antibodies produced by B cells present in the CLN's of immunised mice have the function of protecting mice from systemic infection. Anti-pneumolysin IgG has been shown to protect mice from both pneumonia and intraperitoneal infections (Alexander et al., 1994).

Immunisation with PdB elicited a high titre of specific antibodies and was able to decrease the number of pneumococci present in the nasopharynges. The specific anti-PdB antibodies were able to neutralise the pore forming action of PLY *in vitro*. Since pneumolysin is essential for colonisation of the nasopharynges, inhibition of pneumolysin by neutralising antibodies may prevent a variety of its immuno-modulatory actions. Specific PdB antibodies may bind to PLY released

from invading pneumococci, thus preventing the halting of the cilia. This is a disadvantage to colonising pneumococci, as movement of the epithelium may make it difficult for the bacteria to mediate adhesion and attachment. The specific antibodies may also help prevent the separation of epithelial cell tight junctions, which in turn inhibits the invasion of epithelial cell layers. Specific antibodies against pneumolysin may also prevent inhibition of neutrophil respiratory burst. This may be an important factor if immunity is mediated by CD4+ T cells, as this response is reliant upon the presence of neutrophils.

However, other studies have shown that when PdB was administered subcutaneously it was unable to protect mice from nasopharyngeal carriage (Briles et al., 2003), though, the model used by Briles et al., (2003), differs to the work presented here, as the numbers of pneumococci were enumerated after an acute infection intended to cause invasive pneumonia. The authors found that although a combination of PspA and PdB were able to protect mice from lung carriage, the proteins alone or in combination were unable to prevent carriage in the nasopharynges of these mice.

6. Immunisation with PdB did not reduce the numbers of pneumococci recovered from the nasopharynges of mice colonised with serotypes 3, 4 or 19

The results presented here showed that PdB immunisation was able to decrease nasopharyngeal carriage following a colonisation challenge with D39 (serotype 2). Subsequently, the effect that PdB immunisation had upon colonisation of the nasopharynges with other pneumococcal serotypes was examined. Mice were immunised with PdB as before and then challenged with a colonisation dose of either virulent passaged A66, 19F or TIGR4 pneumococci.

Following colonisation with A66 (serotype 3), mice immunised with PdB had no decrease in the numbers of pneumococci recovered from the nasopharyngeal tissue. However, at time 0, 48 and 168 there was a significant difference in the numbers of IgG antibody producing B cells present in the cervical lymph nodes, in PdB immunised mice. There was also a significant difference in the number of IgA producing B cells present in the cervical lymph nodes of PdB immunised mice. However, increased numbers of IgA and IgG producing B cells did not correlate with a drop in pneumococcal numbers recovered from the nasopharynges.

Previous studies have shown that A66 produces 5 times less cytoplasmic pneumolysin than D39. However, A66 produces 8 times more extracellular pneumolysin than D39 (Benton et al., 1997). Differing amounts of pneumolysin production has been shown to be unimportant in a pneumonia murine model of infection. The growth characteristics of other pneumococcal serotypes producing less pneumolysin have the same inflammatory response by the host. However, pneumolysin production may be important in a colonisation model of disease. Low levels of pneumolysin production could have prevented anti-pneumolysin antibody action. This could explain why there were no decreases in the numbers of A66 pneumococci recovered, from the nasopharynges of PdB immunised mice.

There is controversy over the role pneumolysin plays during colonisation. Some studies suggest that pneumolysin plays no role in colonisation of the upper respiratory tract. One study, with a serotype 14 strain deficient pneumolysin, showed the same ability to colonise the nasopharynges as the wildtype (Rubins et al., 1998). Serotype 3, A66 pneumolysin deficient pneumococci have been shown to colonise the nasopharynges as well as the pneumolysin expressing wildtype strain (Kadioglu et al., 2002). However, serotype 2 D39 pneumococci, deficient in

pneumolysin (PLN-A) were unable to colonise the nasopharynges. These results suggest that the role of pneumolysin in colonisation may also depend on capsular polysaccharide type. Pneumolysin may therefore be important for colonisation in some pneumococcal serotypes but not others. However, this work must be explored further with more pneumococcal serotypes, before a reliable conclusion can be drawn.

A66 pneumococci deficient in pneumolysin were able to colonise the nasopharynges as well as the wildtype pneumococci expressing pneumolysin. Therefore pneumolysin is not necessary for serotype 3 pneumococci to colonise the nasopharynges, and PdB specific antibodies were unable to opsonise pneumococci leading to no reduction in nasopharyngeal carriage of A66.

Following immunisation with PdB, mice were colonised with serotype 4 pneumococci (TIGR4). There was no decrease in the numbers of pneumococci recovered from the nasopharynges of immunised mice. The numbers of pneumococci recovered were similar at all four time points, in both groups of immunised mice, apart from at 168 hours. Although not significant, there was a decrease in the number of pneumococci recovered from the nasopharynges of PdB immunised mice. ELISA results show that there was anti-PdB, secretory IgA antibodies present in the nasal washes of immunised mice. However, the titre of antibodies was low and may have been too low to prevent colonisation. Antibody producing cells were measured from the cervical lymph nodes of both groups of mice. Unlike the significant differences seen in the CLN of mice challenged with D39 pneumococci, there were no significant differences in the numbers of IgG and IgA producing B cells seen at any time point.

Pneumolysin production levels in TiGR4 pneumococci may explain why immunisation with PdB was unable to decrease colonisation in the nasopharynges. Serotype 4 pneumococci have been shown to produce one and a half times less pneumolysin than D39 (Benton et al., 1997). Lower levels of pneumolysin may have inhibited anti-PdB antibodies from binding to the surface of pneumococci, thus, preventing opsonophagocytosis. This may explain why no decrease in the numbers of pneumococci recovered from the nasopharynges was seen following TiGR4 colonisation.

Mice immunised with PdB were subsequently intranasally challenged with serotype 19 pneumococci. The results showed that there was no decrease in pneumococcal numbers recovered from the nasopharynges of PdB immunised mice, in comparison to the control group. Although it was not a significant difference, there was a decrease in pneumococcal numbers at 168 and 336 hours after challenge, in the PdB immunised group, compared to the control immunised group. Unexpectedly, at the endpoint of this experiment, the numbers of pneumococci recovered from the nasopharynges increased in both the PdB immunised group and the control group. This was not seen in the preliminary experiment, where naive mice were colonised with 19F. The numbers of pneumococci recovered from the nasopharynges at 336 hours after infection were decreasing.

The levels of pneumolysin production have not yet been investigated in the 19F strain of pneumococci. Thus far, it is unclear whether the levels of pneumolysin produced by this strain are similar to serotype 2. It may be that the levels of pneumolysin are low in colonisation and this may explain why anti-PdB antibodies were unable to reduce nasopharyngeal carriage of 19F.

Anti-PdB antibodies present in the immunised mice were unable to decrease numbers of A66, 19F or TiGR4 pneumococci in the nasopharynges, as was seen earlier, with D39 challenge. However, in these colonisation experiments a different batch of PdB protein was used to immunise mice, compared to an earlier batch where protection was seen. Results showed that there was a difference in the immune responses elicited by the two different batches of PdB. This may explain why the numbers of A66 were unable to be decreased in the nasopharynges by immunisation with PdB. The difference in the responses elicited by both PdB batches is discussed later in this chapter.

7. PdB batch comparison

Immunisation with the first batch of PdB significantly reduced nasopharyngeal colonisation, in both Balb/c inbred and MF1 outbred mice. The antibody titres induced by immunisation were significantly higher, compared to the control immunised group in both strains of mice. These antibodies were functional with respect to neutralising the activity recombinant pneumolysin *in vitro*.

Since no protection was seen following colonisation with serotypes 3, 4 and 19F, D39 was used once more to investigate if PdB was able to reduce nasopharyngeal colonisation. Two different batches of PdB protein were used for immunisation experiments. These batches were purified at different times, but with the same process. Therefore, the immune responses elicited by each batch of protein used for immunisations were compared. The results showed that immunisations with the second batch of PdB were unable to decrease the numbers of pneumococci recovered from the nasopharynges, compared to control

immunised mice. Unlike the protection seen following immunisation with the first batch of PdB, there was no significant difference in pneumococcal numbers, recovered from the nasopharynges, at any time point following challenge.

Furthermore, antibody production in mice immunised with the second batch of pneumolysin, was significantly decreased in both titre amount and neutralising activity, in comparison to the first batch of protein used for immunisation. The titre of antibody is important in mounting an effective immune response against pneumococci. As well as a decrease in the antibody titre produced, there was also a difference in the numbers of IgG and IgA producing B cells, present in the CLN of mice immunised with the second batch of PdB. There was only one significant difference in the numbers of IgG producing B cells, in mice immunised with the second batch of PdB, at 24 hours following challenge. However, this response was unable to decrease the numbers of pneumococci present in the nasopharynges.

Therefore, the immune response elicited by the second batch of PdB may have been the reason why no protection was seen following challenge with D39, A66, TIGR4 and 19F. This result shows how important it is for the protein antigen to have stringent purification processes, to ensure they are similar enough to elicit the same immune response.

In a study by Roche and Weiser, 2010, six week old, female, mice were colonised with one of three pneumococcal serotypes (4, 6A and 23F). Colonisation was used as an immunising event and so mice were colonised once more with the same pneumococcal strains. The sera from the immunised mice were then analysed for any cross serotype antibodies to three pneumococcal antigens; PspA, PsaA and PpmA. The results showed that mice immunised with the serotypes 4 and 6A had

cross serotype antibodies specific for these proteins. However mice immunised with 23F only had antibodies specific for one of the proteins PpmA. Although colonisation was able to induce some cross serotype protection the antibodies were unable to cross react with all pneumococcal serotypes.

8. Immunisations with PdB were not protective against intravenous challenge

Pneumolysin has been shown to play a role in bacteraemia and is also essential for the survival of pneumococci in the blood. Therefore, PdB immunised mice were challenged intravenously, to investigate whether antibodies elicited by immunisation were able to protect them from sepsis. Three groups of mice were challenged intravenously. Firstly naïve mice which had no prior treatment, secondly PdB immunised mice which had been immunised as described previously, thirdly the control mice which were immunised with PBS at the same time points as the PdB group.

The results showed that at 48 hours after infection, more PdB immunised mice were alive, compared to both the control immunised and naïve mice. However, by the end point of the experiment PdB immunised mice had a survival rate of 40%, which was the same as the survival rate for the naïve mice. Therefore, immunisation with PdB was unable to prevent death from sepsis.

Pneumococci were recovered from the lungs, blood and nasopharynges of mice at the endpoint of the experiment. The numbers of pneumococci recovered from the lungs and blood were similar for all mice in all three groups. This is an unexpected result, as more mice in the PdB group were alive at 48 hours compared to the naïve and control immunised groups. The PdB immunised mice were

expected to have a decreased bacterial load. One explanation for this is that these samples were all taken at the endpoint of the experiment, where the bacterial load in the blood was high enough to cause mice to succumb to infection. Therefore, it is difficult to conclude that the anti-PdB antibodies present in the serum of these mice were the reason that more mice were alive at 48 hours post infection.

Although it was not a significant result, there was a decrease in the numbers of pneumococci recovered from the nasopharynges of PdB immunised mice (mean of log10 3.76 CFU/ml), in comparison to the control immunised group (mean of log10 4.1 CFU/ml). This result showed that there was some type of site specific protection, in the nasopharynges of PdB immunised mice.

This result is in concurrence with work performed by Briles et al., (2003). In the study by Briles et al., (2003), six to eight week old, female, CBA/N mice were subcutaneously immunised with 20μg of PdB three times, fourteen days apart. Fouteen days after the last immunisation mice were challenged intravenously with D39 had a median survival time of 72 hours. However, PdB immunised mice survived for a median time of 8 days following intravenous challenge with serotype 6B. Therefore, PdB immunisation was unable to prevent sepsis and death of mice challenged with D39, but was able to increase survival following challenge with serotype 6B. In the same study PspA was also used as an antigen to immunise mice. The results showed that, immunisations with PspA were able to prevent sepsis of mice following intravenous challenge (Briles et al., 2000). Therefore, PdB was not as effective as PspA at preventing pneumococcal sepsis.

Although, there have been no other studies that determine whether immunisation with PdB, or other pneumolysin toxoids, protect mice from

intravenous challenge, experiments involving anti-pneumolysin antibodies from humans have been performed. Musher et al., (2001) showed that when mice were passively immunised with anti pneumolysin antibodies purified from human serum, protection was seen following intravenous challenge with serotype 4 pneumococci. However, the authors suggest that these antibodies are only able to prevent invasive disease for as long as it takes for the host to produce capsule specific antibodies. Therefore, Musher et al., (2001) proposed that for protection against pneumococcal sepsis it is important to have both protein specific and capsular specific antibodies. At the time of this publication pneumolysin was thought only to be released upon autolysis. New work has now suggested that pneumolysin may be present on the pneumococcal cell surface, even though it has no signal peptide (Price and Camilli, 2009). Therefore, PLY specific antibodies may bind to the outside of the pneumococcal cell. The protective response seen by Musher et al., (2001), could have been due to opsonophagocytosis of pneumococci due to PLY specific antibodies.

In the work presented here it is unclear why anti PdB antibodies were unable to protect mice from invasive disease. The antibodies elicited by immunisation were both of a high titre and functional *in vitro*.

9. PdB immunisation gave no significant additive protection than colonisation alone from intravenous challenge with D39

Colonisation of the nasopharynges of mice has previously been shown to elicit protection against subsequent colonisation (Richards et al., 2010). Immunisation with PdB protein was unable to prevent colonisation of the nasopharynges. However, immunisation with PdB was able to decrease the

numbers of pneumococci recovered from the nasopharynges. Therefore, the effect of both immunisation with PdB and colonisation had on intravenous challenge was investigated. In these experiments there were five differently treated mice that were challenged intravenously or intranasally: PdB immunised mice, control immunised mice, PdB immunised and colonised mice, control immunised and colonised mice as well as naïve mice. Mice in the immunisation and colonisation groups were first immunised twice, fourteen days apart, and then they were colonised with D39, for 21 days. Following this, mice from all treatment groups were challenged with an intravenous or an acute dose of D39 pneumococci.

Following intravenous challenge, PdB immunised and colonised mice had an increased survival rate of 80% compared to 40% in the naïve group. Control immunised and colonised mice had a survival rate of 60%. The numbers of pneumococci recovered from the lungs and blood were significantly lower in mice that were colonised. Although not significant, there was a decrease in the numbers of pneumococci recovered from the blood and nasopharynges of PdB immunised mice that were colonised, compared to the control immunised and colonised group. Therefore, immunisation with PdB followed by colonisation was able to provide additional protection to mice following challenge. Although not significant, there were lower amounts of pneumococci recovered from the lungs of PdB immunised and colonised mice, in comparison to the control immunised and colonised group.

10. PdB immunisation and colonisation reduced the numbers of pneumococci recovered from the nasopharynges following challenge with a high intranasal dose of D39

Following a high dose intranasal challenge, mice that were both immunised with PdB and colonised had a 70% survival rate, compared to 20% in the naïve

group. However, the control immunised and colonised group of mice had a survival rate of 90%. This result showed that colonisation was the most important factor for protection against a high dose intranasal challenge. The numbers of pneumococci recovered from the lungs, blood and nasopharynges of colonised mice were significantly lower, in comparison to mice from both the naïve and immunised alone. There was a significant difference in the numbers of pneumococci recovered from the nasopharynges of mice both immunised with PdB and colonised, compared to the naïve mice. A decrease in the number of pneumococci recovered from the nasopharynges could be a site specific reaction elicited by immunisation by the subcutaneous route. This shows that immunisation with PdB was able confer additive protection to mice that were colonised.

These results both show that colonisation with D39 elicited a better protective response compared to PdB immunisation alone. Recent work by Richards et al., (2010) showed that colonisation, with PLN-A pneumococci, was able to induce protection against subsequent invasive pneumonia with D39 pneumococci. The work by Richards et al., (2010), is in concurrence with the work presented here, where colonisation was able to decrease nasopharyngeal carriage of mice following challenge with a high intranasal dose of D39. The work presented here also shows that colonisation with pneumococci, is able to significantly protect mice from bacteraemia following intravenous challenge with D39.

This result is also in agreement with the work presented by Roche et al., (2007), who showed that colonisation can elicit protection from subsequent invasive pneumonia. In the study by Roche et al., (2007), six week old, female, C57BL/6J mice (which are inbred) were inoculated with a colonisation dose of live attenuated pneumococci, twice, fourteen days apart. Four weeks after colonisation,

mice were challenged with strain 6A at a dose of $1x10^7$ CFU/ml. The results showed that colonisation with live pneumococci were able to protect mice from pneumonia and sepsis. Roche et al., (2007), also showed that colonisation was able to protect mice from infection with a variety of pneumococcal serotypes, following invasive pneumonia.

The studies discussed above by Richards et al., and Roche et al., as well as the work presented here, show that pre-colonisation with pneumococci is able to protect mice from subsequent pneumococcal challenges. The development of immunisation with whole cell pneumococci may be the best strategy to prevent pneumococcal colonisation and infection. However, this strategy needs to be examined for its ability to provide protection from all pneumococcal serotypes.

B. The role of PdB in future pneumococcal vaccine development

Immunisation with PdB has previously been shown to protect mice against acute intranasal, intraperitoneal and intravenous pneumococcal infections. The work presented here has shown that PdB is able to decrease nasopharyngeal colonisation. However, immunisation with PdB is unable to prevent or clear carriage.

Furthermore, there is conflicting data in relation to whether immunisation with PdB or PLY is able to stimulate mucosal immunity in humans. One study showed that PdB was not efficient at stimulating mucosal immunity in humans (Zhang et al., 2002). *In vitro* data using human adenoidal B cells showed that PdB was only able to stimulate the release of secretory IgA, in two out of eight patients. Whereas, Simell at al., (2001), showed that anti-PLY antibodies particularly secretory IgA were present in saliva of children following acute otitis media

infections. These factors are important when considering new vaccine targets. Any future vaccine developed against *Streptococcus pneumoniae* should protect against colonisation by inducing mucosal immunity, as well as invasive pneumococcal disease.

PdB may not be a protective protein alone, however, in combination with other pneumococcal surface proteins, there have been a number of encouraging *in vivo* experiments showing non pneumococcal serotype specific protection (Ogunniyi et al., 2007a, Ogunniyi et al., 2000). Therefore, PdB or other PLY derivatives may be best utilized in combination with other pneumococcal proteins and developed further in the future.

C. Immunisations with NanA

Neuraminidase A has been identified as an important virulence factor of *Streptococcus pneumoniae*. NanA plays a major role both colonisation of the upper and lower respiratory tracts and is essential for the virulence of the pneumococcus (Yesilkaya et al., 2006). NanA is present on the pneumococcal cell surface therefore immunisation with the recombinant protein was hypothesised to be protective.

1. NanA immunisation did not protect mice from intraperitoneal challenge and sepsis

The work presented here shows that mice immunised with recombinant purified NanA were not protected from intraperitoneal challenge and sepsis. Following intraperitoneal immunisation with NanA, mice were subsequently challenged intraperitoneally with D39. The results showed that NanA immunised

mice had no significant increase in survival time (mean survival of 23.6 hours), in comparison to the control immunised group (mean survival of 25.4 hours), following intraperitoneal challenge with virulent D39 pneumococci.

The numbers of pneumococci present in the lungs, blood and nasopharynges, of mice from both immunised groups, were compared, to determine whether immunisation had any effect in the tissues of mice. The results showed that there was no difference in pneumococcal numbers recovered from the blood and lungs of NanA immunised mice compared to the control immunised group following intraperitoneal challenge.

2. NanA immunisation did not protect mice from challenge with a high intranasal dose of D39

Immunisations with NanA were unable to protect mice from peritonitis and sepsis. Therefore, mice intraperitoneally immunised with NanA were subsequently challenged with a high intranasal dose of D39, to examine whether immunisation was able to protect against invasive pneumonia.

The results showed that there was no significant difference in the survival times of NanA immunised mice (mean survival time of 75.8 hours); in comparison to control immunised mice (mean survival time of 67.1 hours). Although, ELISA results showed that NanA immunised mice had a significant increase in specific anti-NanA antibodies present in the sera. However, these antibodies were unable to protect mice from invasive pneumonia.

In view of the fact that there was no protection from intraperitoneal immunisation with NanA mice were subsequently immunised by the subcutaneous route. The adjuvant was not included in the preparation in order to investigate

whether NanA was immunogenic alone. Following challenge with a high intranasal dose of D39, there was no significant difference in the survival times of mice immunised with NanA (mean survival time 61.2 hours), compared to the control group (mean survival time of 56.9). The numbers of pneumococci recovered from the lungs, blood and nasopharynges of both groups of immunised mice were compared. The results showed there were no significant differences between the numbers of pneumococci recovered from NanA or control immunised mice, in any of the tissue samples. Therefore, subcutaneous immunisation with NanA was unable to protect mice from infection with invasive pneumonia.

Both intraperitoneal and subcutaneous immunisations with NanA did elicit a significant increase in specific anti-NanA antibodies in the sera of immunised mice, in comparison to the control group of mice. This significant increase in anti-NanA antibodies was seen after the second immunisation. Although, these antibodies were specific against NanA, they were unable to protect mice from intranasal challenge or intraperitoneal challenge with virulent D39. It is unclear why such high titres of specific anti-NanA antibodies were unable to protect mice from pneumococcal challenge. However, one explanation could be that the antibodies produced from immunisation with NanA were not functional, and were therefore unable to neutralise whole pneumococci. The neutralising activities of anti-NanA antibodies were not tested in the work presented here. Therefore, if anti-NanA antibodies were not functional this could have prevented classical complement activation and inhibited opsonophagocytosis of pneumococci following challenge.

NanA has also been shown to have high sequence diversity (King et al., 2005). Antibodies elicited against one variant of NanA, such as the recombinant protein, may not be cross protective against the NanA expressed by D39, which was

used for challenge in this study. The NanA protein gene used in this study was isolated and amplified from an R6 pneumococcal serotype. Therefore, it is possible that neuraminidase antibodies from the R6 serotype may not be similar in structure to NanA from D39.

Contrary to the work presented here, previous studies which used mutated NanA for immunisation, showed that mice were protected from invasive pneumonia (Yesilkaya et al., 2006). In the study by Yesilkaya et al., (2006), intraperitoneal immunisation with NanA and inactivated mutants of NanA, significantly increased the survival times of mice, compared to the control immunised group following challenge. In the study by Yesilkaya et al. (2006), mutant neuraminidases with no detectable catalytic activity were able to protect mice from challenge, survival time in these groups were higher than active recombinant NanA. However, it is unclear whether immunisation with NanA is cross protective, as no other pneumococcal challenge strains were used in these experiments.

The work presented here showed that immunisation with NanA was unable to elicit protection from invasive pneumonia, in the same outbred MF1 mice. However, one difference between this work and the work presented by Yesilkaya et al., (2006), is that the virulent challenge dose of D39 was one log lower than that used in this study to challenge mice. Yesilkaya et al., (2006) used a challenge dose of $1x10^5$ CFU whereas; in this study a dose of $1x10^6$ CFU was used. This could explain the difference in survival rate of mice as the lower dose may be cleared by the antibodies elicited by immunisation with NanA. Furthermore, the NanA gene used for recombinant protein and mutant expression in the work by Yesilkaya et al., (2006), was amplified from serotype 2 D39 pneumococci. Therefore, NanA

sequence diversity did not factor in to these experiments, as mice were challenged with the same D39 strain that the NanA gene was isolated from.

NanA immunisation has been previously shown to protect chinchillas against acute otitis media infections (Tong et al., 2005). Tong et al., (2005) also showed that significant protection from nasopharyngeal colonisation could be seen in this model of infection. Similarly to Yesilkaya et al, (2006), the work by Tong et al., (2005), used the same NanA gene to amplify and purify the protein that was present in the challenge strain of pneumococci. Tong et al., (2005), suggested that since high specific anti-NanA antibody titres were present in the animals, protection was mediated by these antibodies.

However, recent work suggests that clearance of colonisation in the nasopharynges is mediated by IL-17A producing CD4+ T cells (Lu et al., 2008). IL-17A producing CD4+ T cells have been shown to be induced by immunisation with pneumococcal proteins (Basset et al., 2007). In the study by Basset et al., (2007), no other type of immune response was measured; therefore, it is unclear which immune factor mediated immunity against colonisation in this model of infection.

In contrast, Lock et al., (1988), saw no protection from immunisation with native NanA. In the study by Lock et al., (1988), a group of outbred mice were immunised with either native active NanA or formaldehyde inactivated NanA. Mice were subsequently challenged, intranasally, with virulent D39 pneumococci. The purified active native form of NanA was not immunogenic and did not elicit any protective antibodies, therefore, an inactivated native NanA was used as the antigen. Immunisation with inactivated NanA was able to produce specific

antibodies with functional activity, eleven times higher than that of the control group. Although specific anti-NanA antibodies were elicited in these mice, there was no significant increase in the survival times of mice immunised with active or inactive NanA. The challenge dose used in the study by Lock et al., (1988), was equivalent to the standard dose used to induce invasive pneumonia, in the work presented here. However, Lock et al., used native NanA purified from serotype 1 pneumococci for immunisation, but the challenge strain used was capsular serotype 2. Therefore, if the sequence variability of NanA is different in these two strains, the high antibody titre elicited may not have been cross protective, and was unable to induce protection from pneumococcal infection.

The results demonstrated by Lock et al., (1988), are in accordance with the results presented here. Although there were high titres of specific antibodies against NanA, no protection was seen in the survival times of NanA immunised mice. However, Lock et al., (1988) suggested that native active NanA was unable to induce specific antibodies. In the work presented here, NanA used for immunisation was active, and was able to elicit specific anti-NanA antibodies.

D. The future of NanA for development as a protein vaccine

Theoretically, NanA makes a very good vaccine candidate, as it is present in high amounts on the pneumococcal cell surface, is surface exposed and is an essential virulence factor for both pneumococcal colonisation and lung infection. NanA has been shown to be present in all serotypes of pneumococci tested. However, NanA has high sequence variability in different serotypes of pneumococci (King et al., 2005). This is a disadvantage when considering a vaccine

target as antibodies against one variable of NanA may be unable to elicit protection against other serotypes expressing NanA with a different sequence.

The results presented here show that immunisation with NanA was unable to elicit protection against peritonitis or invasive pneumonia. Immunisation with NanA was unable to increase survival time, compared to the control group, following pneumococcal challenge. NanA immunisation was also unable to decrease the numbers of pneumococci recovered from the tissues of mice after challenge. This is surprising as there were significant increases in specific anti-NanA IgG present in the sera, of all NanA immunised mice. The antibodies elicited may not have been functional and therefore, unable to neutralise pneumococci and subsequently prevent infection.

Another explanation may lie within the sequence variability NanA has in different serotypes of pneumococci. Current publications show that immunisations with NanA were only protective in models which used the same pneumococcal challenge serotype, as the one used to isolate, amplify and purify the NanA protein for immunisation. Therefore, further study of NanA variability is needed, to understand the effect it may have on immunisation. It would be important to discover if any specific NanA sequences are able to provide cross protection against other NanA proteins. This has been seen with other pneumococcal proteins such as PspA, which also has high sequence variability (Moreno et al., 2010).

In the chinchilla model of acute otitis media, immunisation with NanA afforded significant protection, from both inner ear infection and nasopharyngeal colonisation. Therefore, it would be interesting to understand the effect that NanA immunisation has upon nasopharyngeal colonisation in a mouse model of infection.

Yesilkaya et al., (2006), showed that there was an increase in survival time of mice following challenge with a lower pneumococcal dose. This result is promising, as a colonisation challenge also uses a lower dose of pneumococci.

Protection against nasopharyngeal colonisation has been shown by one group to be CD4+T cell dependant, and not antibody dependent as originally thought. This response has been shown to be elicited by pneumococcal proteins, as well as pneumococcal polysaccharides (Basset et al., 2007). If protection was seen with NanA in a colonisation model of infection, the immune response elicited would also need to be investigated. If protection is seen, NanA may be good vaccine target to prevent colonisation.

However, the work presented here does not show that NanA is a valuable protein vaccine candidate for preventing pneumococcal lung infection. Specific NanA antibodies were also unable to prevent sepsis in mice, following peritonitis or invasive pneumonia. Although in this study, antibodies elicited by NanA immunisation were not tested for neutralising function. In future work using NanA immunisations, it would be valuable to test what function these antibodies have and whether they may contribute to the clearance of colonisation.

If NanA immunisation is able to prevent otitis media infections and contributes towards the clearance of pneumococci from the nasopharynges, a combination of pneumococcal proteins may be considered as an alternative vaccine. Since NanA is unable to protect against lung infection, other pneumococcal proteins such as PspA or PspC in combination with NanA may provide an ideal vaccine. Research has shown that combinations of pneumococcal proteins are able

to provide effective protection from pneumococcal infections (Ogunniyi et al., 2000).

E. The future of pneumococcal vaccination

Previous published studies have shown that immunisation with PdB confers protection in some mouse models of infection. The work presented here showed that immunisation with PdB was able to decrease the numbers of pneumococci, recovered from the nasopharynges of immunised mice. However, immunisation with PdB was unable to abolish colonisation of the nasopharynges. Therefore, PdB immunisation alone may not provide enough adequate protection to prevent nasopharyngeal colonisation. The development of a new more effective pneumococcal vaccine should be able to prevent nasopharyngeal colonisation, as well as induce herd immunity reducing horizontal spread within the community (Bogaert et al., 2004a). Protection from nasopharyngeal colonisation would also prevent the development of subsequent invasive pneumococcal infections.

The immune response to pneumococcal infection is complex and protection is mediated by different arms of the immune system. A pneumococcal vaccine must be able to induce CD4+ T cell protection as well as mucosal immunity to prevent nasopharyngeal colonisation. The induction of a specific, effective antibody response against all serotypes of pneumococci is also required (Malley, 2010).

The development of a more effective pneumococcal vaccine than the polysaccharide and conjugate vaccines currently available may lie in a whole cell vaccine approach. Roche et al., (2007), have shown that a live encapsulated, attenuated, strain of pneumococci was able to clear pneumococci from the nasopharynges, after 9 days post challenge.

Another study showed that protection from heat inactivated pneumococci was able to protect mice from subsequent invasive infection (Hvalbye et al., 1999). In the study by Hvalbye et al., (1999), mice were immunised intranasally with heat inactivated serotype 4 pneumococci, and subsequently challenged intraperitoneally. Mice immunised with whole cell pneumococci did not develop sepsis following challenge whereas, all mice in the control immunised group developed sepsis.

Unencapsulated pneumococci have also been shown to protect mice from challenge with encapsulated pneumococci (Malley et al., 2001). In the study by Malley et al., (2001), mice were immunised intranasally with unencapsulated serotype 6 pneumococci. Following immunisation, mice were challenged intranasally with a colonisation dose of encapsulated, 6B pneumococci. Mice immunised with unencapsulated pneumococci were able to clear encapsulated pneumococci from the nasopharynges. However, protection from colonisation with other pneumococcal serotypes was only partial, and around 50% of the mice were unable to clear pneumococci from the nasopharynges. The authors also showed that immunisations with unencapsulated pneumococci were able to induce systemic protection. Rats immunised with the vaccine were protected from sepsis following challenge, with serotype 3 pneumococci. In the immunised group of rats 72% survived whereas, only 17% of rats survived in the control group.

The whole cell vaccine approach has also been studied by (Lu et al., 2010). In the study by Lu et al., (2010), the whole cell vaccine which was adsorbed on alum adjuvant and following challenge, was able to induce both systemic antibody and CD4+ T (IL-17 producing) cells. The antigen was administered subcutaneously and protected mice against colonisation, as well as systemic infection with

pneumococci. The authors hope that this vaccine will go into phase I human trials (Lu et al., 2010).

Combinations of pneumococcal proteins have been shown to provide efficient protection in different models of pneumococcal infection, and have already been discussed in detail. One such combination of pneumococcal proteins has entered phase 1 clinical trials. This vaccine contains three highly conserved proteins called pneumococcal surface antigen A (PsaA), serine threonine kinase (StkP) and protein required for cell separation (PcsB) (Schmid, 2011). These antigens are able to protect against nasopharyngeal colonisation, by mediating $CD4^+$ T cells to release IL-17, as well as inducing a systemic antibody response. The efficacy of this vaccine in humans is still under investigation and clinical trials continue.

Both the whole cell vaccine and protein vaccine approach to pneumococcal vaccine development, show promising results so far. These vaccines should prevent the problems of serotype replacement, as immunity induced by immunisation should be effective against all pneumococcal serotypes. As well as this, these vaccines have been developed to induce immunity against colonisation and subsequently should prevent invasive infection.

F. Final Remarks

The work presented here shows that immunisation with NanA is not protective against peritonitis or invasive pneumonia. NanA immunisations were able to elicit specific high titres of antibodies. However, this response seemed unable to decrease the numbers of pneumococci, in any tissue samples tested, following pneumococcal challenge. Although protection has been shown following

NanA immunisation previously, these studies have only shown limited protective ability. Based on the work presented here and published articles, NanA may not be the best pneumococcal protein for inclusion in future vaccines developed against *Streptococcus pneumoniae*.

PdB has previously been shown to be protective in a variety of pneumococcal infection models. However, the work presented here, shows that PdB is partially protective against nasopharyngeal colonisation. Immunisation with PdB was able to decrease the numbers of pneumococci recovered from the nasopharynges of mice, following challenge. PdB was shown to be immunogenic when administered without any adjuvant and was able to elicit specific, protective antibodies, which were also able to neutralise PLY *in vitro*. Subcutaneous immunisation with PdB induces a specific response in the nasopharynges, including an increase in antibody producing B cells in the CLN, contributing to a decrease in the numbers of pneumococci. Decreasing or clearing colonisation is extremely important in preventing all other pneumococcal infections, as well as horizontal spread in the community. Therefore, due its protective efficacy, PdB or other pneumolysoids, in combination with other pneumococcal proteins, should be included within future pneumococcal protein vaccines that may be developed.

V. Appendix

Buffers for PdB and PLY purification

Acidic Buffer - 0.02M sodium acetate, 0.5M NaCl, 200ml dH_2O, pH 4

Equilibration buffer – 0.05M Sodium phosphate, 0.5M sodium chloride, 200ml dH_2O, pH 7

10mM Imidazole – 10mM imidazole with 0.02M sodium phosphate, 200ml dH_2O, pH 7

20mM Imidazole – 20mM imidazole with 0.02M sodium phosphate, 200ml dH_2O, pH 7

100mM Imidazole –100mM imidazole with 0.02M sodium phosphate, 200ml dH_2O, pH 7

Auto induction media for Neuraminidase induction- Metals mix (1000x) – 0.1M $FeCl_3$-$6H_2O$ dissolved in 0.1M HCl, 1M $CaCl_2$, 1M $MnCl_2$-$4H_2O$, 1M $ZnSO_4$-$7H_2O$, 0.2M $CoCl_2$-$6H_2O$, 0.1M $CuCl_2$-$2H_2O$, 0.2M $NiCl_2$-$6H_2O$, 0.1M Na_2MoO_4-$4H_2O$, 0.1M Na_2SeO_3-$5H_2O$, 0.1M H_3BO_3 and 36ml H_2O and 5052 (50x) - 0.5% Glycerol, 0.05% Glucose and 0.2% α-lactose and NPS (20x) – 0.5M $(NH_4)_2SO_4$, 1M KH_2PO_4 and 1M Na_2HPO_4

Buffers used for Neuraminidase purification

NPI-10 buffer –50mM NaH_2PO_4, 300mM NaCl and 10mM Imidazole, pH 8

NPI-20 buffer –50mM NaH_2PO_4, 300mM NaCl and 20mM Imidazole, pH 8

NPI -250 buffer - 50mM NaH_2PO_4, 300mM NaCl and 250mM Imidazole, pH 8

Buffers used for SDS PAGE gels and Western blot analysis

TGS buffer – (10X)- 250mM Tris, 10% SDS, 2M glycine, 1L dH_2O

Transfer buffer – 0.02M Tris, 0.03M glycine, 20% methanol, 0.03% SDS, 1L dH_2O

TBST –10mM tris, 0.15M $NaCl_2$, 0.05% tween 20, 400ml dH_2O pH- 8

Cholesterol removal solution – 1% dextralip 50 (Sigma), 0.03mM $MgCl_2.6H_2O$, 10ml dH_2O.

VI. References

ABEYTA, M., HARDY, G. G. & YOTHER, J. (2003) Genetic alteration of capsule type but not PspA type affects accessibility of surface-bound complement and surface antigens of Streptococcus pneumoniae. *Infection & Immunity,* 71**,** 218-25.

ADAMOU, J. E., HEINRICHS, J. H., ERWIN, A. L., WALSH, W., GAYLE, T., DORMITZER, M., DAGAN, R., BREWAH, Y. A., BARREN, P., LATHIGRA, R., LANGERMANN, S., KOENIG, S. & JOHNSON, S. (2001) Identification and characterization of a novel family of pneumococcal proteins that are protective against sepsis. *Infection & Immunity,* 69**,** 949-58.

ADAMOU, J. E., WIZEMANN, T. M., BARREN, P. & LANGERMANN, S. (1998) Adherence of Streptococcus pneumoniae to human bronchial epithelial cells (BEAS-2B). *Infection & Immunity,* 66**,** 820-2.

ALEXANDER, J. E., BERRY, A. M., PATON, J. C., RUBINS, J. B., ANDREW, P. W. & MITCHELL, T. J. (1998) Amino acid changes affecting the activity of pneumolysin alter the behaviour of pneumococci in pneumonia. *Microbial Pathogenesis,* 24**,** 167-74.

ALEXANDER, J. E., LOCK, R. A., PEETERS, C. C., POOLMAN, J. T., ANDREW, P. W., MITCHELL, T. J., HANSMAN, D. & PATON, J. C. (1994) Immunization of mice with pneumolysin toxoid confers a significant degree of protection against at least nine serotypes of Streptococcus pneumoniae. *Infection & Immunity,* 62**,** 5683-8.

ALLEGRUCCI, M., HU, F. Z., SHEN, K., HAYES, J., EHRLICH, G. D., POST, J. C. & SAUER, K. (2006) Phenotypic characterization of Streptococcus pneumoniae biofilm development. *Journal of Bacteriology,* 188**,** 2325-35.

ALTAMURA, M., CARADONNA, L., AMATI, L., PELLEGRINO, N. M., URGESI, G. & MINIELLO, S. (2001) Splenectomy and sepsis: the role of the spleen in the immune-mediated bacterial clearance. *Immunopharmacology & Immunotoxicology,* 23**,** 153-61.

ANDERSON, B., J. DAHMEN, T., FREJD, H., LEFFLER, G., MAGNUSSON, G., NOORI, C. & EDEN. (1983) Identification of an active dissacharide unit of a glycoconjugate receptor for pneumococcal attaching to human pharyngeal epithelial cell. *J. Exp. Med.,* 158**,** 559-570.

AUSTRIAN, R. (1960) The Gram stain and the etiology of lobar pneumonia, an historical note. *Bacteriological Reviews,* 24**,** 261-5.

AUSTRIAN, R. (1981) Pneumococcus: the first one hundred years. *reviews of infectious diseases,* 3**,** 183-9.

BASSET, A., THOMPSON, C. M., HOLLINGSHEAD, S. K., BRILES, D. E., ADES, E. W., LIPSITCH, M. & MALLEY, R. (2007) Antibody-independent, CD4+ T-cell-dependent protection against pneumococcal colonization elicited by intranasal immunization with purified pneumococcal proteins. *Infection & Immunity,* 75**,** 5460-4.

BAUERNFEIND, F., ABLASSER, A., BARTOK, E., KIM, S., SCHMID-BURGK, J., CAVLAR, T. & HORNUNG, V. (2011) Inflammasomes: current understanding and open questions. *Cellular & Molecular Life Sciences,* 68**,** 765-83.

BENTON, K. A., PATON, J. C. & BRILES, D. E. (1997) Differences in virulence for mice among Streptococcus pneumoniae strains of capsular types 2, 3, 4, 5, and 6 are not attributable to differences in pneumolysin production. *Infection & Immunity,* 65**,** 1237-44.

BERGERON, Y., OUELLET, N., DESLAURIERS, A. M., SIMARD, M., OLIVIER, M. & BERGERON, M. G. (1998) Cytokine kinetics and other host factors in response to pneumococcal pulmonary infection in mice. *Infection & Immunity,* 66**,** 912-22.

BERGMANN, S. & HAMMERSCHMIDT, S. (2006) Versatility of pneumococcal surface proteins. *Microbiology,* 152**,** 295-303.

BERGMANN, S., ROHDE, M., CHHATWAL, G. S. & HAMMERSCHMIDT, S. (2001) alpha-Enolase of Streptococcus pneumoniae is a plasmin(ogen)-binding protein displayed on the bacterial cell surface. *Molecular Microbiology,* 40**,** 1273-87.

BERGMANN, S., ROHDE, M., PREISSNER, K. T. & HAMMERSCHMIDT, S. (2005) The nine residue plasminogen-binding motif of the pneumococcal enolase is the major cofactor of plasmin-mediated degradation of extracellular matrix, dissolution of fibrin and transmigration. *Thrombosis & Haemostasis,* 94**,** 304-11.

BERRY, A. M. & PATON, J. C. (1996) Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of Streptococcus pneumoniae. *Infection & Immunity,* 64**,** 5255-62.

BERRY, A. M. & PATON, J. C. (2000) Additive attenuation of virulence of Streptococcus pneumoniae by mutation of the genes encoding pneumolysin and other putative pneumococcal virulence proteins. *Infection & Immunity,* 68**,** 133-40.

BLACK, S., SHINEFIELD, H., FIREMAN, B., LEWIS, E., RAY, P., HANSEN, J. R., ELVIN, L., ENSOR, K. M., HACKELL, J., SIBER, G., MALINOSKI, F., MADORE, D., CHANG, I., KOHBERGER, R., WATSON, W., AUSTRIAN, R. & EDWARDS, K. (2000) Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. *Pediatric Infectious Disease Journal,* 19**,** 187-95.

BOGAERT, D., DE GROOT, R. & HERMANS, P. W. M. (2004a) Streptococcus pneumoniae colonisation: the key to pneumococcal disease. *The Lancet Infectious Diseases,* 4**,** 144-54.

BOGAERT, D., HERMANS, P. W. M., ADRIAN, P. V., RUMKE, H. C. & DE GROOT, R. (2004b) Pneumococcal vaccines: an update on current strategies. *Vaccine,* 22**,** 2209-20.

BONEV, B. B., GILBERT, R. J., ANDREW, P. W., BYRON, O. & WATTS, A. (2001) Structural analysis of the protein/lipid complexes associated with pore formation by the bacterial toxin pneumolysin. *Journal of Biological Chemistry,* 276**,** 5714-9.

BOULNOIS, G. J. (1992) Pneumococcal proteins and the pathogenesis of disease caused by Streptococcus pneumoniae. *Journal of General Microbiology,* 138**,** 249-59.

BRANGER, J., KNAPP, S., WEIJER, S., LEEMANS, J. C., PATER, J. M., SPEELMAN, P., FLORQUIN, S. & VAN DER POLL, T. (2004) Role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. *Infection & Immunity,* 72**,** 788-94.

BRAUN, J. & SUBLETT JE, F. D., MITCHELL TJ, CLEVELAND JL, TUOMANEN EI, WEBER JR. (2002) Pneumococcal pneumolysin and H(2)O(2) mediate brain cell apoptosis during meningitis. *J Clin Invest.,* 109**,** 19-27.

BRILES, D. E., ADES, E., PATON, J. C., SAMPSON, J. S., CARLONE, G. M., HUEBNER, R. C., VIROLAINEN, A., SWIATLO, E. & HOLLINGSHEAD, S. K. (2000) Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of Streptococcus pneumoniae. *Infection & Immunity,* 68**,** 796-800.

BRILES, D. E., HOLLINGSHEAD, S. K., PATON, J. C., ADES, E. W., NOVAK, L., VAN GINKEL, F. W. & BENJAMIN, W. H., JR. (2003) Immunizations with pneumococcal surface protein A and pneumolysin are protective against pneumonia in a murine model of pulmonary infection with Streptococcus pneumoniae. *Journal of Infectious Diseases,* 188**,** 339-48.

BROWN, E. J., HOSEA, S. W., HAMMER, C. H., BURCH, C. G. & FRANK, M. M. (1982) A quantitative analysis of the interactions of antipneumococcal antibody and complement in experimental pneumococcal bacteremia. *Journal of Clinical Investigation,* 69**,** 85-98.

BROWN, E. J., JOINER, K. A., COLE, R. M. & BERGER, M. (1983) Localization of complement component 3 on Streptococcus pneumoniae: anti-capsular antibody causes

complement deposition on the pneumococcal capsule. *Infection & Immunity,* 39**,** 403-9.

BROWN, J. S., GILLILAND, S. M. & HOLDEN, D. W. (2001a) A Streptococcus pneumoniae pathogenicity island encoding an ABC transporter involved in iron uptake and virulence. *Molecular Microbiology,* 40**,** 572-85.

BROWN, J. S., HUSSELL, T., GILLILAND, S. M., HOLDEN, D. W., PATON, J. C., EHRENSTEIN, M. R., WALPORT, M. J. & BOTTO, M. (2002) The classical pathway is the dominant complement pathway required for innate immunity to Streptococcus pneumoniae infection in mice. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 16969-74.

BROWN, J. S., OGUNNIYI, A. D., WOODROW, M. C., HOLDEN, D. W. & PATON, J. C. (2001b) Immunization with components of two iron uptake ABC transporters protects mice against systemic Streptococcus pneumoniae infection.[Erratum appears in Infect Immun. 2004 Nov;72(11):6755]. *Infection & Immunity,* 69**,** 6702-6.

BURNAUGH, A. M., FRANTZ, L. J. & KING, S. J. (2008) Growth of Streptococcus pneumoniae on human glycoconjugates is dependent upon the sequential activity of bacterial exoglycosidases. *Journal of Bacteriology,* 190**,** 221-30.

CAMPOS, I. B., DARRIEUX, M., FERREIRA, D. M., MIYAJI, E. N., SILVA, D. A., AREAS, A. P. M., AIRES, K. A., LEITE, L. C. C., HO, P. L. & OLIVEIRA, M. L. S. (2008) Nasal immunization of mice with Lactobacillus casei expressing the Pneumococcal Surface Protein A: induction of antibodies, complement deposition and partial protection against Streptococcus pneumoniae challenge. *Microbes & Infection,* 10**,** 481-8.

CANVIN, J. R., MARVIN, A. P., SIVAKUMARAN, M., PATON, J. C., BOULNOIS, G. J., ANDREW, P. W. & MITCHELL, T. J. (1995) The role of pneumolysin and autolysin in the pathology of pneumonia and septicemia in mice infected with a type 2 pneumococcus. *Journal of Infectious Diseases,* 172**,** 119-23.

CAO, J., GONG, Y., LI, D., YIN, N., CHEN, T., XU, W., ZHANG, X. & YIN, Y. (2009) CD4(+) T lymphocytes mediated protection against invasive pneumococcal infection induced by mucosal immunization with ClpP and CbpA. *Vaccine,* 27**,** 2838-44.

CHENG, Q., FINKEL, D. & HOSTETTER, M. K. (2000) Novel purification scheme and functions for a C3-binding protein from Streptococcus pneumoniae. *Biochemistry,* 39**,** 5450-7.

COSTERTON, J. W., STEWART, P. S. & GREENBERG, E. P. (1999) Bacterial biofilms: a common cause of persistent infections. *Science,* 284**,** 1318-22.

CRAIN, M. J., WALTMAN, W. D., 2ND, TURNER, J. S., YOTHER, J., TALKINGTON, D. F., MCDANIEL, L. S., GRAY, B. M. & BRILES, D. E. (1990) Pneumococcal surface protein A (PspA) is serologically highly variable and is expressed by all clinically important capsular serotypes of Streptococcus pneumoniae. *Infection & Immunity,* 58**,** 3293-9.

CUNDELL, D., MASURE, H. R. & TUOMANEN, E. I. (1995a) The molecular basis of pneumococcal infection: a hypothesis. *Clinical Infectious Diseases,* 21 Suppl 3**,** S204-11.

CUNDELL, D. R., GERARD, N. P., GERARD, C., IDANPAAN-HEIKKILA, I. & TUOMANEN, E. I. (1995b) Streptococcus pneumoniae anchor to activated human cells by the receptor for platelet-activating factor. *Nature,* 377**,** 435-8.

CUNDELL, D. R. & TUOMANEN, E. I. (1994) Receptor specificity of adherence of Streptococcus pneumoniae to human type-II pneumocytes and vascular endothelial cells in vitro. *Microbial Pathogenesis,* 17**,** 361-74.

CUNDELL, D. R., WEISER, J. N., SHEN, J., YOUNG, A. & TUOMANEN, E. I. (1995c) Relationship between colonial morphology and adherence of Streptococcus pneumoniae. *Infection & Immunity,* 63**,** 757-61.

CUTTS, F. T., ZAMAN, S. M. A., ENWERE, G., JAFFAR, S., LEVINE, O. S., OKOKO, J. B., OLUWALANA, C., VAUGHAN, A., OBARO, S. K., LEACH, A., MCADAM, K. P., BINEY, E., SAAKA, M., ONWUCHEKWA, U., YALLOP, F., PIERCE, N. F., GREENWOOD, B. M., ADEGBOLA, R. A. & GAMBIAN PNEUMOCOCCAL VACCINE TRIAL, G. (2005) Efficacy of nine-valent pneumococcal conjugate vaccine against pneumonia and invasive pneumococcal disease in The Gambia: randomised, double-blind, placebo-controlled trial.[Erratum appears in Lancet. 2005 Jul 2-8;366(9479):28]. *Lancet,* 365**,** 1139-46.

DALIA, A. B., STANDISH, A. J. & WEISER, J. N. (2010) Three surface exoglycosidases from Streptococcus pneumoniae, NanA, BgaA, and StrH, promote resistance to opsonophagocytic killing by human neutrophils. *Infection & Immunity,* 78**,** 2108-16.

DAVE, S., CARMICLE, S., HAMMERSCHMIDT, S., PANGBURN, M. K. & MCDANIEL, L. S. (2004) Dual roles of PspC, a surface protein of Streptococcus pneumoniae, in binding human secretory IgA and factor H. *Journal of Immunology,* 173**,** 471-7.

DEIBEL, R. S., HW JR. (1974) Family II: Streptococcaceae. Bergy's manual of determinative bacteriology. . 405-517.

DIAVATOPOULOS, D. A., SHORT, K. R., PRICE, J. T., WILKSCH, J. J., BROWN, L. E., BRILES, D. E., STRUGNELL, R. A. & WIJBURG, O. L. (2010) Influenza A virus facilitates Streptococcus pneumoniae transmission and disease. *FASEB Journal,* 24**,** 1789-98.

DINARELLO, C. A. (2009) Immunological and inflammatory functions of the interleukin-1 family. *Annual Review of Immunology,* 27**,** 519-50.

DINTILHAC, A., ALLOING, G., GRANADEL, C. & CLAVERYS, J. P. (1997) Competence and virulence of Streptococcus pneumoniae: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. *Molecular Microbiology,* 25**,** 727-39.

DOUCE, G., ROSS, K., COWAN, G., MA, J. & MITCHELL, T. J. (2010) Novel mucosal vaccines generated by genetic conjugation of heterologous proteins to pneumolysin (PLY) from Streptococcus pneumoniae. *Vaccine,* 28**,** 3231-7.

EHRLICH, G. D., VEEH, R., WANG, X., COSTERTON, J. W., HAYES, J. D., HU, F. Z., DAIGLE, B. J., EHRLICH, M. D. & POST, J. C. (2002) Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. *JAMA,* 287**,** 1710-5.

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

FELDMAN, C., ANDERSON, R., COCKERAN, R., MITCHELL, T., COLE, P. & WILSON, R. (2002) The effects of pneumolysin and hydrogen peroxide, alone and in combination, on human ciliated epithelium in vitro. *Respiratory Medicine,* 96**,** 580-5.

FELDMAN, C., COCKERAN, R., JEDRZEJAS, M. J., MITCHELL, T. J. & ANDERSON, R. (2007) Hyaluronidase augments pneumolysin-mediated injury to human ciliated epithelium. *International Journal of Infectious Diseases,* 11**,** 11-5.

FERREIRA, D. M., OLIVEIRA, M. L. S., MORENO, A. T., HO, P. L., BRILES, D. E. & MIYAJI, E. N. (2010) Protection against nasal colonization with Streptococcus pneumoniae by parenteral immunization with a DNA vaccine encoding PspA (Pneumococcal surface protein A). *Microbial Pathogenesis,* 48**,** 205-13.

FUKUYAMA, Y., KING, J. D., KATAOKA, K., KOBAYASHI, R., GILBERT, R. S., OISHI, K., HOLLINGSHEAD, S. K., BRILES, D. E. & FUJIHASHI, K. (2010) Secretory-IgA

antibodies play an important role in the immunity to Streptococcus pneumoniae. *Journal of Immunology,* 185**,** 1755-62.

GEELEN, S., BHATTACHARYYA, C. & TUOMANEN, E. (1993) The cell wall mediates pneumococcal attachment to and cytopathology in human endothelial cells. *Infection & Immunity,* 61**,** 1538-43.

GHAFFAR, F., FRIEDLAND, I. R. & MCCRACKEN, G. H., JR. (1999) Dynamics of nasopharyngeal colonization by Streptococcus pneumoniae. *Pediatric Infectious Disease Journal,* 18**,** 638-46.

GILBERT, R. J., JIMENEZ, J. L., CHEN, S., TICKLE, I. J., ROSSJOHN, J., PARKER, M., ANDREW, P. W. & SAIBIL, H. R. (1999) Two structural transitions in membrane pore formation by pneumolysin, the pore-forming toxin of Streptococcus pneumoniae. *Cell,* 97**,** 647-55.

GILLESPIE, S. H. (1989) Aspects of pneumococcal infection including bacterial virulence, host response and vaccination. *Journal of Medical Microbiology,* 28**,** 237-48.

GINGLES, N. A., ALEXANDER, J. E., KADIOGLU, A., ANDREW, P. W., KERR, A., MITCHELL, T. J., HOPES, E., DENNY, P., BROWN, S., JONES, H. B., LITTLE, S., BOOTH, G. C. & MCPHEAT, W. L. (2001) Role of genetic resistance in invasive pneumococcal infection: identification and study of susceptibility and resistance in inbred mouse strains. *Infection & Immunity,* 69**,** 426-34.

GIUDICELLI, S. & TOMASZ, A. (1984) Attachment of pneumococcal autolysin to wall teichoic acids, an essential step in enzymatic wall degradation. *Journal of Bacteriology,* 158**,** 1188-90.

GRAY, B. M., CONVERSE, G. M., 3RD & DILLON, H. C., JR. (1980) Epidemiologic studies of Streptococcus pneumoniae in infants: acquisition, carriage, and infection during the first 24 months of life. *Journal of Infectious Diseases,* 142**,** 923-33.

GRAY, B. M. & DILLON, H. C., JR. (1989) Natural history of pneumococcal infections. *Pediatric Infectious Disease Journal,* 8**,** S23-5.

GRIFFITHS, F. (1928) The Significance of Pneumococcal Types. *The journal of hygiene,* 27**,** 113-59.

GUT, H., KING, S. J. & WALSH, M. A. (2008) Structural and functional studies of Streptococcus pneumoniae neuraminidase B: An intramolecular trans-sialidase. *FEBS Letters,* 582**,** 3348-52.

GUTCHER, I. & BECHER, B. (2007) APC-derived cytokines and T cell polarization in autoimmune inflammation. *Journal of Clinical Investigation,* 117**,** 1119-27.

HAKANSSON, A., KIDD, A., WADELL, G., SABHARWAL, H. & SVANBORG, C. (1994) Adenovirus infection enhances in vitro adherence of Streptococcus pneumoniae. *Infection & Immunity,* 62**,** 2707-14.

HAMMERSCHMIDT, S., TALAY, S. R., BRANDTZAEG, P. & CHHATWAL, G. S. (1997) SpsA, a novel pneumococcal surface protein with specific binding to secretory immunoglobulin A and secretory component. *Molecular Microbiology,* 25**,** 1113-24.

HARAGUCHI, S., DAY, N. K., NELSON, R. P., JR., EMMANUEL, P., DUPLANTIER, J. E., CHRISTODOULOU, C. S. & GOOD, R. A. (1998) Interleukin 12 deficiency associated with recurrent infections. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 13125-9.

HENRICHSEN, J. (1995) Six newly recognized types of Streptococcus pneumoniae. *Journal of Clinical Microbiology,* 33**,** 2759-62.

HIRST, R. A., GOSAI, B., RUTMAN, A., GUERIN, C. J., NICOTERA, P., ANDREW, P. W. & O'CALLAGHAN, C. (2008) Streptococcus pneumoniae deficient in pneumolysin or autolysin has reduced virulence in meningitis. *Journal of Infectious Diseases,* 197**,** 744-51.

HO, C.-F. & LIN, T.-Y. (2005) Pneumococcal vaccines. *Chang Gung Medical Journal,* 28**,** 765-72.

HOLMES, A. R., MCNAB, R., MILLSAP, K. W., ROHDE, M., HAMMERSCHMIDT, S., MAWDSLEY, J. L. & JENKINSON, H. F. (2001) The pavA gene of Streptococcus pneumoniae encodes a fibronectin-binding protein that is essential for virulence. *Molecular Microbiology,* 41**,** 1395-408.

HURLIMANN, J., THORBECKE, G. J. & HOCHWALD, G. M. (1966) The liver as the site of C-reactive protein formation. *Journal of Experimental Medicine,* 123**,** 365-78.

HUSS, A., SCOTT, P., STUCK, A. E., TROTTER, C. & EGGER, M. (2009) Efficacy of pneumococcal vaccination in adults: a meta-analysis.[Erratum appears in CMAJ. 2009 May 12;180(10):1038]. *CMAJ Canadian Medical Association Journal,* 180**,** 48-58.

HVALBYE, B. K., AABERGE, I. S., LOVIK, M. & HANEBERG, B. (1999) Intranasal immunization with heat-inactivated Streptococcus pneumoniae protects mice against systemic pneumococcal infection. *Infection & Immunity,* 67**,** 4320-5.

HYAMS, C., CAMBERLEIN, E., COHEN, J. M., BAX, K. & BROWN, J. S. (2010) The Streptococcus pneumoniae capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. *Infection & Immunity,* 78**,** 704-15.

IINUMA, H. & OKINAGA, K. (1989) Prevention of pneumococcal bacteremia by immunization with type 6 pneumococcal capsular polysaccharide vaccine in splenectomized rats. *Journal of Infectious Diseases,* 160**,** 66-75.

JANEWAY, C. A., JR. & MEDZHITOV, R. (2002) Innate immune recognition. *Annual Review of Immunology,* 20**,** 197-216.

JEDRZEJAS, M. J. (2001) Pneumococcal virulence factors: structure and function. *Microbiology & Molecular Biology Reviews,* 65**,** 187-207 ; first page.

JEDRZEJAS, M. J., HOLLINGSHEAD, S. K., LEBOWITZ, J., CHANTALAT, L., BRILES, D. E. & LAMANI, E. (2000) Production and characterization of the functional fragment of pneumococcal surface protein A. *Archives of Biochemistry & Biophysics,* 373**,** 116-25.

JEDRZEJAS, M. J., MELLO, L. V., DE GROOT, B. L. & LI, S. (2002) Mechanism of hyaluronan degradation by Streptococcus pneumoniae hyaluronate lyase. Structures of complexes with the substrate. *Journal of Biological Chemistry,* 277**,** 28287-97.

JOHNSTON, J. (1991) Pathogenesis of pneumococcal pneumonia. *Reviews of Infectious Diseases,* 13.

JOHNSTON, J. W., MYERS, L. E., OCHS, M. M., BENJAMIN, W. H., JR., BRILES, D. E. & HOLLINGSHEAD, S. K. (2004) Lipoprotein PsaA in virulence of Streptococcus pneumoniae: surface accessibility and role in protection from superoxide. *Infection & Immunity,* 72**,** 5858-67.

KADIOGLU, A. & ANDREW, P. W. (2005) Susceptibility and resistance to pneumococcal disease in mice. *Briefings in Functional Genomics & Proteomics,* 4**,** 241-7.

KADIOGLU, A., BREWIN, H., X00E, RTEL, T., BRITTAN, J. L., KLEIN, M., HAMMERSCHMIDT, S. & JENKINSON, H. F. (2010) Pneumococcal protein PavA is important for nasopharyngeal carriage and development of sepsis. *Molecular Oral Microbiology,* 25**,** 50-60.

KADIOGLU, A., COWARD, W., COLSTON, M. J., HEWITT, C. R. A. & ANDREW, P. W. (2004) CD4-T-lymphocyte interactions with pneumolysin and pneumococci suggest a crucial protective role in the host response to pneumococcal infection. *Infection & Immunity,* 72**,** 2689-97.

KADIOGLU, A., GINGLES, N. A., GRATTAN, K., KERR, A., MITCHELL, T. J. & ANDREW, P. W. (2000) Host cellular immune response to pneumococcal lung infection in

mice.[Erratum appears in Infect Immunol 2000 Apr;68(4):2390]. *Infection & Immunity,* 68**,** 492-501.

KADIOGLU, A., SHARPE, J. A., LAZOU, I., SVANBORG, C., OCKLEFORD, C., MITCHELL, T. J. & ANDREW, P. W. (2001) Use of green fluorescent protein in visualisation of pneumococcal invasion of broncho-epithelial cells in vivo. *FEMS Microbiology Letters,* 194**,** 105-10.

KADIOGLU, A., TAYLOR, S., IANNELLI, F., POZZI, G., MITCHELL, T. J. & ANDREW, P. W. (2002) Upper and lower respiratory tract infection by Streptococcus pneumoniae is affected by pneumolysin deficiency and differences in capsule type. *Infection & Immunity,* 70**,** 2886-90.

KADIOGLU, A., WEISER, J. N., PATON, J. C. & ANDREW, P. W. (2008) The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. *Nature Reviews,* Microbiology. 6**,** 288-301.

KADIOGLU, A. D. F., K. BANGERT, M. FERNANDES, VE. RICHARDS, L. JONES, K. ANDREW, PW. HOGG, N. (2011) The Integrins Mac-1 and {alpha}4{beta}1 Perform Crucial Roles in Neutrophil and T Cell Recruitment to Lungs during Streptococcus pneumoniae Infection. *journal of Immunology,* 186**,** 5907-15.

KANCLERSKI, K., X00F & LLBY, R. (1987) Production and purification of Streptococcus pneumoniae hemolysin (pneumolysin). *Journal of Clinical Microbiology,* 25**,** 222-5.

KEMP, K., X00E, RE, BRUUNSGAARD, H., SKINH, X00F, J, P. & KLARLUND PEDERSEN, B. (2002) Pneumococcal infections in humans are associated with increased apoptosis and trafficking of type 1 cytokine-producing T cells. *Infection & Immunity,* 70**,** 5019-25.

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

KING, S. J., HIPPE, K. R. & WEISER, J. N. (2006) Deglycosylation of human glycoconjugates by the sequential activities of exoglycosidases expressed by Streptococcus pneumoniae. *Molecular Microbiology,* 59**,** 961-74.

KING, S. J., WHATMORE, A. M. & DOWSON, C. G. (2005) NanA, a neuraminidase from Streptococcus pneumoniae, shows high levels of sequence diversity, at least in part through recombination with Streptococcus oralis. *Journal of Bacteriology,* 187**,** 5376-86.

KLUGMAN, K. P., MADHI, S. A., HUEBNER, R. E., KOHBERGER, R., MBELLE, N., PIERCE, N. & VACCINE TRIALISTS, G. (2003) A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection. *New England Journal of Medicine,* 349**,** 1341-8.

KOLBERG, J., AASE, A., BERGMANN, S., HERSTAD, T. K., X00F, DAL, G., FRANK, R., ROHDE, M. & HAMMERSCHMIDT, S. (2006) Streptococcus pneumoniae enolase is important for plasminogen binding despite low abundance of enolase protein on the bacterial cell surface. *Microbiology,* 152**,** 1307-17.

KOMAI-KOMA, M., JONES, L., OGG, G. S., XU, D. & LIEW, F. Y. (2004) TLR2 is expressed on activated T cells as a costimulatory receptor. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 3029-34.

LAWRENCE, M. C., PILLING, P. A., EPA, V. C., BERRY, A. M., OGUNNIYI, A. D. & PATON, J. C. (1998) The crystal structure of pneumococcal surface antigen PsaA reveals a metal-binding site and a novel structure for a putative ABC-type binding protein. *Structure,* 6**,** 1553-61.

LI, Y., WANG, S., XIN, W., SCARPELLINI, G., SHI, Z., GUNN, B., ROLAND, K. L. & CURTISS, R., 3RD (2008) A sopB deletion mutation enhances the immunogenicity and

protective efficacy of a heterologous antigen delivered by live attenuated Salmonella enterica vaccines. *Infection & Immunity,* 76**,** 5238-46.

LOCK, R. A., ZHANG, Q. Y., BERRY, A. M. & PATON, J. C. (1992) Sequence variation in the Streptococcus pneumoniae pneumolysin gene affecting haemolytic activity and electrophoretic mobility of the toxin. *Microb Pathog. 1996 Aug;21(2):71-83.*

LU, Y.-J., GROSS, J., BOGAERT, D., FINN, A., BAGRADE, L., ZHANG, Q., KOLLS, J. K., SRIVASTAVA, A., LUNDGREN, A., FORTE, S., THOMPSON, C. M., HARNEY, K. F., ANDERSON, P. W., LIPSITCH, M. & MALLEY, R. (2008) Interleukin-17A mediates acquired immunity to pneumococcal colonization. *PLoS Pathogens,* 4**,** e1000159.

LU, Y.-J., LEITE, L., GONCALVES, V. M., DIAS, W. D. O., LIBERMAN, C., FRATELLI, F., ALDERSON, M., TATE, A., MAISONNEUVE, J.-F., ROBERTSON, G., GRACA, R., SAYEED, S., THOMPSON, C. M., ANDERSON, P. & MALLEY, R. (2010) GMP-grade pneumococcal whole-cell vaccine injected subcutaneously protects mice from nasopharyngeal colonization and fatal aspiration-sepsis. *Vaccine,* 28**,** 7468-75.

LYNCH, J. P., 3RD & ZHANEL, G. G. (2009a) Streptococcus pneumoniae: does antimicrobial resistance matter? *Seminars in Respiratory & Critical Care Medicine,* 30**,** 210-38.

LYNCH, J. P., 3RD & ZHANEL, G. G. (2009b) Streptococcus pneumoniae: epidemiology, risk factors, and strategies for prevention. *Seminars in Respiratory & Critical Care Medicine,* 30**,** 189-209.

LYNCH, J. P., 3RD & ZHANEL, G. G. (2010) Streptococcus pneumoniae: epidemiology and risk factors, evolution of antimicrobial resistance, and impact of vaccines. *Current Opinion in Pulmonary Medicine,* 16**,** 217-25.

MAGEE, A. D. & YOTHER, J. (2001) Requirement for capsule in colonization by Streptococcus pneumoniae. *Infection & Immunity,* 69**,** 3755-61.

MALLEY, R. (2010) Antibody and cell-mediated immunity to Streptococcus pneumoniae: implications for vaccine development. *Journal of Molecular Medicine,* 88**,** 135-42.

MALLEY, R., HENNEKE, P., MORSE, S. C., CIESLEWICZ, M. J., LIPSITCH, M., THOMPSON, C. M., KURT-JONES, E., PATON, J. C., WESSELS, M. R. & GOLENBOCK, D. T. (2003) Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proceedings of the National Academy of Sciences of the United States of America,* 100**,** 1966-71.

MALLEY, R., LIPSITCH, M., STACK, A., SALADINO, R., FLEISHER, G., PELTON, S., THOMPSON, C., BRILES, D. & ANDERSON, P. (2001) Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci. *Infection & Immunity,* 69**,** 4870-3.

MALLEY, R., TRZCINSKI, K., SRIVASTAVA, A., THOMPSON, C. M., ANDERSON, P. W. & LIPSITCH, M. (2005) CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 4848-53.

MANCO, S., HERNON, F., YESILKAYA, H., PATON, J. C., ANDREW, P. W. & KADIOGLU, A. (2006) Pneumococcal neuraminidases A and B both have essential roles during infection of the respiratory tract and sepsis. *Infection & Immunity,* 74**,** 4014-20.

MARRA, A., LAWSON, S., ASUNDI, J. S., BRIGHAM, D. & HROMOCKYJ, A. E. (2002) In vivo characterization of the psa genes from Streptococcus pneumoniae in multiple models of infection. *Microbiology,* 148**,** 1483-91.

MARRIOTT, H. M., MITCHELL, T. J. & DOCKRELL, D. H. (2008) Pneumolysin: a double-edged sword during the host-pathogen interaction. *Current Molecular Medicine,* 8**,** 497-509.

MARTIN-FONTECHA, A., THOMSEN, L. L., BRETT, S., GERARD, C., LIPP, M., LANZAVECCHIA, A. & SALLUSTO, F. (2004) Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nature Immunology,* 5**,** 1260-5.

MARTNER, A., DAHLGREN, C., PATON, J. C. & WOLD, A. E. (2008) Pneumolysin released during Streptococcus pneumoniae autolysis is a potent activator of intracellular oxygen radical production in neutrophils. *Infection & Immunity,* 76**,** 4079-87.

MCNEELA, E. A., BURKE, A., NEILL, D. R., BAXTER, C., FERNANDES, V. E., FERREIRA, D., SMEATON, S., EL-RACHKIDY, R., MCLOUGHLIN, R. M., MORI, A., MORAN, B., FITZGERALD, K. A., TSCHOPP, J., PETRILLI, V., ANDREW, P. W., KADIOGLU, A. & LAVELLE, E. C. (2010) Pneumolysin activates the NLRP3 inflammasome and promotes proinflammatory cytokines independently of TLR4. *PLoS Pathogens,* 6**,** e1001191.

MITCHELL, A. M. & MITCHELL, T. J. (2010) Streptococcus pneumoniae: Virulence factors and variation. *Journal*.

MITCHELL, T. J., ANDREW, P. W., SAUNDERS, F. K., SMITH, A. N. & BOULNOIS, G. J. (1991) Complement activation and antibody binding by pneumolysin via a region of the toxin homologous to a human acute-phase protein. *Molecular Microbiology,* 5**,** 1883-8.

MIZRACHI-NEBENZAHL, Y., LIFSHITZ, S., TEITELBAUM, R., NOVICK, S., LEVI, A., BENHARROCH, D., LING, E. & DAGAN, R. (2003) Differential activation of the immune system by virulent Streptococcus pneumoniae strains determines recovery or death of the host. *Clinical & Experimental Immunology,* 134**,** 23-31.

MORENO, A. T., OLIVEIRA, M. L. S., FERREIRA, D. M., HO, P. L., DARRIEUX, M., LEITE, L. C. C., FERREIRA, J. M. C., JR., PIMENTA, F. C., ANDRADE, A. L., X00FA, CIA, S. S. & MIYAJI, E. N. (2010) Immunization of mice with single PspA fragments induces antibodies capable of mediating complement deposition on different pneumococcal strains and cross-protection. *Clinical & Vaccine Immunology: CVI,* 17**,** 439-46.

MORONA, J. K., MORONA, R. & PATON, J. C. (2006) Attachment of capsular polysaccharide to the cell wall of Streptococcus pneumoniae type 2 is required for invasive disease. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 8505-10.

MU, X00F, OZ-ALMAGRO, C., JORDAN, I., GENE, A., LATORRE, C., GARCIA-GARCIA, J. J. & PALLARES, R. (2008) Emergence of invasive pneumococcal disease caused by nonvaccine serotypes in the era of 7-valent conjugate vaccine. *Clinical Infectious Diseases,* 46**,** 174-82.

MUSHER, D. M., GROOVER, J. E., REICHLER, M. R., RIEDO, F. X., SCHWARTZ, B., WATSON, D. A., BAUGHN, R. E. & BREIMAN, R. F. (1997) Emergence of antibody to capsular polysaccharides of Streptococcus pneumoniae during outbreaks of pneumonia: association with nasopharyngeal colonization. *Clinical Infectious Diseases,* 24**,** 441-6.

NAKAYAMA, S., GEWURZ, H., HOLZER, T., DU CLOS, T. W. & MOLD, C. (1983) The role of the spleen in the protective effect of C-reactive protein in Streptococcus pneumoniae infection. *Clinical & Experimental Immunology,* 54**,** 319-26.

NELSON, A. L., ROCHE, A. M., GOULD, J. M., CHIM, K., RATNER, A. J. & WEISER, J. N. (2007) Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance. *Infection & Immunity,* 75**,** 83-90.

OGGIONI, M. R., TRAPPETTI, C., KADIOGLU, A., CASSONE, M., IANNELLI, F., RICCI, S., ANDREW, P. W. & POZZI, G. (2006) Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis. *Molecular Microbiology,* 61**,** 1196-210.

OGUNNIYI, A. D., FOLLAND, R. L., BRILES, D. E., HOLLINGSHEAD, S. K. & PATON, J. C. (2000) Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with Streptococcus pneumoniae. *Infection & Immunity,* 68**,** 3028-33.

OGUNNIYI, A. D., GRABOWICZ, M., BRILES, D. E., COOK, J. & PATON, J. C. (2007a) Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of Streptococcus pneumoniae. *Infection & Immunity,* 75**,** 350-7.

OGUNNIYI, A. D., LEMESSURIER, K. S., GRAHAM, R. M. A., WATT, J. M., BRILES, D. E., STROEHER, U. H. & PATON, J. C. (2007b) Contributions of pneumolysin, pneumococcal surface protein A (PspA), and PspC to pathogenicity of Streptococcus pneumoniae D39 in a mouse model. *Infection & Immunity,* 75**,** 1843-51.

OGUNNIYI, A. D., WOODROW, M. C., POOLMAN, J. T. & PATON, J. C. (2001) Protection against Streptococcus pneumoniae elicited by immunization with pneumolysin and CbpA. *Infection & Immunity,* 69**,** 5997-6003.

OISHI, K., KOLES, N. L., GUELDE, G. & POLLACK, M. (1992) Antibacterial and protective properties of monoclonal antibodies reactive with Escherichia coli O111:B4 lipopolysaccharide: relation to antibody isotype and complement-fixing activity. *Journal of Infectious Diseases,* 165**,** 34-45.

PAI, R., MOORE, M. R., PILISHVILI, T., GERTZ, R. E., WHITNEY, C. G., BEALL, B. & ACTIVE BACTERIAL CORE SURVEILLANCE, T. (2005) Postvaccine genetic structure of Streptococcus pneumoniae serotype 19A from children in the United States. *Journal of Infectious Diseases,* 192**,** 1988-95.

PARK, I. H., PRITCHARD, D. G., CARTEE, R., BRANDAO, A., BRANDILEONE, M. C. C. & NAHM, M. H. (2007) Discovery of a new capsular serotype (6C) within serogroup 6 of Streptococcus pneumoniae. *Journal of Clinical Microbiology,* 45**,** 1225-33.

PARKER, D., SOONG, G., PLANET, P., BROWER, J., RATNER, A. J. & PRINCE, A. (2009) The NanA neuraminidase of Streptococcus pneumoniae is involved in biofilm formation. *Infection & Immunity,* 77**,** 3722-30.

PASTEUR, L. (1881) Note sur la maladie nouvelle provoque´e par la salive d'un multienfant mort de la rage. *Comptes Rendus,* 92**,** 159-65.

PATERSON, G. K. & MITCHELL, T. J. (2006) Innate immunity and the pneumococcus. *Microbiology,* 152**,** 285-93.

PATON, J. C., ANDREW, P. W., BOULNOIS, G. J. & MITCHELL, T. J. (1993) Molecular analysis of the pathogenicity of Streptococcus pneumoniae: the role of pneumococcal proteins. *Annual Review of Microbiology,* 47**,** 89-115.

PATON, J. C., BERRY, A. M. & LOCK, R. A. (1997) Molecular analysis of putative pneumococcal virulence proteins. *Microbial Drug Resistance-Mechanisms Epidemiology & Disease,* 3**,** 1-10.

PATON, J. C. & FERRANTE, A. (1983) Inhibition of human polymorphonuclear leukocyte respiratory burst, bactericidal activity, and migration by pneumolysin. *Infection & Immunity,* 41**,** 1212-6.

PATON, J. C., LOCK, R. A., LEE, C. J., LI, J. P., BERRY, A. M., MITCHELL, T. J., ANDREW, P. W., HANSMAN, D. & BOULNOIS, G. J. (1991) Purification and immunogenicity of genetically obtained pneumolysin toxoids and their conjugation to Streptococcus pneumoniae type 19F polysaccharide. *Infection & Immunity,* 59**,** 2297-304.

PATON, J. C., ROWAN-KELLY, B. & FERRANTE, A. (1984) Activation of human complement by the pneumococcal toxin pneumolysin. *Infection & Immunity,* 43**,** 1085-7.

PEPPOLONI, S., RICCI, S., ORSI, C. F., COLOMBARI, B., DE SANTI, M. M., MESSINO, M., FABIO, G., ZANARDI, A., RIGHI, E., BRAIONE, V., TRIPODI, S., CHIAVOLINI, D., CINTORINO, M., ZOLI, M., OGGIONI, M. R., BLASI, E. & POZZI, G. (2010) The encapsulated strain TIGR4 of Streptococcus pneumoniae is phagocytosed but is resistant to intracellular killing by mouse microglia. *Microbes & Infection,* 12**,** 990-1001.

PETTIGREW, M. M., FENNIE, K. P., YORK, M. P., DANIELS, J. & GHAFFAR, F. (2006) Variation in the presence of neuraminidase genes among Streptococcus pneumoniae isolates with identical sequence types. *Infection & Immunity,* 74**,** 3360-5.

PRACHT, D., ELM, C., GERBER, J., BERGMANN, S., ROHDE, M., SEILER, M., KIM, K. S., JENKINSON, H. F., NAU, R. & HAMMERSCHMIDT, S. (2005) PavA of Streptococcus pneumoniae modulates adherence, invasion, and meningeal inflammation. *Infection & Immunity,* 73**,** 2680-9.

PRICE, K. E. & CAMILLI, A. (2009) Pneumolysin localizes to the cell wall of Streptococcus pneumoniae. *Journal of Bacteriology,* 191**,** 2163-8.

QUAN, C. P., BERNEMAN, A., PIRES, R., AVRAMEAS, S. & BOUVET, J. P. (1997) Natural polyreactive secretory immunoglobulin A autoantibodies as a possible barrier to infection in humans. *Infection & Immunity,* 65**,** 3997-4004.

QUIN, L. R., MOORE, Q. C., 3RD & MCDANIEL, L. S. (2007) Pneumolysin, PspA, and PspC contribute to pneumococcal evasion of early innate immune responses during bacteremia in mice. *Infection & Immunity,* 75**,** 2067-70.

RAM, S., SHARMA, A. K., SIMPSON, S. D., GULATI, S., MCQUILLEN, D. P., PANGBURN, M. K. & RICE, P. A. (1998) A novel sialic acid binding site on factor H mediates serum resistance of sialylated Neisseria gonorrhoeae. *Journal of Experimental Medicine,* 187**,** 743-52.

RATNER, A. J., LYSENKO, E. S., PAUL, M. N. & WEISER, J. N. (2005) Synergistic proinflammatory responses induced by polymicrobial colonization of epithelial surfaces. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 3429-34.

RICHARDS, L., FERREIRA, D. M., MIYAJI, E. N., ANDREW, P. W. & KADIOGLU, A. (2010) The immunising effect of pneumococcal nasopharyngeal colonisation; protection against future colonisation and fatal invasive disease. *Immunobiology,* 215**,** 251-63.

RILEY, I. D. & DOUGLAS, R. M. (1981) An epidemiologic approach to pneumococcal disease. *Reviews of Infectious Diseases,* 3**,** 233-45.

ROMANELLO, V., MARCACCI, M., DAL MOLIN, F., MOSCHIONI, M., CENSINI, S., COVACCI, A., BARITUSSIO, A. G., MONTECUCCO, C. & TONELLO, F. (2006) Cloning, expression, purification, and characterization of Streptococcus pneumoniae IgA1 protease. *Protein Expression & Purification,* 45**,** 142-9.

ROMERO, P., LOPEZ, R. & GARCIA, E. (2007) Key role of amino acid residues in the dimerization and catalytic activation of the autolysin LytA, an important virulence factor in Streptococcus pneumoniae. *Journal of Biological Chemistry,* 282**,** 17729-37.

ROSENOW, C., RYAN, P., WEISER, J. N., JOHNSON, S., FONTAN, P., ORTQVIST, A. & MASURE, H. R. (1997) Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of Streptococcus pneumoniae. *Molecular Microbiology,* 25**,** 819-29.

ROSSJOHN, J., GILBERT, R. J., CRANE, D., MORGAN, P. J., MITCHELL, T. J., ROWE, A. J., ANDREW, P. W., PATON, J. C., TWETEN, R. K. & PARKER, M. W. (1998) The molecular mechanism of pneumolysin, a virulence factor from Streptococcus pneumoniae. *Journal of Molecular Biology,* 284**,** 449-61.

RUBINS, J. B., CHARBONEAU, D., FASCHING, C., BERRY, A. M., PATON, J. C., ALEXANDER, J. E., ANDREW, P. W., MITCHELL, T. J. & JANOFF, E. N. (1996) Distinct roles for pneumolysin's cytotoxic and complement activities in the pathogenesis of pneumococcal pneumonia. *American Journal of Respiratory & Critical Care Medicine,* 153**,** 1339-46.

RUBINS, J. B., CHARBONEAU, D., PATON, J. C., MITCHELL, T. J., ANDREW, P. W. & JANOFF, E. N. (1995) Dual function of pneumolysin in the early pathogenesis of murine pneumococcal pneumonia. *Journal of Clinical Investigation,* 95**,** 142-50.

RUBINS, J. B., DUANE, P. G., CHARBONEAU, D. & JANOFF, E. N. (1992) Toxicity of pneumolysin to pulmonary endothelial cells in vitro. *Infection & Immunity,* 60**,** 1740-6.

RUBINS, J. B., DUANE, P. G., CLAWSON, D., CHARBONEAU, D., YOUNG, J. & NIEWOEHNER, D. E. (1993) Toxicity of pneumolysin to pulmonary alveolar epithelial cells. *Infection & Immunity,* 61**,** 1352-8.

RUBINS, J. B., PADDOCK, A. H., CHARBONEAU, D., BERRY, A. M., PATON, J. C. & JANOFF, E. N. (1998) Pneumolysin in pneumococcal adherence and colonization. *Microbial Pathogenesis,* 25**,** 337-42.

RUBINS, J. B. & POMEROY, C. (1997) Role of gamma interferon in the pathogenesis of bacteremic pneumococcal pneumonia. *Infection & Immunity,* 65**,** 2975-7.

SACKS, D. & NOBEN-TRAUTH, N. (2002) The immunology of susceptibility and resistance to Leishmania major in mice. *Nature Reviews,* Immunology. 2**,** 845-58.

SCHMID, P. S., S. KELLER, M. LUHAN, B. MAGYARICS, Z. SEIDAL, S. SCHLICK, P. REINISCH, C. LINGNAU, K. NAGY, E. GRUBECK-LOEBENSTEIN, B. (2011) Th17/Th1 biased immunity to the pneumococcal proteins PcsB, StkP and PsaA in adults of different age. *vaccine,* 29**,** 3982-9.

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

SHAKHNOVICH, E. A., KING, S. J. & WEISER, J. N. (2002) Neuraminidase expressed by Streptococcus pneumoniae desialylates the lipopolysaccharide of Neisseria meningitidis and Haemophilus influenzae: a paradigm for interbacterial competition among pathogens of the human respiratory tract. *Infection & Immunity,* 70**,** 7161-4.

SHAPER, M., HOLLINGSHEAD, S. K., BENJAMIN, W. H., JR. & BRILES, D. E. (2004) PspA protects Streptococcus pneumoniae from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin [corrected].[Erratum appears in Infect Immun. 2004 Dec;72(12):7379]. *Infection & Immunity,* 72**,** 5031-40.

SINGLETON, R. J., HENNESSY, T. W., BULKOW, L. R., HAMMITT, L. L., ZULZ, T., HURLBURT, D. A., BUTLER, J. C., RUDOLPH, K. & PARKINSON, A. (2007) Invasive pneumococcal disease caused by nonvaccine serotypes among alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. *JAMA,* 297**,** 1784-92.

SØRENSEN, U. B. S., HENRICHSEN, J., CHEN, H.-C. & SZU, S. C. (1990) Covalent linkage between the capsular polysaccharide and the cell wall peptidoglycan of Streptococcus pneumoniae revealed by immunochemical methods. *Microbial Pathogenesis,* 8**,** 325-334.

STEINFORT, C., WILSON, R., MITCHELL, T., FELDMAN, C., RUTMAN, A., TODD, H., SYKES, D., WALKER, J., SAUNDERS, K., ANDREW, P. W. & ET AL. (1989) Effect of Streptococcus pneumoniae on human respiratory epithelium in vitro. *Infection & Immunity,* 57**,** 2006-13.

STERNBERG, G. (1881) A fatal form of septicaemia in the rabbit, produced by the subcutaneous injection of human saliva. An experimental research. *National board of health bull,* 2**,** 781-3.

TAI, S. S. (2006) Streptococcus pneumoniae protein vaccine candidates: properties, activities and animal studies. *Critical Reviews in Microbiology,* 32**,** 139-53.

TALBOT, U. M., PATON, A. W. & PATON, J. C. (1996) Uptake of Streptococcus pneumoniae by respiratory epithelial cells. *Infection & Immunity,* 64**,** 3772-7.

THIBODEAU, K. P. & VIERA, A. J. (2004) Atypical pathogens and challenges in community-acquired pneumonia. *American Family Physician,* 69**,** 1699-706.

THOMAS-RUDOLPH, D., DU CLOS, T. W., SNAPPER, C. M. & MOLD, C. (2007) C-reactive protein enhances immunity to Streptococcus pneumoniae by targeting uptake to Fc gamma R on dendritic cells. *Journal of Immunology,* 178**,** 7283-91.

TILLEY, S. J., ORLOVA, E. V., GILBERT, R. J. C., ANDREW, P. W. & SAIBIL, H. R. (2005) Structural basis of pore formation by the bacterial toxin pneumolysin. *Cell,* 121**,** 247-56.

TONG, H. H., LI, D., CHEN, S., LONG, J. P. & DEMARIA, T. F. (2005) Immunization with recombinant Streptococcus pneumoniae neuraminidase NanA protects chinchillas against nasopharyngeal colonization. *Infection & Immunity,* 73**,** 7775-8.

TONG, H. H., WEISER, J. N., JAMES, M. A. & DEMARIA, T. F. (2001) Effect of influenza A virus infection on nasopharyngeal colonization and otitis media induced by transparent or opaque phenotype variants of Streptococcus pneumoniae in the chinchilla model. *Infection & Immunity,* 69**,** 602-6.

TORRES, J. M., CARDENAS, O., VASQUEZ, A. & SCHLOSSBERG, D. (1998) Streptococcus pneumoniae bacteremia in a community hospital. *Chest,* 113**,** 387-90.

TRAPPETTI, C., KADIOGLU, A., CARTER, M., HAYRE, J., IANNELLI, F., POZZI, G., ANDREW, P. W. & OGGIONI, M. R. (2009) Sialic acid: a preventable signal for pneumococcal biofilm formation, colonization, and invasion of the host. *Journal of Infectious Diseases,* 199**,** 1497-505.

TSENG, H.-J., MCEWAN, A. G., PATON, J. C. & JENNINGS, M. P. (2002) Virulence of Streptococcus pneumoniae: PsaA mutants are hypersensitive to oxidative stress. *Infection & Immunity,* 70**,** 1635-9.

TUOMANEN, E., RICH, R. & ZAK, O. (1987) Induction of pulmonary inflammation by components of the pneumococcal cell surface. *American Review of Respiratory Disease,* 135**,** 869-74.

TUOMANEN, E. I. (1997) The biology of pneumococcal infection. *Pediatric Research,* 42**,** 253-8.

UNKELESS, J. C., SCIGLIANO, E. & FREEDMAN, V. H. (1988) Structure and function of human and murine receptors for IgG. *Annual Review of Immunology,* 6**,** 251-81.

VAN DE WIJGERT, J. H., VERHEUL, A. F., SNIPPE, H., CHECK, I. J. & HUNTER, R. L. (1991) Immunogenicity of Streptococcus pneumoniae type 14 capsular polysaccharide: influence of carriers and adjuvants on isotype distribution. *Infection & Immunity,* 59**,** 2750-7.

WALTMAN, W. D., MCDANIEL, L. S., GRAY, B. M. & BRILES, D. E. (1990) Variation in the molecular weight of PspA (pneumococcal surface protein A) among Streptococcus pneumoniae. *Microbial Pathogenesis,* 8**,** 61-9.

WANG, S., LI, Y., SHI, H., SCARPELLINI, G., TORRES-ESCOBAR, A., ROLAND, K. L. & CURTISS, R., 3RD (2010) Immune responses to recombinant pneumococcal PsaA antigen delivered by a live attenuated Salmonella vaccine. *Infection & Immunity,* 78**,** 3258-71.

WARDLAW, T. M. J., EMILY WHITE; HODGE, MATTHEW; WORLD HEALTH ORGANIZATION; UNICEF (2006) Pneumonia:the forgotten killer of children. *WHO website*.

WATSON, D. A. & MUSHER, D. M. (1990) Interruption of capsule production in Streptococcus pneumonia serotype 3 by insertion of transposon Tn916. *Infection & Immunity,* 58**,** 3135-8.

WEISER, J. N., AUSTRIAN, R., SREENIVASAN, P. K. & MASURE, H. R. (1994) Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. *Infection & Immunity,* 62**,** 2582-9.

WEISER, J. N. B., D. FASCHING, C. SCAMURRA, RW. RATNER, AJ. JANOFF, EN. (2003) Antibody -enhanced pneumococcal adherence requires IgA1 protease. *Proceedings of the National Academy of Sciences of the United States of America,* 100**,** 4215-20.

WELLMER, A., ZYSK, G., GERBER, J., KUNST, T., VON MERING, M., BUNKOWSKI, S., EIFFERT, H. & NAU, R. (2002) Decreased virulence of a pneumolysin-deficient strain of Streptococcus pneumoniae in murine meningitis. *Infection & Immunity,* 70**,** 6504-8.

WHO (2009) Estimated Hib and pneumococcal deaths for children under 5 years of age. *Online article*.

WHO (2010) World health statistics. *WHO website*.

YESILKAYA, H., SOMA-HADDRICK, S., CRENNELL, S. J. & ANDREW, P. W. (2006) Identification of amino acids essential for catalytic activity of pneumococcal neuraminidase A. *Research in Microbiology,* 157**,** 569-74.

YOSHIMURA, A., LIEN, E., INGALLS, R. R., TUOMANEN, E., DZIARSKI, R. & GOLENBOCK, D. (1999) Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *Journal of Immunology,* 163**,** 1-5.

YUSTE, J., SEN, A., TRUEDSSON, L., JONSSON, G., TAY, L.-S., HYAMS, C., BAXENDALE, H. E., GOLDBLATT, F., BOTTO, M. & BROWN, J. S. (2008) Impaired opsonization with C3b and phagocytosis of Streptococcus pneumoniae in sera from subjects with defects in the classical complement pathway. *Infection & Immunity,* 76**,** 3761-70.

ZHANG, J. R., MOSTOV, K. E., LAMM, M. E., NANNO, M., SHIMIDA, S., OHWAKI, M. & TUOMANEN, E. (2000) The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells. *Cell,* 102**,** 827-37.

ZHANG, Q., CHOO, S. & FINN, A. (2002) Immune responses to novel pneumococcal proteins pneumolysin, PspA, PsaA, and CbpA in adenoidal B cells from children. *Infection & Immunity,* 70**,** 5363-9.

ZWIJNENBURG, P. J., VAN DER POLL, T., FLORQUIN, S., VAN DEVENTER, S. J., ROORD, J. J. & VAN FURTH, A. M. (2001) Experimental pneumococcal meningitis in mice: a model of intranasal infection. *Journal of Infectious Diseases,* 183**,** 1143-6.

ZYSK, G., SCHNEIDER-WALD, B. K., HWANG, J. H., BEJO, L., KIM, K. S., MITCHELL, T. J., HAKENBECK, R. & HEINZ, H. P. (2001) Pneumolysin is the main inducer of cytotoxicity to brain microvascular endothelial cells caused by Streptococcus pneumoniae. *Infection & Immunity,* 69**,** 845-52.