Investigating the role of *Streptococcus pneumoniae* serotype 1 virulence factors in nasopharyngeal carriage and invasive disease

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by

Laura Claire Jacques

200400448

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Abstract

Streptococcus pneumoniae serotype 1 displays some unusual epidemiological, clinical and microbiological characteristics. In Africa, serotype 1 is the leading serotype causing invasive pneumococcal diseases, including, pneumonia, meningitis and sepsis. Unusually, serotype 1 is rarely detected during routine nasopharyngeal swabbing, even in areas of high disease burden. *S. pneumoniae* are natural commensals of the nasopharynx and colonisation of this area has been described as a pre-requisite for invasive disease, hence little is understood about why serotype 1 carriage rates are so low, whilst burden of disease is so high. This body of work sought to identify key virulence factors associated with serotype 1 disease pathogenesis and the effect these have on nasopharyngeal colonisation and progression from carriage to invasive disease. Murine models of nasopharyngeal carriage, pneumonia and sepsis were used to study serotype 1 pathogenesis and identified the bacterial toxin pneumolysin, and autolysin as key virulence factors associated with disease pathogenesis. In addition, the influence of serotype 1 infection on the host immune responses in murine models of pneumococcal infection was also addressed.

Current pneumococcal vaccines (PCV13) include serotype 1 however, the impact of this vaccination in reducing serotype 1 disease burden is still unknown. Findings here suggest that pneumolysin, not bacterial capsule should be a target for strong consideration in future vaccine design. New therapeutics should also be designed to target pneumolysin as it is in the main driver of pathogenesis in the context of pneumococcal disease, particularly in serotype 1 infection.

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Abbreviations

Ab	Antibody
APC	Allophycocyanin
ATP	Adenosine triphosphate
BAB	Blood agar base
BHI	Brain heart infusion
BSA	Bovine serum albumins
C3	Complement component 3
CAP	Community acquired pneumonia
CD	Cluster of differentiation
CDC	Cholesterol-dependant cytolysins
CFU	Colony forming units
CLN	Cervical lymph nodes
CPS	Capsule polysaccharide locus
CSF	Cerebral spinal fluid
DMEM	Dulbecco's Modified Eagle' Medium
DMSO	Dimethyl-sulphoxide
DPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FI	Fluorescent index
FITC	Fluorescein isothiocyanate
Flincr	Florescent index increase
FOXP3	Forkhead box P3
PFGE	Pulsed field gel electrophoresis
HBSS	Hanks' Balanced Salt Solution
HU	Haemolytic units

Ig	Immunoglobulin
IL	Interleukin
INF	Interferon
IPD	Invasive pneumococcal disease
IVC	Individually ventilated cages
IVIG	Intravenous immunoglobulin
LDH	Lactate dehydrogenase
Lyt	Autolysin
M-CSF	Macrophage colony-stimulating factor
MFI	Mean fluorescence intensity
MLEC	Mink lung epithelial cells
MLST	Multi locus sequence type
MHC	Major histocompatibility complex
NaOH	Sodium hydroxide
NCTC	The National Collection of Type Cultures
NK	Natural Killer cells
NLRP3	NOD-like receptor family pyrin domain containing 3 inflammasome
NP	Nasopharynx
OB	Olfactory bulb
OD	Optical density
OE	Olfactory epithelium
OPKA	Opsonophagocytic killing assay
OR	Odds ratio
PAI-1	Plasminogen activator inhibitor-1
PavA	Pneumococcal adherence and virulence factor A
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCV	Pneumococcal conjugate vaccine
PE	Phycoerythrin
PE-CY7	Phycoerythrin/Cyanine dye7

PiuA	Pneumococcal iron uptake A
PiaA	Pneumococcal iron acquisition A
PLN-A	Pneumolysin-deficient pneumococcus
Ply	Pneumolysin
PPSV23	23-valent pneumococcal polysaccharide vaccine
PsaA	Pneumococcal surface adhesion
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
RBC	Red blood cell
RORyT	Retinoic acid receptor-related orphan receptor gamma
RPMI	Roswell Park Memorial Institute medium
ST	Sequence type
TEER	Transepithelial/transendothelial electrical resistance
TGF	Transforming growth factor
Th	T- helper cells
TO	Thiazole orange
TOD	Time of death
UK	United Kingdom
USA	United States of America
V/V	Volume/volume %
ZPS	Zwitterionic polysaccharides

Introduction

Chapter 1

Introduction

1.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae, or the pneumococcus is a gram-positive bacterium and a major cause of human infections ranging from relatively minor middle ear infections such as otitis media, to severe infections such as pneumonia, meningitis and septicemia [1].

It was first isolated by Pasteur in 1881 from the saliva of a patient who had died from rabies. In 1883, Friedlander and Talamon were the first to describe an association between the pneumococcus and pneumonia. However, pneumococcal pneumonia was often confused with other types of pneumonia until the development of the Gram stain in 1884. Between 1915 and 1945 extensive research was done on the pneumococcus which included the discovery of the chemical structure of bacterial capsule polysaccharide and its role in human disease. This led to more than 80 serotypes of pneumococci described by 1940[2]. Several years on, we now understand that *S. pneumoniae* is also a commensal bacteria that resides on the mucosal surface of the upper respiratory tract, specifically the nasopharynx of humans.[3] Colonisation of this site is predominantly asymptomatic but occasionally the bacteria can disseminate into what are normally considered sterile sites such as the lungs and blood. This causes a rapid inflammatory response leading to the many manifestations of pneumococcal disease [4].

S. pneumoniae causes invasive pneumococcal disease (IPD) predominantly in children, the elderly and the immunocompromised (Centres for Disease Control 2008). IPD is defined as isolation of *S. pneumoniae* from a normally sterile body site such as blood, cerebrospinal or pleural fluid [5]. In Europe, the pneumococcus is the most common bacterial respiratory pathogen and causes between 30-50% of community-acquired pneumonia (CAP) requiring hospitalization and leads to mortality rates of around 20% in patients with concurrent septicemia [6] [7,8]. The pneumococcus is an even bigger burden in the developing world where pneumococcal septicemia is a major cause of infant mortality and leads to approximately 25% of all preventable deaths in children under the age of 5 and more than 1.2 million infant deaths per year [9,10].

1.2 Epidemiology

All pneumococcal disease begins with the establishment of nasopharyngeal colonisation [3]. Pneumococci are known to spread from person to person through droplets or aerosols from a colonised individual. Once acquired, a strain can be carried from weeks to months before eventual clearance by the host immune system. Colonisation by pneumococci is most common in children, peaking around 2-3 years of age, and subsequently diminishes to less than 10% in the adult population in Western Europe. However, in poor resource settings, up to 90% of children and over half of adults are colonised. [11] [12] [13] [14].

During colonisation, bacteria enter the nasal cavity and adhere to nasopharyngeal epithelial cells. From this point, depending on the serotype

and immune status of the host, bacteria either stay as colonisers or spread further to organs such as the ears, lungs or penetrate the mucosal barrier to enter the blood stream. Occasionally, bacteria may be able to cross the blood-brain barrier to cause meningitis (Figure 1) [15] [13].

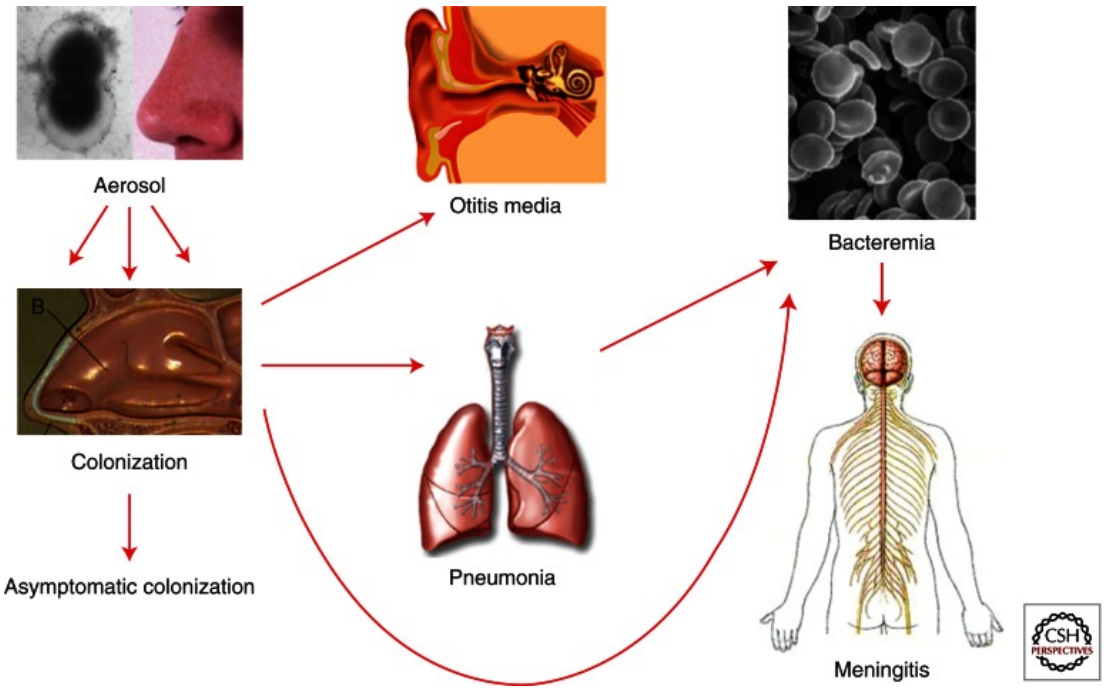


Figure 1 Progression of pneumococcal disease. Pneumococci are spread between individuals by aerosol and enter the nasal cavity. Here bacteria can attach to the nasopharyngeal epithelial cells and colonise the nasopharynx asymptomatically until eventual clearance by the host immune system. However, dissemination of the bacteria to the inner ear is common in children and leads to the onset of middle ear infection known as Otitis media. Invasive disease can occur when bacteria spread to the lungs and bloodstream. Once in the bloodstream, bacteria can occasionally cross the blood-brain barrier, leading to development of meningitis. Adapted from Henriques-Normark et al, 2013

Tracing the global spread of pneumococcal infection is done by serotyping of the capsular polysaccharide. To date, 98 different serotypes have been characterised by the chemical structure of the capsular polysaccharide [16] [17]. Most serotypes are capable of colonising a healthy human nasopharynx, however, there are some serotypes, for example, serotype 1 and 7F, that are rarely found in carriage but frequently cause high rates of IPD [18] [19].

Several methods are used to serotype pneumococci and to study genetic relationships between clinical isolates. The pneumococcus is known to be able to take DNA up from other bacteria and there is evidence that bacteria can switch capsular phenotype [20]. One sequence-based method that is commonly used to identify different serotypes is MLST (multilocus sequence typing) in which seven housekeeping genes are sequenced and depending on the allelic variations present, strains are assigned a sequence type (ST). Other molecular techniques include pulsed field gel electrophoresis (PFGE), whereby bacterial chromosomal DNA is cleaved using restriction enzymes and run on a pulsed field gel which separates the bands by size and charge [21].

1.2.1 Disease burden and mortality rates in adults and children

Streptococcus pneumoniae is a leading cause of invasive disease (IPD) in children, the elderly, and those with certain underlying medical conditions. People with immunodeficiency, such as HIV-positive individuals and those with sickle cell anaemia, are also predisposed to IPD. Other risk factors are poor nutrition, recent influenza epidemics and climate [1]. The pneumococcus is the leading cause of morbidity and mortality due to infectious disease globally and particularly in developing countries such as those in sub-Saharan Africa where it remains a major contributor to childhood death [1].

Adults:

Pneumococcal disease burden in adults is high, with an estimated 600,000 to 800,000 deaths each year from pneumococcal pneumonia, meningitis and sepsis [22]. Disease burden was found to be highest in low-income countries such as sub-Saharan Africa with disease rates as high as 416 per 100,000

population compared to high income countries with incidence rates as low as 2.4 per 100,000 population [23] [24]. In developing countries, pneumonia is one of the leading causes of death in patients over 60 years of age and in South Africa, invasive pneumococcal disease is 3-fold higher in the elderly population than in the younger population [25]. Mortality rates from IPD are also higher too [26].

In 2011, IPD accounted for over 36,000 cases in the USA. The incidence of IPD is strongly related to age with 38% of cases occurring in children under the age of two years and 54% in adults over 50 [27]. In Europe, IPD burden ranges from 11 to 27 per 100,000 people and in Asia up to 216 cases per 100,000 are reported annually [28] [29].

Surveys done in the United States of America in 2000 before the introduction of pneumococcal conjugate vaccine (PCV7), found the incidence of pneumococcal disease in the over 65 populations were second only to that of children under the age of two[30]. Analysis of 1258 cases requiring hospitalisation showed that 76.6% of disease manifested as pneumonia, 3.6% meningitis and 17.5% sepsis. Case fatality rates among those 80 years and over was 20.6% and persons aged 65 or older accounted for 51% of all deaths due to IPD[31]. In high-income countries, pneumococcal disease burden is high in the elderly due to a range of comorbidities and waning immune system, contributing to an increased risk of pneumococcal pneumonia. Many studies have concentrated on rates of community acquired pneumonia (CAP) in elderly patients where *S. pneumoniae* is the most commonly isolated pathogen (40-50%). In Germany, overall incidence of CAP was 7.7 per 1000 people, rising to 35.8 in adults over 90 years of age. Among adults over 50

years in the US, approximately 30,000 cases of pneumococcal disease and over 500,000 cases of pneumococcal pneumonia were estimated to occur yearly and resulted in more than 25,000 pneumococcus-related deaths.

Children:

Streptococcus pneumoniae is the leading bacterial cause of pneumonia, sepsis and meningitis in children under the age of five worldwide. The World Health Organisation (WHO) estimates around 800,000 children die due to pneumococcal disease each year, with 90% of these deaths occurring in developing countries [1]. Prior to the introduction of PCV7 for US children under 5 years in 2000, approximately 65,000 cases of IPD occurred annually. Shortly after the introduction of the vaccine, IPD incidence declined rapidly, and 7 years after the vaccine was licensed for use, overall disease rates were 45% lower in all age groups and 76% lower for children under five years of age [32]. The effectiveness of PCV7 and the more recent PCV13 vaccination in high income countries such as the USA and UK have been well documented. However, the effectiveness of these vaccines in low to middle income countries is not yet published. Incidence of IPD varies among populations and countries due to underlying differences in healthcare, poverty and HIV status. These factors account for why large amounts of disease and high mortality rates are seen in Africa [33]. Case control studies have been performed in South Africa to assess the effectiveness of PCV13 against IPD in HIV-positive and HIV-negative children [34]. Results showed that the 2+1 schedule was 85% effective for preventing IPD caused by serotypes included in PCV13 in children without HIV. However, PCV13 was less effective (54%) for overall IPD in HIV-negative children. However, this study is likely to have underestimated the

effectiveness of PCV13 due to small sample size and failure to examine herd effect [35]. New data produced from the Gambia and South Africa show results comparable with those from high-income countries and should encourage remaining countries to include pneumococcal conjugate vaccines into their immunization programs[34]. Figure 2 demonstrates global pneumococcal mortality rates; the majority of deaths in HIV negative children occurred in ten African and Asian countries [1].

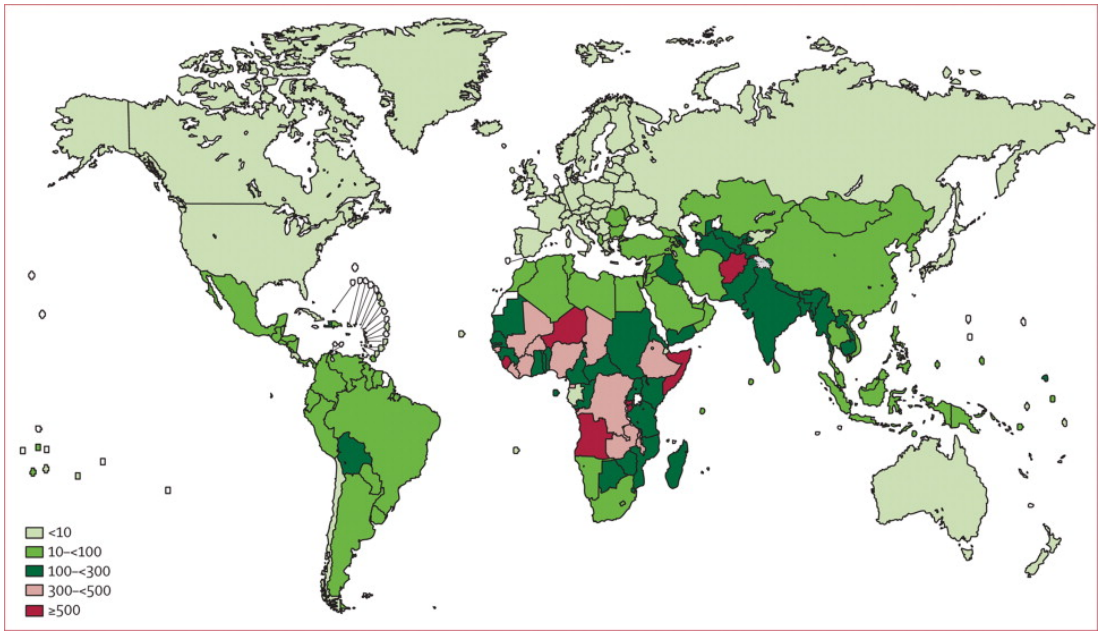


Figure 2 Global Pneumococcal mortality rates. Pneumococcal deaths in children under the age of 5 per 100,000 children (HIV Negative deaths only). Highest rates of death seen in Africa. Taken from O'Brien et al, 2009.

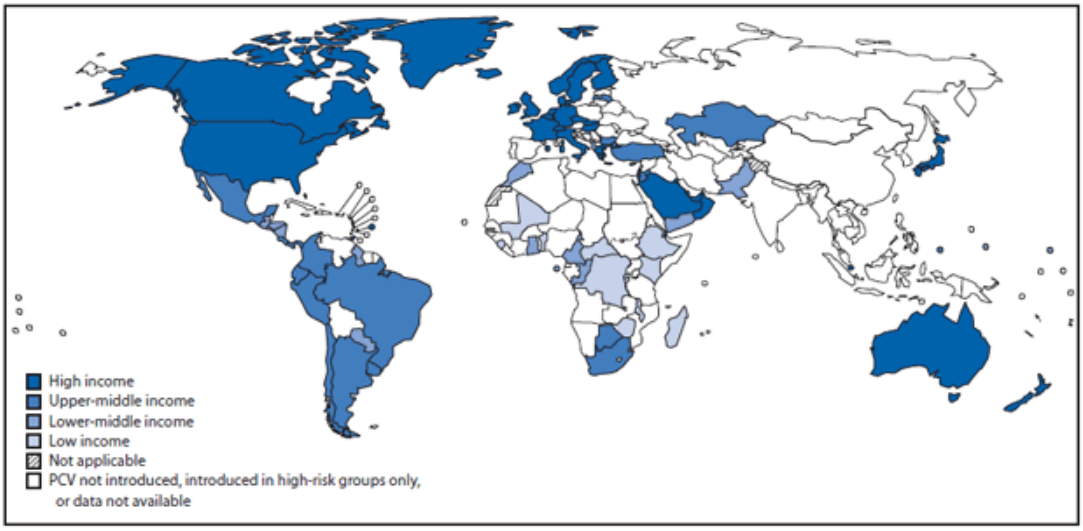


Figure 3 Countries that have introduced the pneumococcal conjugate vaccine (PCV) in their national immunisation programs, by income status in 2012. 73% of 50 high-income countries introduced PCV whereas 37% of low income, 35% of lower-middle income, and 34% of upper-middle countries. Taken from Progress in Introduction of Pneumococcal Conjugate vaccine – worldwide, 2000-2012 (cdc.gov).

Figure 2 and 3 show the current situation with IPD mortality rates and global vaccine use. There is a lack of vaccine use in many of the areas with the highest rates of IPD where vaccines are needed most.

The Pneumococcal Global Serotype Project is a systematic evaluation of serotypes causing IPD globally among children under the age of 5. The review

covers studies from 1980 to 2007, after PCV vaccination had been introduced. The study examined which serotypes were most commonly causing IPD. Serotype 14 was found to be the most common cause of IPD in every region and serotypes 1, 5, 6A, 6B, 14, 19F and 23F collectively accounted for over half of IPD in all regions [36]. Based on WHO Global Burden of Disease (GBD) Project incidence for the year 2000, the seven global serotypes listed above accounted for approximately 9 million cases and 500,000 deaths in children under the age of five[36].

It was reported that globally and regionally, between 6 and 11 serotypes accounted for over 70% of IPD. Epidemiological studies such as these have been essential to inform vaccine design. As the global distribution of serotypes had been well characterised, informed selection of serotypes for inclusion in the next generation of PCV (PCV13) was possible, enabling coverage of serotypes that previously accounted for 49%-88% of deaths in Africa and Asia[36]. Rational vaccine design will help to reduce the 800,000 child deaths per year, 90% of which occur in developing countries[36].

1.2.2 Global pneumococcal carriage rates

Carriage rates vary globally; in Europe, pneumococcal carriage is common in early life, with a 30-60% prevalence in young children and only 1-10% in adults[3]. However, using a systematic review, carriage in children under 5 in sub-Saharan Africa was found to be 63.5%, 42.6% in ages 5 to 15 and 28% in adults [11]. Lower carriage rates in adults suggest that colonisation in early life provides some immunological protection in adulthood[37].

Interestingly, concurrent carriage of up to six serotypes has been observed in approximately 95% of children by the age of two [38] [39] but little is currently understood about co-colonisation with multiple serotypes or the clinical significance this may have.

The distribution of colonising serotypes differs in children compared to adults. Studies found that, prior to PCV7 introduction, serotypes 19F, 6A, 6B and 23F were highly prevalent in children under the age of 5, whereas serotypes 3 and 23F were the most common in adults [40-42]. Post PCV7 vaccination, it was found that 19A, 6C, 11A, 15A, and 15B/C non-vaccine serotypes are now the most prevalent in carriage; a phenomenon called serotype replacement whereby serotypes covered by vaccination are replaced by non-vaccine serotypes [43].

The duration of carriage can vary significantly (from days to months) between serotypes. For example, serotype 1 is rarely found causing any nasopharyngeal carriage, whereas serotype 6B can colonise for over 19 weeks [4,44]. The mechanisms of nasopharyngeal colonisation will be discussed in detail later in this introduction.

1.2.3 Invasive disease potential

As mentioned previously, some serotypes are commonly found causing asymptomatic nasopharyngeal carriage whereas others are more commonly associated with invasive disease. Several methods have been used to calculate the invasive disease potential of specific serotypes. The most common of these is the invasive odds ratio (OR). The OR calculation is as follows:

Invasive odds ratio (OR) = (ad)/(bc)

Whereby, a is the number of invasive A serotypes, b is the number of carriage A serotypes, c is the number of invasive non-A serotypes and d is the number of carriage non-A serotypes[42,45]. Table 1 shows the serotype-specific invasive disease potential of *Streptococcus pneumoniae* by country.

Study author	Brueggemann *et al* [45]	Hanage *et al* [46]	Sa-Leao *et al* [47]	Shouval *et al* [48]	Kronenburg *et al* [42]	Rivera-Olivero *et al* [49]
Study Period (Year)	1994-2001	1995-1999	2001-2003	2000-2004	2002-2004	2003-2009
Study Area	UK	Finland	Portugal	Israel	Swiss	Venezuela
Highly invasive serotypes	**1,**4,14,18C	6B, 14, 18C, 19A	**1**,3,4,5,7F,8,9N,9L,12B, 14,18C,20	**1**,5,12F	**1**,4,5,7F,8,9V,14	**1**,5,7F,18

Table 1 Capsular serotype-specific invasive disease potential of *Streptococcus pneumoniae*. OR, odds ratio. Adapted from Song, Y.N *et al* 2013

Serotype 1, 4, 5 and 14 appear to be some of the significantly invasive serotypes in a variety of geographical locations before the introduction of PCV7 [50]. Little has been documented on the invasive odds ratio of serotypes present in developing countries such as Africa. Use of the Invasive OR is a useful tool for mapping prevalence of serotypes causing high rates of IPD, which is critical for highlighting serotypes needed to be covered in new vaccines.

1.2.3 Clinical presentations

Different pneumococcal serotypes are known to be associated withs differences in clinical presentations of pneumococcal disease. *Streptococcus pneumoniae* is the most common cause of community acquired pneumonia (CAP) worldwide [51]. Bacteraemic community acquired pneumonia is more

severe than non-bacteraemic and they are therefore considered to be different clinical entities [52]. Table 2 shows the top three serotypes associated with different types of pneumonia.

Authors	Gentile et al[53]	Bender et al[54]	Resti et al[55]	Bewick et al[56]	Cilloniz et al[57]
Study design	Meta-analysis	Retrospective study	Observational Study	Prospective observational study	Prospective observational study
Study period (year)	1980-2008	1997-2006	2007-2009	2008-2010	2006-2009
Study Area	Latin American & Caribbean	USA	Italy	UK	Spain
Age of study population	<5 yrs	<18yrs	0-16yrs	>16yrs	Adult
Pneumonia type	CAP	Non-bacteraemic pneumonia	Bacteraemic CAP	CAP	Bacteraemic CAP
Serotypes	14 (33.0%) **1 (11%)** 5 (10%)	**1 (22.6%)** 3 (11.3%) 19A (10.5%)	**1 (32.5%)** 19A (15%) 3 (12.5%)	14 (18.6%) **1 (16.5%)** 8 (14.5%)	**1 (32.1%)** 19A (17.9%) 7F (6.0%)

Table 2. Serotype distribution of patients with pneumococcal pneumonia. Adapted from Song, Y.N *et al* 2013.

In all studies listed in table 2, serotype 1 is amongst the top two serotypes causing pneumonia in both adults and children, suggesting this is one of the most invasive serotypes found across the world. In addition to pneumonia, serotypes 1, 3, 7F and 14 are commonly associated with empyema. Empyema is a serious complication characterized by pus and bacteria in the pleural space [58]. This condition is more commonly found in adults but incidence but has been increasing in the child population over the last decade[59].

S. pneumoniae is a major pathogenic cause of meningitis and results in over 60,000 deaths and long term disabilities in children under the age of five worldwide[1]. Studies performed in the African meningitis belt have identified serotype 1 as the cause of 60-80% of pneumococcal meningitis [60]. Unusually,

adults and older children were more likely to be affected and high case fatality rates were observed.

Pneumococcal epidemiology of different capsular types varies geographically and temporally. Little data are currently available regarding the serotype distribution after the introduction of the PCV13 vaccine, which includes serotype 1. Data between the 1980s and 2010 have shown serotype 1 to be a prevalent serotype globally and one of the major serotypes causing pneumonia and meningitis. Unlike some other serotypes, disease caused by serotype 1 can often be found in older children and adults, suggesting serotype 1 is one of the most virulent *S. pneumoniae* serotypes [44].

1.3 Pneumococcal virulence factors

The pneumococcus produces a wide range of protein virulence factors that make major contributions to pathology and the induction of immune responses and that also have potential for use as vaccine candidates that may offer broad serotype coverage.

Over recent years, many new virulence factors and their importance in not only carriage versus invasive disease, but also their role in different niches of the host have been widely discussed. The main pneumococcal virulence factors are shown in figure 4 and each will be discussed in turn.

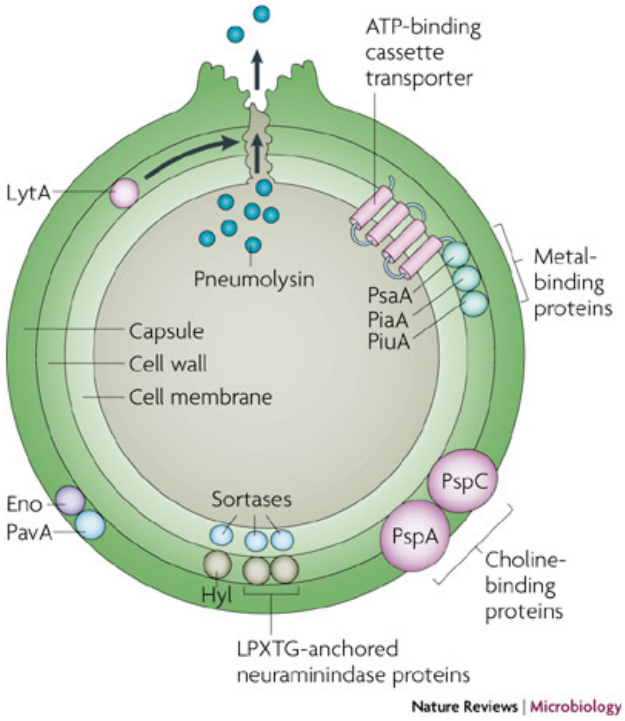


Figure 4 Pneumococcal virulence factors. Selection of important virulence factors and their location in relation to the structure of the pneumococcus. Virulence factors listed here include; the capsule, choline-binding proteins, metal-binding proteins, PavA, LytA and, pneumolysin. Figure taken from Kadioglu *et al,* 2008.

1.3.1 Polysaccharide capsule

One of the major virulence factors of *Streptococcus pneumoniae* is the extracellular capsular polysaccharide (CPS). Forming the outermost layer of *S. pneumoniae*, the capsule is 200-400nm thick and consists of chains of monosaccharides [61]. Pneumococcal serotypes can carry structurally distinct capsular polysaccharides composed of repeating oligosaccharide units joined together by glycosidic linkages [16]. The genes for capsule synthesis are located on the CPS operon between *dexB* and *aliA* genes. The first four genes (*cpsA* to *cpsD*) are downstream of serotype-specific genes and play a role in the regulation of capsule expression [62]. These regulatory genes are highly conserved across serotypes of *S. pneumoniae*.

The importance of the capsule for virulence is clear. Firstly, the majority of clinical isolates causing invasive disease are encapsulated, secondly, different capsular serotypes vary in their ability to cause invasive disease, and finally, loss of capsule by genetic mutation or enzymatic degradation dramatically reduces virulence in murine models of infection [19,62-66]. In addition, swapping capsular serotypes between strains can affect virulence in animal models of infection [67 68 69]. Capsule can also protect the bacteria from the host immune response by affecting susceptibility to neutrophil extracellular traps (NETs), killing by defensins and clearance by mucus [70 71 72].

Variations in susceptibility to host immune defences and invasive disease potential are attributable to differences in capsular serotypes. The main function of the polysaccharide capsule is to protect the bacteria from the host immune response by forming a shield around the bacteria that is strongly anti-phagocytic and blocks complement binding. An effective immune response against pneumococci is largely dependent on the deposition of C3b complement, and the level of complement deposition is largely influenced by the capsular serotype [73]. For example, studies have shown that significantly more complement is deposited on the surface of the highly carried serotype 6B, compared to 19F which is associated with invasive disease. In addition, significantly higher concentrations of polysaccharide-specific antibody are required for opsonophagocytic killing of 19F isolates [74]. Thus, resistance to complement and phagocytosis appears to be commonly associated with capsular polysaccharides found on invasive isolates.

It appears that the amount of capsule expressed and the metabolic demand of its synthesis can affect invasiveness. Many reports have shown that a thick

capsule protects the bacteria from phagocytosis after invasion, however, a thick capsule will also reduce ability to adhere to epithelial cells and therefore hamper colonisation of the nasopharynx [75] [76] [77]. Bacteria isolated from the blood have large capsules which are able to reduce complement deposition and aid bacterial survival in the blood [78]. In addition, in an infant rat model of meningitis, when comparing 6B and 7F isolates, it was found that 6B caused severe disease and inflammation in the CSF and had a significantly thicker capsule than 7F [76].

Weinburger et al have suggested that polysaccharide capsules can differ in the metabolic demand that their synthesis places on the bacteria. They described an inverse correlation between the number of carbons and high-energy bonds with colonisation preference [79]. Further work by *Hathaway et al* suggests that serotypes prevalent in carriage produce capsule with lower metabolic demand, aiding their survival in a low nutrient environment. In contrast, invasive capsular serotypes need more nutrients to produce their capsule and so have a preference for more nutrient-rich areas such as the blood [75].

The majority of clinical isolates of *Streptococcus pneumoniae* consist of two heterogeneous populations of clonal opacity [80]. Opaque (O) and transparent (T) colony variants of the same strain can differ in the amount of CPS produced, with opaque colonies producing between 1.2 to 5.6 fold greater amounts of capsule [81]. Studies of these phase variations in animal models revealed that transparent variants persist for longer in the nasopharynx and show greater ability to adhere to epithelial cells *in vitro*. In contrast, murine sepsis models showed a strong selection for opaque (O) variants during

invasive disease [80]. However, phase variation during colonisation or invasive disease does not appear to be as definitive as this. Increased adherence of T variants to lung and pharyngeal epithelial cells was due to decreased thickness of capsule [80]. Following successful colonisation, a thin capsule may no longer be advantageous as it is associated with increased susceptibility to complement deposition and phagocytosis, which is not beneficial for survival in either the nasopharynx or blood. These observations suggest that the pneumococcus varies between the T variant with increased adherence to cells which is optimal for establishing carriage, and the O form which, with its thicker capsule, is better adapted to survive inflammation and the host immune responses in nasopharynx or invasive sites [82].

In summary, the presence of capsule is essential for isolates to cause disease, hence it is used as a target for pneumococcal vaccination. In addition, the thickness and type of capsule present can determine the virulence of a specific strain. However, there are many other virulence factors that are also important for the pneumococcus, and these will now be discussed.

1.3.2. Pneumolysin

Pneumolysin is a 53kd protein produced by virtually all clinical isolates of *Streptococcus pneumoniae* [83]. It belongs to the family of cholesterol-dependant cytolysins (CDCs) which are a group of pore-forming toxins expressed by over twenty species of Gram-positive organisms [84]. Pneumolysin has the capacity to induce pores in cholesterol-rich membranes, and this action equates to haemolytic activity [85].

Structurally, pneumolysin is described as an elongated, rod-like molecule consisting of four domains (D1-D4). Domain 1 (D1) compromises six α-helices surrounding a five-stranded β-sheet. D2 connects D1 to D4 by four β-strands while D3 consists of a five stranded anti-parallel β-sheet surrounded by two helix bundles [86]. (Figure 5)

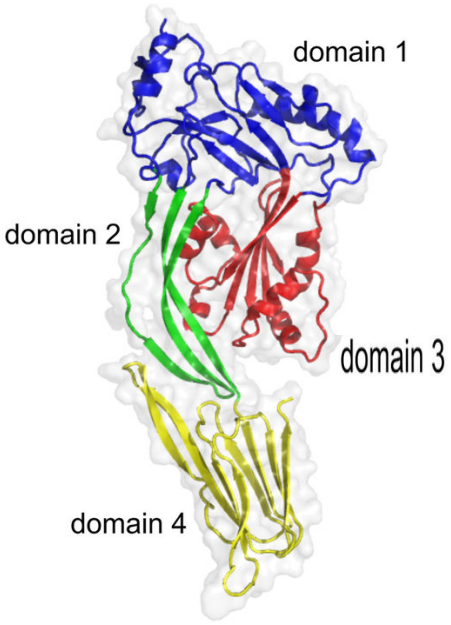


Figure 5 Domain structures of Pneumolysin. Taken from Marshall *et al*, 2015

Domain 4 contains a conserved tryptophan-rich motif that is critical for binding to cholesterol-containing membranes [87]. Once domain 4 has successfully interacted with mammalian membranes, domain 3 is responsible for pore formation.

Pore formation by CDCs follows a series of steps. Firstly, soluble monomeric pneumolysin binds to cholesterol-containing membranes. Once toxin is bound, ring shaped oligomers form and a subtle change in shape increases D4 interaction with the membrane and favours formation of a 'pre-pore'. In the

final step, lipids are removed from within the 'pre-pore' ring and forms the pore complex which leads to lysis of the mammalian cell [86].

Pneumolysin lacks an N-terminal secretion signal so cannot be actively transported from the cytoplasm to the surface of the bacteria. The traditional paradigm states that release of pneumolysin is dependent on the activation of autolysin (LytA) [88]. Autolysis is characterised by degradation of the cell wall to release virulence factors present in the cytoplasm including pneumolysin. However, this has been challenged by several observations. Firstly, mutants that lack autolysin are still able to release pneumolysin and can be as virulent as wild-type bacteria of the same strain in murine models of infection [89]. Secondly, bacteriophages may harbour autolysin-like genes that cause cell death and trigger the release of pneumolysin [90]. Thirdly, pneumolysin has been detected in the cell wall of pneumococci in the absence of autolysis. Pneumolysin found in the cell wall was shown to be active by haemolytic assays [91]. Finally, studies have shown that high levels of pyruvate oxidase can also contribute to pneumolysin release [92]. Thus, the mechanisms by which pneumolysin is released from the pneumococci remains unclear.

As pneumolysin is a major virulence factor of pneumococci, it is the cause of many disease manifestations associated with pneumococcal disease. At sub-lytic concentrations, pneumolysin aids bacterial virulence by a) inhibiting ciliary beating of respiratory epithelium, b) inhibiting phagocytic respiratory burst in phagocytic cells and c) inducing synthesis of cytokines and promotion of $CD4^+$ T cell activation[93,94].

Experimental evidence shows that when purified pneumolysin is administered into murine lungs, it can mimic an array of features of pneumococcal

pneumonia [95]. Highly haemolytic pneumolysin has been shown to have direct cytotoxic effects on lung epithelial cells and may lead to a high level of cell death. Increasing levels of cell cytotoxicity can lead to the separation of tight junctions between epithelial cells and alterations in alveolar or peritoneal permeability [96]. This ultimately leads to dissemination of bacteria into the bloodstream and the onset of bacteraemia.

Isogenic mutants have been created to assess the activity of pneumolysin. When serotype 2 pneumococcal strain D39 lacking pneumolysin (PLN-A) was intranasally administered to mice, virulence of the bacteria was greatly reduced. Infection with PLN-A resulted in lower inflammation, a reduced rate of bacterial replication and a delayed onset of bacteraemia when compared to the wild type parent [97].

When intravenously administered into the blood of mice, PLN-A caused chronic bacteraemia over 7 days whereas the wild type parent exhibited rapid exponential growth that led to death around 28 hours post infection[98]. These findings suggest that pneumolysin aids rapid bacterial growth in blood and absence of the toxin results in high pneumococcal numbers being tolerated in blood without disease symptoms.

Studies have also shown that pneumolysin plays an essential role in nasopharyngeal colonisation [68]. *In vitro* studies have found pneumolysin-deficient mutants had reduced adherence to respiratory epithelial cells [99]. Further to this, animal models of nasopharyngeal carriage using PLN-A mutants demonstrate reduced density and duration of carriage [68 100 101].

Whilst the role of pneumolysin in pneumonia and carriage is well understood, the effect of pneumolysin in bacterial meningitis is less clear. Meningitis occurs

when bacteria either breach the blood-brain barrier or invade olfactory neurons and migrate into the CSF [102]. Once in the CSF, an inflammatory response is initiated, leading to toxic effects in the brain and disease symptoms occur[103]. When investigating the role of pneumolysin in meningitis, one report found no difference in the levels of inflammation in the brain of rabbits infected with pneumolysin-deficient or wild-type pneumococci[104]. However, *in vitro* cell culture models suggest that pneumolysin is key to breaching the blood-brain barrier and therefore is essential to the development of meningitis[105]. High levels of pneumolysin present in the CSF of patients with pneumococcal meningitis was found to be associated with poor prognosis and high mortality rates [106].

Pneumolysin is an important virulence factor that aids in nasopharyngeal carriage, bacterial survival and replication in the lungs and blood. Cytotoxic effects of pneumolysin causes mammalian cell damage and allows for bacterial dissemination from the lungs, into blood and possibly translocation across the blood-brain barrier to cause meningitis. However, there is a serotype 1 clinical strain (ST306) that has been found to produce a non-haemolytic pneumolysin but is a predominant cause of IPD in continental Europe and the South Pacific [107] [108].

Emergence of ST306 in Europe caused serotype 1 to rise from 10th to 4th most prevalent serotype causing IPD in Scotland between 2001 and 2003 [109] and an increased incidence of empyema has been associated with the emergence of the ST306 clone [110]. In Sweden, serotype 1 IPD increased from 1% in 1992 to 10% in 1997, due to the emergence of ST306 [111]. Prior to the introduction

of PCV13 vaccination, ST306 was the dominant serotype 1 strain in continental Europe.

Between 2000 and 2007 a surveillance system was set up in the South Pacific to monitor pneumococcal serotype prevalence. Interestingly, the most prevalent serotype was found to be serotype 1 strain ST306. In New Caledonia, ST306 was responsible for IPD outbreaks and demonstrated a propensity to cause pneumonia and septicaemia in healthy indigenous people who had no previous risk factors for IPD. All patients with ST306 IPD were hospitalised with pneumonia and/or septicaemia but no deaths, cases of meningitis or referrals to intensive care occurred [107].

Despite expression of a non-haemolytic pneumolysin, ST306 serotype 1 has caused a marked increase in IPD in many European countries such as England, Wales, Scotland, Netherlands and Portugal. Murine studies of ST306 have shown that mild disease occurs in MF1 mice compared to serotype 1 with haemolytic pneumolysin [108]. Using very high doses of ST306, no difference was found in survival times of intravenously infected ST306 and ST227 clone of serotype 1 which expresses a haemolytic pneumolysin, suggesting haemolytic activity of pneumolysin has no effect of virulence in some animal models of infection [108]. Thus, a haemolytically active pneumolysin may not be essential for disease pathogenesis. Previous work suggests that haemolytic activity of pneumolysin is important in the initial stages of pneumonia, which include lung tissue invasion and neutrophil recruitment, but is not essential for bacterial replication in the lung [85 96]. However, it is not yet clear whether expressing non-haemolytic pneumolysin

makes ST306 better adapted to nasopharyngeal colonisation compared to serotype 1 clones expressing haemolytic pneumolysin.

Pneumolysin and the host immune response:

As pneumolysin has been widely accepted as a highly immunogenic pneumococcal virulence factor, studies have been performed to investigate whether antibodies directed against pneumolysin may protect from IPD.

One study examined levels of anti-pneumolysin IgG in hospital admissions of patients [112]. They found that patients with non-bacteraemic pneumococcal pneumonia had higher levels of serum anti-pneumolysin IgG compared to patients with bacteraemic pneumococcal pneumonia or uninfected patients, suggesting that antibodies generated against pneumolysin may offer protection against severe IPD. In addition, intravenous administration of human anti-pneumolysin IgG protected mice from intraperitoneal challenge with serotypes 1 and 4. Antibodies directed against pneumolysin were found to increase survival times of infected mice [96]. Administration of anti-pneumolysin antibodies appeared to reduce inflammation and damage to the peritoneal lining, thus reducing bacterial invasion into the bloodstream. Mice given anti-pneumolysin IgG also had enhanced clearance of bacteria from the bloodstream [112].

Interactions of pneumolysin with the host immune system have been widely described. Pneumolysin is known to activate complement and initiate inflammation but it also influences host innate and adaptive immune responses.

Nasopharyngeal colonisation is an essential prerequisite for pneumococcal disease [3]. Studies using PLN-A have shown that pneumolysin is essential for successful nasopharyngeal colonisation as it is important for adherence to respiratory epithelial cells, as demonstrated by Rubin *et al* who showed pneumolysin-deficient mutants had reduced attachment to cells.

Pneumolysin can also have a direct effect on immune cells in the lung during pneumonia. Upon pneumococcal invasion, neutrophils rapidly infiltrate the lungs and are the main innate cell type responsible for bacterial clearance [113]. The rate of immune cell infiltration into the lung is greatly influenced by pneumolysin. If pneumolysin is absent then there appears to be a delay in immune cell infiltration which results in lower levels of inflammation over the course of infection [114]. However, if pneumolysin is present in the lungs it contributes to tissue damage and results in higher levels of inflammation. In turn, this leads to a faster, greater influx of neutrophils and other immune cells into the lungs. Activation of complement by pneumolysin also increases immune cell infiltration. Evidence from *in vitro* work suggests that when neutrophils arrive into the infected lung, pneumolysin can inhibit the phagocytic ability of these cells due to its cytotoxic activity [114].

T lymphocytes are also involved in clearance of pneumococci from the lungs [114]. When comparing infection of PLN-A with wild-type D39, it was found that recruitment of T cells in the lungs was significantly reduced and delayed in the absence of pneumolysin [94].

During pneumococcal infection, pneumolysin may also be involved in inducing inflammatory cytokines through involvement with Toll-like receptors (TLRs) [115]. The ability of pneumolysin to promote expression of co-stimulatory molecules

on macrophages and to promote the release of inflammatory cytokines was originally thought to be dependent on TLR4 activation but more recent evidence suggests this is independent of TLR-4[116]. Instead, pneumolysin induces NLRP3 inflammasome activation independently of TLR4 activation and leads to the secretion of an array of pro-inflammatory cytokines including, interleukin (IL)-1α, IL-1β, IL-23, tumour necrosis factor (TNF)α and IL-6 [116 117]. Highly haemolytic pneumolysin causes inflammation as a result of cell death. Upon death, cells release lysosomal products which are seen by the host immune system as danger signals and detected by NLRP3 inflammasomes. Upon inflammasome assembly, caspase-1 is activated and the pro-inflammatory cytokine IL-1β is secreted from dendritic cells [116]. Via its ability to induce cytokine production, pneumolysin can also aid in T cell differentiation. For example, IL-6 is needed for downstream Th17 differentiation and pneumolysin-enhanced secretion of IL-12 is important for Th1 differentiation [118].

To conclude, evidence from animal, human and *in vitro* studies provide evidence for a critical role for pneumolysin in disease pathogenesis. Mutants of *S. pneumoniae* lacking pneumolysin are much less virulent and are not found to cause disease in humans. Whilst presence of pneumolysin is essential for virulence, the haemolytic activity appears to be less important as demonstrated by ST306.

The neutralisation of pneumolysin using antibodies appears to have great potential in future therapeutics for treating pneumococcal disease and detoxified pneumolysin variants could be excellent candidates for inclusion in future vaccines.

1.3.3 Autolysin

Autolysis is a process that *S. pneumoniae* undergoes when it reaches stationary growth phase. It is controlled by the product of the *LytA* gene, which degrades the pneumococcal cell wall, resulting in lysis and release of fragmented peptidoglycans and intracellular products, including pneumolysin [119]. LytA is an N-acetyl-muramyl-L-alanine amidase and is the major autolysin found in *S. pneumoniae* [120]. The pneumococcus also encodes two other lytic enzymes known as LytB and LytC. The exact roles of these enzymes is unknown but they are currently thought to be involved in cell wall growth during growth and division [121,122].

Although LytA has been widely accepted as being involved in the release of pneumolysin, not all serotypes depend on LytA for this. For example, serotype 3 was found to express pneumolysin prior to reaching stationary phase [123]. When LytA mutants were created in this serotype, there was little difference in secretion of pneumolysin, thus suggesting that serotype 3 pneumolysin secretion is not dependant on LytA[89]. However, this serotype does express LytB and LytC which may trigger the release of pneumolysin[121]. Of note, this study only found LytA to be irrelevant for serotype 3 and it is not a common finding for all serotypes.

Many studies have shown that pneumococci with mutations in *LytA* are less virulent compared to wild type bacteria[98,124,125]. The precise reason for this is not fully understood but there are several potential explanations. Firstly, activation of autolysis leads to the release of pneumolysin which is a key virulence factor[126,127]. Secondly, products released from the degraded cell wall such as peptidoglycan fragments have been shown to activate toll-like

receptor 2 (TLR2), leading to an inflammatory response including enhanced production of IL-8 [128]. A third explanation is that fragments of bacteria released during autolysis are able to significantly reduce production of phagocyte-activating cytokines[129]. The ability of the host immune response to phagocytose and eliminate bacteria is dependent on the release of TNFα by macrophages, interferon (IFN)-γ production by Natural Killer (NK) cells and T cells and IL-12 production by macrophages[130-133]. In general, infection with Gram positive bacteria induces high titres of these cytokines but around 30% lower titres have been observed during pneumococcal infection [134]. In addition, intact bacteria stimulate IL-12 production by monocytes but fragments of the same bacteria down-regulate IL-12 production in a dose-dependent manner [135]. Evidence suggests that when autolysis of *S. pneumoniae* occurs, cell wall fragments down-regulate the production of IL-12 which has a knock-on effect for IFNγ production and prevents phagocytic cell activation. Further evidence that autolysis prevents phagocytosis is demonstrated by the finding that autolysed bacteria can inhibit the ability of blood cells to eliminate viable pneumococci *in vitro*[129]. It is thought that only around 10% of pneumococci need to autolyse during an infection to significantly down regulate the production of phagocyte-activating cytokines and prevent the elimination of the remaining bacteria [129].

Autolysis has often been referred to as the suicidal characteristic of the pneumococcus [136]. However, evidence suggests a new physiological function of LytA in avoiding anti-capsular immune responses during early stage acute lung infection[137]. Upon interaction with antimicrobial peptides both *in vitro* and

in vivo, the pneumococcus is thought to be able to remove its capsule in a process known as capsule shredding and this process is controlled by LytA. Shredding of its capsule has many advantages for the pneumococcus. It can avoid anti-capsular responses and increase its ability to invade local epithelial cells and escape the host immune system. This finding suggests that LytA does not just cause autolysis but appears to be involved in controlling the amount of capsule expressed on the bacterial surface.

1.3.4 Pneumococcal surface protein A (PspA)

PspA is a member of the choline-binding protein family and is expressed on all known clinical isolates of pneumococcus [138]. As this protein is cross reactive and serologically variable, it has been shown to be a promising candidate antigen for future protein-based vaccines. Research using murine models has shown that passive administration of antibodies to PspA are protective against pneumococcal challenge [139 140 141 142 143].

Current understanding of the main role of PspA is that it protects the pneumococcus from complement-mediated phagocytosis by blocking both C1q and C3 deposition on the bacterial surface [144,145]. In addition, PspA is able to protect pneumococci from the bactericidal effects of apolactoferrin found on host mucosal surfaces [146]. Mutants lacking surface PspA show reduced virulence, greater clearance from the blood and higher levels of complement deposition in serum [147 148]. Recent evidence has also suggested several mechanisms whereby PspA may influence the immunosuppressive activity of *S. pneumoniae*. One study states that PspA can exert immunosuppressive

ability by inhibition of IgG humoral responses therefore aiding bacterial colonisation [149].

1.3.5 Pneumococcal surface protein C (PspC)

PspC is a polymorphic choline-binding protein that is found in 75% of pneumococcal strains[150] and has been described by various research groups based on its different functional properties. This virulence factor has many roles; firstly, it is important in protecting *S. pneumoniae* from complement-mediated killing via binding human factor H to block activation of the alternative complement pathway[151,152]. PspC can also bind to secretory IgA receptor to aid invasion of the mucosa[153,154]. Mutants of *S. pneumoniae* lacking PspC have shown this virulence factor is essential for colonisation and adherence to lung cells[145]. Finally, invasion of the CSF and attachment to the blood-brain barrier is dependant on PspC expression[155,156].

1.3.6 Pneumococcal surface antigen A (PsaA)

PsaA is a member of the metal-binding lipoprotein family expressed by all serotypes of S. *pneumoniae,* and is essential for transportation of metal ions; Mn^{2+}, Zn^{2+} and Fe^{2+}[157]. Studies have shown that loss of PsaA in pneumococci renders them avirulent in murine models of carriage, pneumonia and sepsis[158-160]. Functional PsaA aids resistance against oxidative stress and prevents killing by H_20_2 and superoxide [161]. Recently, PsaA has been used as a pneumococcal antigen in an intranasally administered mucosal vaccine in mice. Using a chitosan delivery method, nanoparticles of chitosan-PsaA successfully induced mucosal and systemic immune responses that increased

protection from acute otitis media and invasive pneumococcal infections [162]. Thus PsaA has promise for use in future vaccine design.

1.3.7 PiuA and PiaA

PiuA (pneumococcal iron uptake A) and PiaA (pneumococcal iron acquisition A) are lipoproteins involved in two separate ion uptake ABC transporter operons. Both of these highly-conserved metal-binding ions are needed for bacterial growth and full virulence in murine models of infection[163].

1.3.8 Pneumococcal adhesion and virulence protein (PavA)

This fibronectin-binding protein is essential for pneumococcal adherence to epithelial cells[164] and has key roles in modulating inflammation to maintain carriage and aid in the development of sepsis[165,166]. In addition, PavA protects pneumococci against recognition and phagocytosis by dendritic cells *in vivo*[167]. PavA mutant pneumococci have been shown to be cleared quickly from the nasopharynx during carriage. However, in a pneumonia murine infection model, pavA mutants persisted in the lungs and caused a chronic infection whereas wild type pneumococci caused systemic infection. When pavA mutant bacteria were intravenously injected into blood, survival times were comparable with wild type pneumococci [166].This suggests that pavA has an important role in bacterial seeding from lungs into blood and also from blood into the brain to cause meningitis. As pavA is involved in bacterial translocation, it is a promising target for future vaccine design that may allow carriage to occur but may stop the dissemination of bacteria to other tissues and therefore prevent the onset of IPD.

1.4 Host immune responses during *S. pneumoniae* infection

1.4.1 Nasopharyngeal carriage

The pneumococcus is an important commensal of the human nasopharynx and colonisation is thought to be a pre-requisite for invasive disease [168] [3] [169]. The initial steps of nasopharyngeal colonisation involve the adherence of pneumococci to local epithelial cells [170]. Presence of pneumococci in the nasopharynx triggers an immune response at the mucosal surface. Initially, a carefully controlled inflammatory response is generated, followed by increased infiltration of immune cells which leads to the clearance of colonising pneumococci and the development of protective immunity[171].

Immune responses initiated at mucosal surfaces such as the nasopharynx need to be carefully balanced between Th17-mediated inflammation and anti-inflammatory T-regulatory (Treg) cells [172] [173] [100]. The suppression of inflammatory responses is important to ensure minimal tissue damage and preservation of the natural flora within the nasopharynx. Recently, a role for transforming growth factor β (TGFβ) and T regulatory cells in maintaining nasopharyngeal carriage by pneumococci has been described[100]. TGFβ has a pivotal immunosuppressive role in both innate and adaptive immunity. This cytokine, released in response to epithelial cell damage, reduces inflammation and promotes tissue healing and remodelling [174]. Experimental evidence using murine carriage models shows low density of pneumococcal colonisation in the nasopharynx induces immunoregulatory responses with elevated T regulatory cells and TGFβ levels, whilst higher bacterial loads induce a strong inflammatory response resulting in rapid clearance of pneumococci from the

nasopharynx[100]. Low pneumococcal density and high immune-regulatory responses result in maintenance of carriage for prolonged periods without the onset of invasive disease. Inhibition of TGFβ-1 resulted in increased dissemination of bacteria from the nasopharynx into the lungs, reduced pneumococcal carriage and a failure to induce T regulatory responses in the nasopharynx. This highlights the role of TGFβ-1 and T regulatory cells in allowing the persistence of pneumococcal carriage by limiting pro-inflammatory responses and tissue damage that would allow for bacterial dissemination into the lungs. Pneumococcal pneumolysin appears to be the key to activating host immune modulatory responses during carriage, through a process dependent upon epithelial cells and the NLRP3 inflammasome [100].

Innate immune responses are also important in controlling nasopharyngeal carriage of *S. pneumoniae*. Infiltrating monocytes and resident macrophages play major roles in the containment and clearance of pneumococci [175] and macrophages expressing characteristic markers of alternative activation accumulate in the nasopharynx and draining cervical lymph nodes during carriage[100]. Although neutrophils are rapidly recruited after initial colonisation, their presence in the nasopharynx does not correlate with clearance of pneumococci. In addition, depletion of neutrophils in murine models, has no effect on bacterial burden during carriage [176].

1.4.1.2 Carriage as an immunising event:

Most *S. pneumoniae* infections manifest as asymptomatic colonisation of the human nasopharynx. Colonisation is almost universal in children under the age of five but the incidence and duration of carriage is significantly decreased in the adult population[3]. There have been many studies to examine whether nasopharyngeal carriage results in an effective memory response that protects from future carriage and invasive disease episodes.

Infants are often colonised repeatedly with multiple strains of *S. pneumoniae* and this continues throughout childhood. Successive colonisation even occurs with the same serotypes, suggesting that poor protective immune memory is generated in young children[177]. This apparent lack of immune memory was observed when children under the age of two failed to respond to polysaccharide antigens[178]. They did, however, mount significant antibody responses to pneumococcal protein antigens[179]. Interestingly, the average duration of carriage drops significantly between a child's first and second birthday, suggesting that naturally acquired immunity in children is dependent on $CD4^+$ T cells [180].

HIV infection confers a 50-fold increase in the risk of *S. pneumoniae* infection, highlighting the importance of $CD4^+$ T cells in immunity to pneumococci[181,182]. Experiments performed with mice that have been repeatedly colonised with pneumococci to mimic natural colonisation seen in humans have demonstrated that Th17 cells reduce duration of experimental carriage independently of Th1 or Th2 cells. Also, IL-17A responses in immunised mice correlated with reduced carriage duration[183]. Reductions in carriage density observed between 1 and 2 years of age correlates with the development of

Th17 cells that are found to be present in young children through to adults but are not detectable in newborns[183].

Human studies performed in the Gambia, an area of high pneumococcal colonisation, show that $CD4^+$ T cell immune memory to pneumococcal proteins is high, with robust proliferation of T cells from blood. However, this had no impact on the carriage status of individuals, suggesting carriage-induced immune memory may have potential for pathogen clearance in invasive disease but offers little to no protection from establishment of nasopharyngeal colonisation[184]. Of note, this study did not examine whether immune memory to pneumococcal proteins altered carriage duration in study participants.

There is been strong evidence to suggest that nasopharyngeal carriage in murine models induces total and capsule specific IgA mucosal antibodies and systemic IgG antibodies against PspA, PhtD and PpmA in addition to capsular polysaccharide-specific IgM[101,185]. These antibodies have been shown to correlate with reduced nasopharyngeal bacterial numbers and are protective against pneumococci in the lungs.

Mice pre-colonised with *S. pneumoniae* had significantly increased survival times following invasive pneumococcal challenge due to a combination of anti-PspA and capsular polysaccharide antibodies[101]. The presence of anti-pneumococcal antibodies, plus, increased levels of IL-17A and $CD4^+$ T cells in pre-colonised mice led to a reduction in bacterial load in both lungs and blood of infected mice, resulting in significant increases in survival rates[101]. Mouse models depleted of CD4 T cells or IL-17A abrogated the effects of experimental carriage and protection from re-colonisation[101]. Protection during

sepsis in pre-colonised mice was found to be reliant on antibodies and was not affected by $CD4^+$ T cell depletion[101].

Experimental human carriage experiments have shown that carriage induced cross reactive antibodies against pneumococcal protein antigens and protected from re-acquistion of carriage up to a year after first episode of experimental carriage. In addition, the serum antibodies produced as a result of carriage protected mice against invasive bacteraemic pneumonia[186].

Human and mouse data collectively conclude that colonisation episodes are immunising events that induce cellular, Th17 mediated and humoral adaptive immune responses to *S. pneumoniae* and can aid faster clearance of bacteria in lung and blood infections as well as reducing the duration of subsequent nasopharyngeal colonisation.

1.4.2 Pneumonia

Pneumococcal pneumonia causes more deaths than the combined mortalities of HIV, malaria and tuberculosis [187]. Outcomes of pneumonia are often related to the control of inflammation in the lung, with inflammation being described as a double-edged sword in pneumonia [188]. In humans, incidence of pneumococcal pneumonia has been shown to be higher in males compared to females in all age groups [189]. In addition, disease severity appears to be greater in males with significantly higher cases of pneumococcal septicaemia, and meningitis compared to females [190 4 191 192 193]. This phenomenon of increased male susceptibility to IPD has also been observed in mice. In the context of pneumonia, male mice exhibited higher levels of neutrophil recruitment into the lungs in the first 24 hours after infection, accompanied by

significant increases in proinflammatory cytokines [194]. Thus, inflammation is needed to effectively eliminate bacteria from the lungs but excessive or uncontrolled inflammation leads to lung injury and is often associated with poor patient outcomes [195]. Therefore, controlled inflammatory responses, driven through immune-modulatory cells such as T regulatory cells and cytokines such as IL-10 are key to improving host survival [173].

Lung airways are protected from bacteria by a series of mechanisms such as cough reflex, mucus and a mucocillary escalator [196]. Cell mediated responses are also crucial in the defence against bacterial infection. Lung-resident alveolar macrophages clear finite pneumococci from the lungs by phagocytosis. However, these cells quickly become exhausted and overwhelmed if pneumococcal numbers are high [197]. Neutrophils are recruited into the lungs within 12 hours in response to cytokines and chemokines released from macrophages and epithelial cells [114]. Once infiltration of neutrophils occurs, clinical symptoms of pneumonia become apparent. Neutrophils are highly effective in reducing pneumococcal numbers in the lungs through the production of reactive oxygen species (ROS) and release of anti-bacterial and lytic enzymes inside the phagosome during phagocytosis of pneumococci [195]. DNA-based neutrophil extracellular traps (NETS) are generated by neutrophils through release of genomic DNA, elastase and anti-microbial proteins. These NETS can trap and kill bacteria within the lung [198]. Neutrophils have control over the immune clearance of pneumococci in the lungs through production of chemokines and cytokines that control recruitment of innate immune cells and T cells. Resolution of inflammation has been shown

to correlate with rates of programmed cell death (apoptosis) of recruited neutrophils and rates of apoptotic cell clearance by macrophages during pneumococcal pneumonia infection [199,200]. As neutrophils are the primary controllers of inflammation, they are often the cause of dysregulation of inflammation, leading to excessive lung injury which allows pneumococci to seed from the lungs into the bloodstream, leading to the onset of bacteraemic pneumonia.

The definitive role of each cytokine in lung immune responses during pneumonia is still not fully understood. However, cytokines are classified into pro- and anti-inflammatory groups. Tissue resident alveolar macrophages (AM) produce a variety of pro-inflammatory cytokines including, TNFα, IL-1, IL-6, IL-8, IL-12, IL-17/IL-23 and IFN-γ [201] [202] [203]. TNFα is involved in the recruitment of inflammatory cells and activates respiratory burst in phagocytes [204]. Pneumococcal pneumolysin triggers the production of IL-1β through activation of NLRP3 inflammasome and promotes macrophage activation [116]. Excessive production of these cytokines can result in uncontrolled inflammation that can have deleterious effects on the host. To create a balance, IL-4, TGFβ and IL-10 cytokines are also produced to inhibit production of pro-inflammatory cytokines [202]. Release of inflammatory cytokines and chemokines recruits NK T cells and Th17 lymphocytes into the lung to aid in bacterial clearance [205] [206]. If pneumococci overcome the host immune defences in the lungs, bacteria enter the bloodstream and it is primarily anti-capsular antibodies, not cellular responses that are responsible for protection in the blood [207].

As discussed, a fine balance is needed to create enough inflammation for immune cell recruitment and clearance of bacteria. Unfortunately, excessive inflammation often occurs and can lead to systemic and severe inflammation which can result in multi organ failure and poor patient outcome [195].

1.4.3 Murine models as a tool to understand host immune responses to pneumococcal infection

Murine models are commonly used to study host immune responses to pneumococcal infections. Inbred and outbred mouse strains differ in their susceptibilities to pneumococcal pneumonia, which prompted comparisons of the immune responses in these mouse strains to further understand the role of the host immune system in influencing resistance or susceptibility to invasive pneumococcal disease [208].

The mouse strains used in pneumococcal research can be categorised into resistant and susceptible groups based upon their survival rates in acute infection challenge experiments and the numbers of bacteria they harbour in infected organs [208]. BALB/c mice have been shown to be highly resistant to pneumococcal pneumonia with 100% survival rates against a range of pneumococcal serotypes and the ability to confine infection to the lung without developing sepsis. Bacteria are then cleared from the lungs around 7 days post infection. CBA/Ca mice on the other hand are highly susceptible to pneumococcal lung infection and develop bacteraemia by 6 hours post infection which rapidly progresses to sepsis. Mice succumb to infection very quickly and median survival times are around 24 hours post infection [208].

The difference in resistance to disease severity in these mice prompted investigation into host genetic components that control the immune response

to pneumococci. Numbers of infiltrating neutrophils in the lungs of BALB/c compared to CBA/Ca mice was one of the first differences to be identified. By 12 and 24 hours post infection with serotype 2 (strain D39) pneumococci, BALB/c mice were found to have significantly higher numbers of neutrophils compared to CBA/Ca mice [208 209]. Greater quantities of TNFα were also found in the lungs of BALB/c mice by 6 hours post infection, compared to CBA/Ca, and this difference was maintained up to 72 hours post-infection [209]. TNFα is important in pneumococcal infection as it thought to contribute to early chemotaxis of phagocytic cells into the lung to control bacterial numbers and to prime cells for antimicrobial activity [210]. TNFα knockout mice have shown that TNFα is necessary to control infection in lungs [211]. Rapid increases in TNFα in BALB/c and not CBA/Ca lungs may result from higher numbers of mast cells observed in BALB/c lungs immediately after infection [209]. Mast cells contribute to clearance of pneumococci, produce large amounts of TNFα and are likely to contribute to the rapid increases in levels of the cytokine seen in BALB/c mice [212,213]. Alternatively, rapid increases in TNFα in BALB/c could reflect a difference in the cellular responses of macrophages between BALB/c and CBA/Ca mouse strains [214].

Infected lungs in CBA/Ca mice were found to have significantly high levels of IL-6 which has been shown to be associated with lethargy in pneumococcal pneumonia whilst BALB/c mice had barely detectable levels of IL-6 throughout infection [209]. In addition, production of IFN-γ and rates of apoptosis were found to be significantly higher in CBA/Ca mice compared to BALB/c [173]. Presence of inflammatory cytokines such as IFNγ and IL-6 are associated with increased

inflammation which causes tissue damage and bacterial dissemination into the bloodstream within 6 hours which leads to high mortality rates.

Thus, it appears that resistance to pneumococcal pneumonia is associated with early increases in TNFα and rapid infiltration of neutrophils into the lung. These cells phagocytose pneumococci and bacterial load is significantly reduced in the lungs which prevents the onset of systemic disease. Mice susceptible to pneumococcal pneumonia have lower levels of TNFα and neutrophils in the lungs so bacterial numbers increase over time and disseminate into the bloodstream.

Recently, it has been demonstrated that T regulatory cells have an important role in resistance against pneumococcal infection through regulation of inflammation [173]. Linkage mapping of the offspring of BALB/c and CBA/Ca intercrosses revealed a major locus on chromosome 7 that influences survival post pneumococcal infection [215]. The gene for *Tgfb1* gene is located on chromosome 7 and prompted investigation into the role of immune-modulatory cells and cytokines in resistance to infection with *S. pneumoniae* [173]. T regulatory cells are both a source and target of TGFβ and BALB/C mice were found to have significantly higher numbers of $Foxp3^+$ Treg cells in the lungs within 12 hours of infection with *S. pneumoniae* compared to CBA/Ca mice [173]. TGFβ appears to protect against the development of bacteraemia by maintaining lung epithelial cell barrier integrity which stops bacterial dissemination into the bloodstream. Inhibition of TGFβ resulted in fewer numbers of T regulatory cells in the lungs and development of bacteraemia in 50% of BALB/c mice. In addition, when T regulatory cells were adoptively transferred into CBA/Ca mice shortly before infection with *S. pneumoniae,*

bacterial loads in blood were lower and there was a decrease in IFNγ^+ cells in the lungs, all of which resulted in increased survival rates [173].

Observation of differing rates of resistance to pneumococcal infection in murine strains has led to a further understanding of the role of the host immune response in influencing pathogenesis of *S. pneumoniae* in the context of lung infection. Resistant BALB/c mice have an immune response characterised by an early influx of neutrophils, driven by TNFα, which significantly reduces bacterial load in the lungs. In addition to this, high levels of TGFβ and Foxp3 T regulatory cells limit cytokine and immune-mediated lung tissue damage in these mice, to contain bacteria within the lungs and avoid bacterial dissemination into the bloodstream and the onset of bacteraemia[173].

1.5 Pneumococcal vaccination

Pneumococcal vaccines have been available for over 100 years with the first development of a pneumococcal whole cell vaccine in 1911[216]. However, improvements in vaccine designs were halted with the introduction of penicillin in the 1940s. Unfortunately, many patients still died despite antibiotic treatment which prompted the development of polysaccharide vaccines such as the 23-valent pneumococcal polysaccharide vaccine (PPSV23) in 1983[217]. This vaccine exhibited 65% efficacy in immunocompetent adults and was shown to reduce rates of IPD but had no effect on pneumococcal carriage[218]. Unfortunately, the vaccine also failed to induce an immune response in children under the age of two years, the group with the highest rate of IPD[219]. Pure polysaccharide vaccines such as PPSV23, were found to induce poor immunoglobulin memory responses due to polysaccharide antigens failing to

bind to MHC Class II for presentation to T cells, thus rendering polysaccharide antigens T-independent antigens. As a result, B cell activation is incomplete and poor immunoglobulin responses are generated[220].

Improvement in vaccine design included the development of conjugate vaccines which have capsular polysaccharide antigens chemically bound to a diphtheria protein[219]. As a result, carrier protein peptides are presented to T cells and MHC Class II on the B cell surface to signal for activation of T-helper cells. This combination of T and B cell activation resulted in production of high titres of antibody against polysaccharide capsule. T-dependent immune responses also initiate the production of good immunological memory responses against serotypes included in the vaccine in children under the age of two years[221-223].

Three protein-conjugate vaccines are currently available; PCV7, PCV10 and PCV13.

Vaccine	Company	Year licenced for use	Serotypes included
Prevnar (PCV7)	Wyeth	2000	4, 6B, 9V, 14, 18C, 19F, 23F
Synflorix (PCV10)	GlaxoSmithKline	2008	1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F,
Prevnar 13 (PCV13)	Pfizer	2010	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A 19F, 23F,

Table 3 Pneumococcal vaccines and their serotype coverage. Different pneumococcal protein-conjugate vaccines and the serotype coverage by each. Adapted from Daniels *et al*, 2016 and Torres *et al* , 2015.

After the introduction of PCV7, there was a significant decline in the global burden of IPD in children. The USA reported a 77% reduction in IPD rates and 98% reduction in IPD caused by serotypes covered by vaccination in children under the age of five[224]. Rates of antibiotic resistance and nasopharyngeal carriage of vaccine serotypes also declined significantly since the introduction of PCV7[225]. Evidence of herd immunity for unvaccinated individuals over the age of 65 and for infants under 60 days old was also apparent in the USA and Canada[226,227]. However, PCV7 led to changes in serotype distribution with a significant global rise in serotype 19A. This prompted the development of vaccines covering more serotypes and now PCV13 is the latest vaccine available and covers 13 of the most prevalent serotypes causing IPD globally[216]. In the years since the availability of PCV13, there has been a decline in IPD in children in the UK[216]. PCV13 has had excellent results in high risk groups such as elderly patients with chronic medical conditions such as cardiovascular and pulmonary disease, with immune responses comparable with non-high-risk individuals[228].

Despite significant declines in IPD with vaccine serotypes in both the developed and developing world after the introduction of polysaccharide conjugate vaccines, there is a continuing weakness in the design of these vaccines. The efficacy of current vaccinations in preventing hospitalisation correlates with encapsulation of bacteria. Recent evidence shows changes in capsule expression and evidence of capsule shredding occurring in some *S. pneumoniae* to avoid capsule-targeted immune responses[137]. In addition, there is an emergence of IPD caused by unencapsulated strains and a pressing concern about serotype replacement whereby vaccine-serotypes are

replaced by potentially more virulent serotypes not included in the vaccine[229,230]. Limited serotype coverage in PCV vaccines means there will always be new serotypes emerging that may cause IPD and new vaccines will need to be designed to cover these emerging serotypes. Longitudinal studies from the USA have noted that serotype 35B infection rates have increased since the introduction of PCV13, demonstrating that serotype replacement will be a common occurrence until a vaccine can be developed to cover all known serotypes of *S. pneumoniae*[223].

New vaccine antigens

The high incidence of serotype replacement and rise in antibiotic resistant pneumococci highlights the need for future vaccines to cover a broader range of serotypes. One strategy to achieve this would be to use conserved pneumococcal surface protein antigens as vaccine targets.

One such antigen in contention is Pneumococcal surface protein A (PspA) which is an antibody-accessible protein attached to the pneumococcal cell surface[231]. PspA has been found on all clinical isolates and aids virulence by escaping complement-mediated phagocytosis[232]. When humans are immunized with this protein, they produce antibodies that are protective against IPD in mice [142]. Phase I clinical trials showed promise as immunisation of healthy adults elicited good antibody responses to PspA [140]. However, concerns were raised as low sequence homology between PspA and human cardiac myosin was detected, raising the possibility of inducing cardiac autoimmunity. Further evaluation of PspA as a vaccine candidate has since been placed on hold [233].

Pneumolysin is another potential vaccine protein antigen. Again, this is expressed by all clinical isolates and is essential for disease pathogenesis. Due to toxicity of pneumolysin, it is not safe to use in humans without being mutated to create non-toxic but still highly immunogenic variants. Murine studies have shown vaccination with pneumolysin protects mice against infection, and when combined with PspA, protection against IPD is increased[234]. Immunisation with pneumolysin toxoid B (PdB) which has 0.1% of haemolytic activity of wild type pneumolysin, has been shown to be highly protective against invasive pneumococcal disease in murine models especially in the presence of low pneumococcal carriage density, suggesting that PdB could significantly enhance and augment existing memory immune responses [235].

Phase I human clinical trials with attenuated pneumolysin showed safety, immunogenicity and efficacy and when in combination with PspA and this may be an effective future vaccine although issues still remain about cross reactivity with PspA antigen[236].

Ongoing research aims to identify protein antigens suitable for inclusion in the next generation of pneumococcal vaccines. The idea of a multi-variant protein antigen vaccine encompassing multiple pneumococcal antigens may have great potential in future effective vaccine design[237].

1.6 Serotype 1

Streptococcus pneumoniae exists in over 93 serotypes, each of which is defined by the chemical structure of its capsular polysaccharide. Serotype 1 was one of the first serotypes to be described and displays some unusual epidemiological, clinical and microbiological characteristics[44].

1.6.1 Epidemiology of Serotype 1

Multi-locus sequence typing of serotype 1 has clustered sequence types into three major lineages (A, B and C). These lineages are separated geographically with lineage A predominantly found in Europe, the United States and Canada. Lineage B isolates are found in Africa and Israel whereas lineage C are from South America[238]. Specific sequence types are dominant in each lineage and details of these can be found in table 4.

Lineage	Geographical area	Sequence types
A	Europe, Canada & North America	ST227, ST306, ST304, ST288
B	Africa and Israel	**ST217**
C	South America	ST615

Table 4 Major lineages of Serotype 1. Adapted from Brueggemann *et al*, 2003

Serotype 1 is one of the top 5 serotypes associated with IPD in Europe, Asia, Africa and Oceania [238]. This serotype is classically described as highly invasive despite being rarely found in nasopharyngeal carriage, even in areas of high disease incidence [44]. Compared to other serotypes, rates of antibiotic resistance in serotype 1 are lower; this is likely due to shorter durations of

carriage compared to other serotypes, suggesting that serotype 1 will undergo fewer recombination events and therefore will be slower to respond to antibiotic selective pressure than other, highly-recombinogenic serotypes [239].

Incident rates of serotype 1 IPD fluctuate year on year and often disease is associated with outbreaks in close knit communities [240] [107]. Sequence types found in Africa are highly invasive and tend to affect older children and otherwise healthy adults. There is also a lower disease prevalence in HIV-infected patients compared to other serotypes, which suggests that serotype 1 is highly invasive and can cause disease in healthy individuals with no co-morbidities [240] [241].

Epidemiological studies in South Africa over ten years between 2003 and 2013 found that generally serotype 1 caused less severe pneumococcal disease which manifested as pneumonia and bacteraemia. The study also found that children under the age of 5 were less likely to die with serotype 1 IPD compared to infection with other serotypes [242].

In sub-Saharan Africa, *S. pneumoniae* is one of the most common causes of bacterial meningitis. Figure 6 shows the African meningitis belt which is an area from Ethiopia to Senegal, which, for over the past 100 years has had meningitis epidemics occurring at a frequency of every 8 to 12 years [243]. Between 1998 and 2003, it was found that incidence of meningitis caused by *S. pneumoniae* in this area was over ten times higher than in Western Europe and the United States [244]. Serotype 1 accounted for 76% of all isolates causing pneumococcal meningitis and 43.6% case fatality rates were observed. Serotype 1, ST217 was found in 80% of cases in older children and adults

while only causing 33% of pneumococcal meningitis in children under the age of 4 years [244].

Figure 6. Location of the African Meningitis belt.

Source: Control of epidemic meningococcal disease, WHO practical guidelines, World Health Organization, 1998, 2nd edition, WHO/EMC/BAC/98.3

It appears that the meningitis belt in Africa is unique in its disease epidemiology, even within Africa. In Europe and the USA, the bimodal meningitis age distribution has peaks in infants and the elderly, however within the African meningitis belt, disease is found occurring in outbreaks, usually during the dry season, and serotype 1 disease prevalence is highest in older children and working age adults [245] [244]. Over 35 years of surveillance data concluded that pneumococcal meningitis within the meningitis belt results in a high burden of disease and mortality, with 59% of cases occurring in people aged between 5 and 59 years [60]. Unfortunately, little is known about pneumococcal pneumonia disease prevalence in this area and whether there is a correlation between meningitis and pneumonia outbreaks. Recently, it has been suggested that climatic factors may play a role in meningitis outbreaks

[246]. The Harmattan winds come across the African meningitis belt from November to May and correlate with timings of disease outbreaks. These winds carry dust particles which have been shown to increase pneumococcal carriage density and dissemination of bacteria into the lungs and even the brain [247] [246]. Neutrophils and macrophages exposed to these dust particles have a reduced capacity to phagocytose pneumococci [246]. Temperatures rising to over 39.5^{0}C is common during the dry season and *in vitro* assays have shown that high temperatures increase autolysis rates in serotype 1 bacteria and also bacterial dissemination into lung tissue [246]. Therefore, seasonal climate changes in Africa are thought to contribute to outbreaks in pneumococcal meningitis cases.

1.6.2 Nasopharyngeal colonisation by serotype 1

As early as 1937, Heffon found that serotype 1 was responsible for approximately 22% of pneumococcal pneumonia cases in children and 33% in adults. Despite this high prevalence, he found that serotype 1 asymptomatically colonised only around 2% of adults and children. Studies of nasopharyngeal carriage in people in close contact with outbreak patients have concluded that serotype 1 is able to initiate colonisation and pass between people in close contact, but colonisation is much shorter in duration compared to other serotypes [248] [249] [250]. Despite limited duration of carriage, the odds ratio for serotype 1 IPD is high, that is, it has a high attack rate [19]. Studies of serotype 1 nasal colonisation have been limited due to the rarity of detection in the human population, but future work in murine models and

experimental human carriage may further understanding of why asymptomatic colonisation is rarely detected.

1.6.3 Clinical disease presentations

Different serotypes often have different presentations of pneumococcal disease. Serotype 1 has been found to cause a variety of uncommon disease phenotypes compared to other serotypes of *S. pneumoniae*. For example there is a longstanding association between the development of empyema and serotype 1 infection. The UK has seen a rise in the incidence of empyema over the last 15 years[251]. This has been linked to the emergence of ST306 and a rise in serotype 1 IPD around Europe[252,253]. As patients are treated with antibiotics prior to pleural aspiration, it is difficult to fully assess rates of empyema caused by serotype 1[254].

Different serotype 1 lineages differ in their ability to cause meningitis. In Africa's meningitis belt, lineage B serotypes including ST217, cause approximately 80% of pneumococcal meningitis and are associated with high mortality levels[60]. In Europe, pneumococcal meningitis caused by serotypes in lineage A is much less common. A study in Denmark found that serotype 1 caused 15% of all IPD cases over the age of 5 years but only accounted for 7% of meningitis cases[255]. Similar findings were observed in the UK suggesting that genetic differences between lineages may account for differences in disease phenotypes seen[256].

Overall clinical outcome from serotype 1 IPD is varied in the literature. Reports of serotype 1 pneumococcal disease in Europe indicate high levels of

empyema and low mortality rates, which is due to ST306 being the dominant sequence type[252]. ST306 however is an unusual strain as it expresses a non-haemolytic pneumolysin and has been shown to be significantly less virulent in murine models of pneumococcal disease compared to serotype 1 sequence types expressing a fully haemolytic pneumolysin such as ST217[108,257].

Serotype 1 is often associated with outbreaks in close knit communities and affects healthy children and young adults[245]. One such outbreak of serotype 1 pneumococcal disease took place at a military training camp where 128 cases of pneumonia were recorded with approximately 20% developing bacteraemic pneumonia. Further investigation found that around 25% of people at the camp had been colonised with serotype 1 which resulted in an attack rate of around 12%[44].

1.6.4 Experimental work with serotype 1

Pneumococcal models of infection in mice and rats have been widely used to study bacterial pathogenesis. There are limited data published on serotype 1 infections in murine models so little is currently understood about mechanisms of serotype 1 colonisation of the nasopharynx or virulence in invasive pneumococcal disease.

Serotype 1 has been found to have a weak ability to colonise the nasopharynx of mice compared to other serotypes. In addition, when a large dose of serotype 1 is given intranasally, mice were found to develop persistent bacteraemia with no disease symptoms [258]. However, these experiments were performed with European serotype 1 isolates, which included ST306, hence, this is not a general representation of serotype 1 disease pathogenesis. In

contrast, infections with African ST217 have shown high rates of mortality as ST217 is able to rapidly divide and survive in the lungs and blood of mice[259]. This contrasts with ST304 European isolate which can colonise the nasopharynx but is cleared from the lungs and is not able to survive in blood. ST217 was found to induce strong type 1 interferon levels in the lungs of mice which was associated with increased invasion into the blood and low survival rates[259]. Data from animal models strongly suggest that there are significant differences in the different clonal types of serotype 1 with African serotype 1 isolates demonstrating high virulence, whereas European isolates have lower mortality rates in experimental pneumococcal disease models.

1.6.5 Serotype 1 virulence factors

Capsule

Complete sequences for the capsular polysaccharide locus (cps) have been published for all known serotypes[16]. Upon detailed analysis, it was found that serotype 1 capsule differed significantly compared to other serotypes of *S. pneumoniae*. A frameshift mutation in the *rmlC* gene resulted in an incorporation of a sugar known as AAT-Galp, forming the initial carbohydrate of the capsular polysaccharide[260]. The mutations seen in serotype 1 capsule have given rise to alternating positive and negative chains of polysaccharides, termed zwitterionic[261]. Interestingly, zwitterionic capsules are known as T cell –dependent antigens as they can be presented on MHC Class II molecules to T cells and influence T cell activation and differentiation[262]. In the context of infection, zwitterionic capsules are associated with increased abscess formations in murine models after intraperitoneal injection[261]. Little is currently

understood about how serotype 1 capsule influences host immune responses in humans during infection and how they may influence serotype 1 vaccine efficacy.

In vitro assays have found serotype 1 capsule to be significantly more resistant to opsonisation and complement deposition, possibility due to zwitterionic capsule, but it is not known how this may affect resistance to phagocytosis during infection and influence carriage duration or invasiveness compared to other serotypes[263].

Pneumolysin

Very little investigation has been done on non-capsular virulence factors and their role in serotype 1 disease pathogenesis. As previously discussed, a serotype 1 sequence type, known as ST306, expresses a non-haemolytic form of pneumolysin[108]. Infection with ST306 results in empyema, pneumonia and meningitis with low mortality rates across Europe[251,252,254]. The mechanistic interactions with the host of non-haemolytic pneumolysin in the context of nasopharyngeal carriage and invasive disease have not been explored.

1.6.6 Vaccination

Serotype 1 is one of the thirteen serotypes included in the current PCV13 vaccine which was introduced in 2010[264]. This vaccine has been shown to be effective in inducing antibodies against the capsule in children. Post-vaccine epidemiological studies are not yet available to ascertain the efficacy of PCV13 against serotype 1 carriage and rates of invasive disease. Initial clinical trial data found that vaccine failure was observed in 5 out of 12 cases of serotype 1 disease in a vaccinated group in Africa[265,266]. Given serotype 1 capsule is significantly more resistant to complement deposition and opsonisation, it

remains to be seen whether serotype 1 IPD will be effectively abolished with routine vaccination or whether an alternative means of vaccination will be required.

1.7 Research Aims

Serotype 1 is a highly diverse serotype with several lineages found across the world. Serotype 1 strains prevalent in Europe produce non-hemolytic pneumolysin and are associated with high rates of empyema and low mortality. African serotype 1 on the other hand is found to be highly invasive, particularly in sub-Saharan Africa where rates of meningitis, sepsis and pneumonia are leading causes of death in both adults and children.

Serotype 1 has some unusual characteristics including a zwitterionic capsule which is thought to be a T cell-dependant antigen and so interaction between serotype 1 and the host immune system will likely differ compared to other serotypes.

The aim of this PhD project is to understand the mechanisms of disease pathogenesis of African serotype 1 using *in vitro* and *in vivo* models of infection.

Aims:

1. **Investigate the role serotype 1 capsule and pneumolysin have in invasive disease pathogenesis.**
    a. Study the role of serotype 1 capsule in carriage and invasive disease
    b. Compare effects of African ST217 pneumolysin with European ST306 non-hemolytic pneumolysin in murine models of invasive pneumococcal disease.

2. **Determine host – serotype 1 interactions during nasopharyngeal carriage and invasive disease.**
    a. Define and compare the phenotypic characteristics of ST217 serotype 1 isolates from the nasopharynx, CSF and blood in their respective ability to establish nasopharyngeal colonisation and cause invasive disease.
    b. Monitor the density and duration of serotype 1 (ST217) nasopharyngeal carriage and its interaction with host immune cells during carriage.

Materials and Methods

Chapter 2

Materials and Methods

2.1 Microbiology

2.1.1 Bacterial strains:

Table 5 details the strain and type of pneumococci used in this thesis. For each serotype a single culture broth was batch processed and pneumococci stored in single–use aliquots for up to six months. Both laboratory and clinically derived strains of pneumococci were used.

Serotype 1 Isolates:

A)

Sample ID	Carriage/ Invasive	Blood / CSF/ Nasopharynx	Sequence type	Location	Year	Age of patient	Serotype
C9471	Invasive	CSF	217	Blantyre, Malawi	2004	3	1
D25796	Invasive	Blood	217	Blantyre, Malawi	2004	8	1
W000168	Carriage	nasopharynx	217	Karonga, Malawi	2008	26	1
ST3081	Clinical isolate from Gambia	Blood	3081	The Gambia	2011	9	1
ST306	Clinical isolate - Europe	Unknown	306	Europe	Unknown	Unknown	1

B)

Serotype	Type/Source
2 (D39)	Laboratory (NCTC 7466)
5	Clinical isolate B101.38 (Bern, Switzerland)
6B (ST138)	Clinical carriage isolate (BHN418)
6B	Clinical isolate, nasopharynx #106.66 (Bern, Switzerland)
19A	Clinical isolate, human (Hungary)
23F	Liverpool school of Tropical Medicine
23F	Clinical isolate, nasopharynx #B103.57 (Bern, Switzerland)
7F	208.41 (Bern, Switzerland)
7F	Clinical isolate, nasopharynx
14 (ST124)	Invasive clinical isolate, BHN78
14	Clinical isolate, blood #B103.66 (Bern, Switzerland)
19F (ST162)	Clinical carriage isolate BHN100

Table 5 List of Streptococcus pneumoniae serotypes/sequence types used.

Serotype 1, ST217 isolates used in this thesis were isolated from HIV negative individuals in Malawi, both invasive isolates (C9471 and D25796) were from children in Blantyre, whilst the nasopharyngeal carriage isolate was from a young adult in Karonga. The ST217 isolates were selected for this work because they have all been fully sequenced and therefore gives the option for future genomic analysis.

2.1.2 Standard Media

Blood agar base (BAB) culture plates

16 grams of BAB medium (Sigma) was added to 400ml of distilled water and then autoclaved at 15psi (103kPA) for 30 minutes. After autoclaving and the media cooled to ~56^{o}C, 20 mls of Sterile Debrinated Horse Blood (Sigma) was added and gently mixed. The medium was then poured into sterile petri dishes (90mm), left to dry overnight and the next day stored inverted at either room temperature or 4 ^{o}C. One bottle of 400ml medium would make approximately 25 BAB culture plates which could be stored for up to 14 days before use.

Blood agar base (BAB) culture plates + 5% V/V horse blood with gentamicin

BAB culture plates were prepared as above with a minor modification: when 20mls of horse blood was added to 400ml of autoclaved BAB culture medium, 2ug/ml of gentamicin (Sigma) was also added to the medium and gently mixed.

Brain heart infusion (BHI) broth

14.8 grams of BHI medium (Sigma) was added to 400ml of distilled water and autoclaved at 15psi (103kPA) for 30 minutes. Following autoclaving, the medium was stored at room temperature for up to 4 weeks.

BHI Serum broth

BHI broth was prepared as above with a minor modification. After autoclaving, 20mls of Fetal Bovine Serum (FBS) (Sigma) was added to 80mls of medium to make 80% v/v BHI medium with 20% v/v FBS. This BHI serum broth was prepared fresh for each use.

Cryopreservation media

To cryopreserve cells from either tissue culture or isolated from murine tissue, a freezing media was prepared using the ratios 75% RPMI medium (Sigma), 15% Foetal bovine serum (FBS) (Sigma) and 10% dimethyl-sulphoxide (DMSO) (Sigma).

2.1.3 Miles and Misra method to determine viable counts of bacteria

The Miles and Misra method is a technique used to determine the number of colony forming units (CFU) in bacterial suspension or homogenate. It relies on serial dilutions, culturing and counting CFU. The number of colony forming units seen is used to determine the CFU/ml of a sample [267].

20μl of sample (e.g. tissue homogenate, blood or stock solution) was added to 180μl of sterile PBS and serially diluted 10 fold, until dilutions of 10^6 were reached. The BAB culture plates were divided into six sections and 60μl (3x20μl) of each dilution was plated onto each corresponding sector. When dry, the plates were inverted and incubated at 37^0C 5%CO_2 for 16-18 hours.

The following day, sections in which 20-200 CFU were visible were counted and the CFU/ml or CFU/mg of tissue were calculated as follows:

To calculate the numbers of CFU in blood or infection dose:

CFU/ml = number of colonies in sector x dilution factor x (1000/60)

CFU/mg of tissue = CFU/ml x 3 mls /tissue weights (mg)

2.1.4 Preparing stocks of pneumococci

Pneumococci from laboratory bead collections were streaked onto BAB culture plates and grown overnight at 37^0C 5%CO_2. In addition, an optochin antibiotic disk (Oxoid) was placed on a site of inoculation. After culture, a zone of bacterial growth inhibition around the optochin disk indicates susceptibility to optochin and this is a common method to ensure only pneumococci is present.

For serotype 1 stock preparation, after overnight incubation of BAB plates, a full sweep of colonies was used to inoculate 10mls of BHI Serum broth. The inoculated broth was then placed on ice in a water bath with a timer set to allow for 9-12 hours incubation at 37^0C until OD_{500} 0.9-1.1 was reached.

For all other serotypes, following overnight culture of sweep on BAB plates, a sweep full of colonies was used to inoculate 10mls of BHI broth in a sterile universal tube. The inoculum was then incubated for 16-18 hours at 37^0C5%CO_2. until OD_{500} 1.4 -1.6 was reached.

When the BHI pneumococcal broth culture reached the desired OD, it was centrifuged at 1500g for 10 minutes, the supernatant was removed and the remaining pellet of bacteria re-suspended in 1ml BHI Serum broth. 700μl of

the re-suspended pellet was added to 10mls of BHI Serum and sub-cultured at 37^0C5%CO_2. until OD_{500} 1.4 -1.6 was reached.

The resulting serum broth containing pneumococci was then divided into 1ml single use aliquots in sterile cryotubes and kept at -80^0C. After 24 hours in the freezer, aliquots were thawed, centrifuged 10,000xg for 2 minutes, re-suspended in sterile PBS and the CFU/ml of pneumococci was determined using the Miles and Misa Method.

2.1.5 Growth curves for relative fitness determination

The OD_{500} of 2000l of a solution of 10^3 cfu/ml of bacteria in 20% BHI serum was determined every 30 minutes for a total of 12 hours using a BMG Labtech FLUOstar OMEGA microplate reader (BMG Labtech, Aylesbury, UK).

2.2 Quantification of capsule using Fluorescence isothiocyanate (FITC)-dextran exclusion assay

Capsule thickness was determined using fluorescence labelled FITC-dextran (2000kDa, Sigma), based on a published method by Gates et al[268].

Bacterial colonies were inoculated into BHI culture medium and cultured overnight at 37^0C 5%CO_2 until OD_{600}nm = 0.5. 200μl of the overnight culture was taken and sub-cultured into 10mls of fresh, pre-warmed BHI culture medium and incubated again at 37^0C 5%CO_2 until OD_{600}nm = 0.4 - 0.6. The pneumococcal culture was then centrifuged at 1000xg for 10 minutes. The supernatant was carefully poured off and the pellet re-suspended in 1ml of pre-chilled PBS. To prepare each slide, 10μl of bacterial suspension was mixed with 2μl of FITC-dextran solution (10mg/ml), 10μl of this was added to

the slide and coverslip applied. The slides were viewed using a Nikon Eclipse 80i fluorescent microscope with 100x objective lens and photographed using a Hamamatsu C4742-95 camera. The mean area of each bacterium (pixels) was determined using Image J software.

2.3 Opsonophagocytosis killing assay

Opsonophagocytic assays were performed to assess differences in the ability of different bacterial strains to resist phagocytosis by human neutrophils. The protocol has been modified by that published by Romero-Steiner in 2003[269]. Frozen bacterial stocks were thawed, washed with PBS and re-suspended to a final concentration of 10^5 cfu/ml in HBSS (Invitrogen). 10μl of bacteria (10^3 cfu) were then incubated with 20μl of opsonin for 30 minutes at 37^0C, shaking at 175 rpm. The opsonin used in this assay was pooled human IgG (IVIG, Grifols Therapeutics, USA). Whilst the opsonisation step was taking place, human neutrophils (HL-60 cell line) were prepared. Cell were centrifuged at 250g for ten minutes and resuspended in 1ml of $DPBS^{-/-}$ (Invitrogen). The concentration of cells was determined using cell counts on a Neubauer chamber and trypan blue staining. The cells were adjusted to a final concentration of 1.125 $x10^7$ cells/ml. After opsonisation, 40μl of neutrophils ($4x10^5$ cells) were added to opsonised bacteria, in addition to 10μl of baby rabbit complement (Invitrogen) and incubated for 30 minutes at 37^0C, shaking at 175 rpm. Finally, 10μl of the suspension was plated out onto 5% BAB plates and incubated overnight at 37^0C to determine the number of viable bacteria. Experiments were performed in triplicate.

2.4 C3b Complement deposition assay

This assay measures the amount of complement deposition on the bacterial surface. Bacterial cells are incubated with human serum which allows complement deposition (C3) on the bacterial surface. A primary (mouse-anti-human C3) and secondary antibody (Anti-mouse-APC) are subsequently used to detect C3; bacterial cells are analysed using flow cytometry.

iC3b deposition were measured using previously described[270]

Firstly, to prepare the bacteria samples for C3b deposition assays, frozen aliquots were thawed quickly and a 5ml stock solution of $1x10^5$CFU/ml in BHI was made and left to incubate at 37^{o}C for 15 minutes. After incubation, the bacteria suspension was centrifuged at 3000 x g for 10 minutes to pellet the bacteria. Supernatant was discarded and pellet re-suspended in 5mls of PBS. Bacteria were then distributed into Eppendorf tubes for the assay. Following centrifugation at 17,000xg for 3 minutes, supernatant was removed and pellet re-suspended in 100ul of 20% human serum (diluted in FBS). Pooled human serum was obtained from unvaccinated normal human volunteers. Sera were stored in single use aliquots at -70^{o}C. 1% Gelatin Veronal buffer in PBS was also added to aid complement deposition. Samples were then incubated at 37^{o}C for 30 minutes. After a wash step and brief centrifuging at 17,000 x g for 3 minutes, supernatant was discarded and 1:300 dilution of Mouse anti human mAb C3 (Abcam ab17455) was added to bacteria. After samples were incubated at 37^{o}C for 30 minutes, centrifugation was repeated and a secondary binding antibody Anti Mouse IgG2a APC (eBioscience 17-4210-80) was added to bacteria. Samples were incubated at 4^{o}C for 30 minutes in the absence of light. Once incubated, samples were centrifuged and re-

suspended in 500μl of PBS. At this stage, 1 in 100 dilution of Thiazole Orange (TO) (BD, 42uMol/L) was added to each sample to isolate bacteria. Flow cytometry assays of C3b deposition were performed on a BD Accuri C6 flow cytometer. Once the sample had been acquired, the Fluorescence Index Increase (FIincr) was calculated using the following:

FIincr = Fluorescence Index Increase

FIincr =% of TO^{+ve} and APC^{+ve} bacteria ×(MFI of TO^{+ve} and APC^{+ve} bacteria) – (MFI of TO^{+ve} and APC^{-ve} bacteria)

2.5 T cell differentiation assay

2.5.1 Macrophage differentiation from murine bone-marrow derived monocytes

The first step in this assay is to differentiate macrophages from bone marrow derived cells. This is done by collecting both tibias and femurs from 7-10week old naive BALB/c mice (Harlan). Bone marrow was flushed from the leg bones into DMEM medium supplemented with 10% foetal bovine serum and 1% P/S. The media was also supplemented with M-CSF (20ng/ml, R&D Systems). Bone marrow – derived cells were then counted and $6.25x10^5$ cells/ well were plated into 48 well tissue culture treated plates (Greiner). Cells were incubated for 7 days at 37°C 5%CO_2 with a change of media on the 4^{th} day of culture. After allowing the monocytes to differentiate for 7 days, the media on the cells was changed to DMEM +10%FBS with no antibiotics. Pneumococci were then

added to the macrophages in a 1:10 cells to bacteria ratio and cultured together at 37^{o}C 5%CO_2 for 24 hours.

2.5.1 Isolation of $CD4^+$ T cells.

$CD4^+$ T cells were isolated using mouse $CD4^+$ T cell isolation kit II (Miltenyi Biotec). Firstly, spleens were collected from 5-10 naïve female BALB/c mice (7-10 weeks old). Spleens were passed through a 40μm cell strainer (BD Biosciences) and washed through twice with DMEM media to create a single cell suspension. Cells were then counted, pelleted and re-suspended in 40μl of buffer (0.5%BSA in PBS) per 10^7 cells. 10μl of Biotin-Antibody cocktail (Miltenyi Biotec) was added per 10^7 total cells. This was incubated for 5 minutes at 2-8^{0}C. After short incubation, 30μl of buffer (0.5%BSA in PBS) and 20μl of Anti-Biotin Microbeads (Miltenyi Biotec) was added per 10^7 total cells. After mixing, cell suspensions were incubated at 2-8^{0}C for ten minutes.

LS columns and MACS separator (Miltenyi Biotec) were used to isolate $CD4^+$ T cells. 5 mls of buffer was rinsed through the LS Column prior to adding the cell suspension. The flow-through was collected which contained the $CD4^+$ T cell population. Additional 3 mls of buffer was added after the cell suspension and was collected and added to the T cell population. The total number of $CD4^+$ T cells collected was counted and 6.25 x 10^6 cells/well were added to the bone-marrow derived macrophages.

2.5.2 Preparation of infected macrophages

Whilst the $CD4^+$ T cells were being collected from the LS Column, the infected macrophages were prepared for culture with T cells. Firstly, supernatant (500μl) was removed from the wells containing macrophages and centrifuged at 13,000 rpm for 2 minutes to pellet bacteria. Macrophages were carefully washed with 200μl of PBS and 450μl of the supernatant was added produced during pneumococci infection. 50μl of isolated $CD4^+$ T cells and 5μl of penicillin/streptomycin were added. Cells were incubated at 37^0C 5% CO_2 for 3-5 days before performing flow cytometry on cells to assess T cell differentiation (see 2.8.3). 5-18 hours prior to flow cytometry staining, 5μl of 1X Cell Stimulation Cocktail (eBioscience) was added to cells to boost T cell activation. 5μl of 1X Protein Transport Inhibitor cocktail (eBioscience) was used as a control on unstimulated cells.

2.6 MLEC Luciferase TGF-β reporter assay

This bioassay is used to quantify TGF-β activity using mink lung epithelial cells (MLEC) permanently transfected with the expression construct p800neoLUC which contains a PAI-1 promoter fused to the firefly luciferase reporter gene. TGF-β is quantified based on its ability to upregulate PAI-1. [271].

2.6.1 Cell culture

MLEC cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 1% Pen/strep and 200μg/ml G418 (Geneticin, Thermo Fisher Scientific) Cells were seeded for the assay by removing the media from flask, detaching the cells using Trypsin EDTA AT 37^0C for up to 10 minutes. Cells were then re-suspended to give $1.6x10^5$ cells/ml in complete growth medium. Cells were seeding in a 96 well flat-bottom tissue culture plate (100μl/well) and incubated for 3 hours to allow cells to attach.

2.6.2 Standard Luciferase assay

Culture medium was carefully removed from 96 well plate and 100μl of test samples (lung or nasopharyngeal homogenates) were added. Purified TGF-β (diluted in DMEM 0.1% BSA) was used as a positive control and standard concentrations were from 0-2,500pg/ml. Cells, samples and positive controls were incubated overnight at 37^oC 5%CO_2 .

The following day, culture media/supernatant was removed and wells were washed three times with 100μl of cold PBS/well. 50μl of Lysis buffer (Promega Kit) was added to each well and incubated for 30 minutes at room temperature on a rocker to complete the lysis step. 30μl of cell lysate/well was then transferred into an opaque white fluorescent plate (Greiner Bio-One CELLSTAR plate). Using a BMG Omega Plate reader, 100μl of freshly prepared Luciferase Assay Reagent (800μM luciferin + 750μM ATP) provided in Promega Kit was added. The luciferase activity is recorded as relative light

units (RLU). These RLU values are converted to TGF-β activity (pg/ml) using the TGF-β standard curve.

2.7 Murine models of pneumococcal infection

The details of both mouse strains used in this thesis are detailed in table 6. After arrival into the Biomedical Services Unit, mice could acclimatise for one week. Mice were kept in groups of 5 in individually ventilated GM500 micro-isolator cage (IVC) racks. All murine experiments were conducted following the strict guidelines from the animal welfare committee and under authority of the UK Home Office licence (project licence PPL 40/3602, personal licence PIL 40/10905)

Strain	Age	Sex	Supplier
MF1 (outbred)	7-10 weeks	Female	Charles River UK
BALB/c (inbred)	7-10 weeks	Female	Harlan, UK

Table 6. Murine strains used in models of pneumococcal infection

2.7.1 Dose preparation

Once stock had been prepared and the CFU determined, dosing for infection models could be done. Frozen 1ml aliquots of stock were thawed at room temperature and centrifuged at 10,000xg for 2 minutes. The supernatant was discarded and sterile PBS was added to dilute suspension to desired concentration (Table 7).

2.7.2 Intranasal infection

The intranasal route of infection was used to introduce pneumococci into the lungs to establish invasive pneumonia or nasopharynx for induction of carriage. See table below for doses and volumes:

Model	Inoculum dose	Inoculation volume	CFU/ml
Nasopharyngeal carriage	$1X10^5$ cfu	10μl	$1x10^7$
Invasive pneumonia	$1x10^6$ cfu	50μl	$2x10^7$

Table 7. Details of dose preparation for the different murine models of pneumococcal infection.

Mice were mildly anaesthetized with 2.5% v/v Isofluorane over oxygen (1.4-1.6 litres/min). When sufficiently anaesthetised, 10μl of sterile PBS containing $1x10^5$ cfu of *S. pneumoniae* was equally distributed between both nostrils in a drop-wise fashion. Following the infection, the dose was measured for viable counts as described using the Miles and Misra method.

2.7.3 Intravenous infection

The intravenous route was used to introduce bacteria directly into blood to establish a bacteraemic infection. Mice were injected with 10^6 CFU in 50μl of PBS into the tail vein.

2.7.4 Monitoring murine behaviour using a pain scoring system

Following all infections, mice were monitored closely for physical signs of disease. The scoring system below was used to assess the course of infection and the wellbeing of the mice. In all experiments, once the mice had reached a 'lethargic +/++' they were culled in accordance to the severity limits imposed by the UK Home Office Licence.

Score	Description
Normal (0)	Mouse is moving around cage normally and has a normal coat
Hunched + (1)	Mouse is slightly arched over and walks on tip toes
Hunched ++ (2)	Mouse is very arched over and walks on tip toes
Starry/piloerect + (1)	The fur is upright around the neck area
Starry/ piloerect ++ (2)	The coat is upright all over the mouse
Lethargic + (5)	Mouse is slower moving around the cage
Lethargic ++ (7)	Mouse is not moving around the cage unless encouraged
Moribund	Mouse has stopped moving, coat is start and is upright, breathing is very laboured

Table 8. Details of the pain scoring system to monitor disease severity in mice. Numbers in brackets represent the pain score number given.

2.7.5 Infection studies: Lung, Nasopharyngeal, OB, OE and Brain tissue

Lung, nasopharynx, olfactory bulb (0B), olfactory epithelium (OE) and brain tissue were harvested and placed in bijou tubes containing 3mls of sterile PBS. If needed, the tissue weight was extrapolated by comparing weight of tube before and after the addition of the tissue. After weight was recorded, tissue was mechanically disrupted by homogenising for ~1minute with (IKA T10).

20μl was then removed from the homogenate and used to assess for CFU numbers using the Miles and Misra method. The remaining homogenate was stored at -80^{0}C for any future cytokine analysis.

2.7.6 Blood tissue

Blood was collected by cardiac puncture under terminal anaesthesia or for live mice, a small volume was removed from the tail vein and placed in Eppendorf tubes containing 2μl of Heparin (10units/ml, Sigma) to prevent the blood clotting. 20μl of blood was then taken to assess the CFU levels by Miles and Misra method.

2.8 Cell population studies (NP and Lung, preparation and antibody staining acquisition and analysis)

2.8.1 Tissue preparation (murine lungs)

Lung tissue was harvested, weighed, placed into a petri dish and then cut into smaller pieces using a scalpel blade. To help release immune cells via enzymatic digestion, lung tissue it was placed in 1.5ml Eppendorf tubes containing 1ml of PBS and 10mg/ml of Collagenase D (Roche). The Eppendorf tubes were then incubated at 37^{o}C for 30 minutes. After digestion, tissue was passed through a 40μm cell strainer (BD Biosciences) and washed through twice with 3mls of sterile PBS to create a single cell suspension. Cell suspensions were then centrifuged at 400 x g for 5 minutes and supernatant was taken for storage at -80^{o}C for cytokine analysis.

The cell pellet was re-suspended in 1x Red blood cell lysis buffer (Sigma) to lyse all red blood cells. After 5 minutes incubation, the reaction was quenched with PBS. The cell suspensions were then centrifuged at 400 x g for 5 minutes. Supernatant was then discard and cell pellet re-suspended in cryopreservation media, placed in a slow – freezing jar at -80^{o}C. When needed, aliquots of cells were thawed quickly in the water bath.

2.8.2 Tissue preparation (Nasopharynx)

Nasopharyngeal tissue was harvested and placed into bijou tubes containing 3mls of sterile PBS. The tissue was then mechanically disrupted for ~ 1 minute using a homogeniser (IKA T10). The homogenised tissue was then passed through a 40μm pore cell strainer and washed through twice with 3 mls of PBS to give a total volume of 6mls. The cell suspension was then centrifuged at 400 x g for 5 minutes. Supernatant was aliquoted and stored at -80^{o}C for later cytokine analysis. The cell pellet was either re-suspended in cryopreservation media or used for FACS analysis on the same day.

2.8.3 Flow cytometry

Nasopharynx and lung tissue were collected and prepared as described above (2.8.1 and 2.8.2). For staining and acquisition, samples were either thawed or used fresh from dissection. Cells were plated into a round bottom 96 well plate and washed twice with Facs buffer (PBS 2% FBS) before being incubated with a 1/200 dilution of purified anti- CD16/CD32 Fc blocking antibody (eBiosciences) for 30 minutes at room temperature. Following incubation with blocking antibody, cells were washed with Facs buffer, cell surface markers

were stained using a combination of monoclonal antibodies conjugated with FITC, PE, PE-Cy7 and APC fluorochromes (eBiosciences). An intracellular monoclonal antibody panel was used to detect both intracellular cytokines and transcription factors for different $CD4^+$ T cell subsets. The samples were acquired using a BD FACSCalibur TM flow cytometer (BD Biosciences).

Antibody panels used:

Target Cell	Antibodies used	Clone	Dilution
Neutrophils	CD45 FITC CD11b PE Gr-1 PE-Cyanine	30-F11 M1/70 RB6-8CS	1/200 1/600 1/600
Macrophages	CD45 FITC F4/80 APC CD68 PE-cy7 CD11c PE	30-F11 BM8 FA-11 N418	1/200 1/400 1/250 1/600
T regulatory cells	CD4 PE-CY7 CD45 FITC FoxP3 PE TGFβ APC or IL-10 APC	G.K 1.5 30-F11 NRRF-30 TW7-16B4 JESS-16E3	1/400 1/200 1/400 1/400 1/400
Th17 cells	CD4 PE-CY7 CD45 FITC RORγT PE IL-17A APC	G.K 1.5 30-F11 AFKJS-9 eBio17B7	1/400 1/200 1/400 1/400
Th1 cells	CD4 PE-CY7 CD45 FITC Tbet PE IFNγ- APC	G.K 1.5 30-F11 eBio4B10 XMG102	1/400 1/200 1/400 1/400
Th2 cells	CD4 PE-CY7 CD45 FITC GATA-3 PE IL-4 APC	G.K 1.5 30-F11 TWAJ 11B11	1/400 1/200 1/400 1/400

Table 9. Antibodies and dilutions used for FACS analysis of different cell types.

2.9 T cell re-stimulation assay

Mice were infected with either serotype 1 strain D25796 or serotype 2 strain D39. Mice were infected intranasally with a 10^5 CFU of either strain and left for 28 days during which time nasopharyngeal carriage was established and bacteria eventually cleared by the host immune system (see 2.7.2). After 28 days, mice were sacrificed and cervical lymph nodes (CLN) were removed from each mouse and placed in 1ml of RPMI 5% FBS (Sigma-Aldrich). CLN were passed through 40μM cell strainers and washed through with 5ml of RPMI. Cells were counted and re-suspended to give a concentration of 10^6 cells/100μl. Cells were then centrifuged at 400g for 5 minutes to create a cell pellet. Supernatant was removed and the pellet was re-suspended in fresh media. 100μl of cells were added per well of a 96 well tissue culture plate (Greiner). Bacteria were heat killed by incubating at 60^0C for 1 hour and then were added to cells. $1x10^6$ CFU of serotype 1 strain (ST217 Sepsis) or serotype 2 strain D39 were added to cells along with 2μl/ml of Cell Stimulation Cocktail (eBioscience) and left to incubate at 37^0C 5% CO_2 for 16-18 hours. Staining for cell surface marker CD4 and intracellular cytokines was performed to assess T cell subsets induced from re-stimulation with serotype 1 or serotype 2 pneumococci (see 2.8.3 for FACS staining).

2.10 Pneumolysin ELISA

ELISAs were used to detect the amount of pneumolysin produced by different serotypes of *S. pneumoniae* (10^7 cfu/ml) when bacteria were lysed with antibiotics over an hour at room temperature. The ELISA was also used to detect the amount of pneumolysin released into the supernatant when 10^7 CFU/ml of *S. pneumoniae* were cultured in PBS for one hour. The pneumolysin detection ELISA was performed by coating 96 well flat bottom plates with 1ug/well of IgG1-PLY4 antibody (Abcam ab71810) in PBS. Plates were sealed and incubated overnight at 4^0C. The following day, plates were washed, five times with Peprotech wash buffer (1.0% TWEEN-20 in PBS). Wells were then blocked for two hours at room temperature using 300ul/well of Peprotech Blocking Buffer (1% BSA in PBS). After the blocking stage, samples of bacteria supernatant or lysed bacteria along with the standards were added to the plate (100ul/well). The highest concentration of standard was optimised to be 200ng/ml. All plates contained a PBS negative control. The plate was then incubated for at least two hours at room temperature. After samples had been incubated, the plate was washed five times with wash buffer (1.0% TWEEN-20 in PBS). 1ug/ml of detection antibody (Anti-pneumolysin antibody PLY-4, Abcam ab71810) was added to the wells and incubated for two hours at room temperature. Plate was then washed five times with wash buffer (1.0% TWEEN-20 in PBS). Next, goat anti-rabbit IgG alkaline phosphatase (Abcam Ab97048) (1:5000 dilution) was added to wells for 30 minutes at room temperature. Plate was then washed five times with wash buffer (1.0% TWEEN-20 in PBS). The colour reagent Alkaline Phosphatase yellow pNNP (Sigma p7998) was added and plate incubated for 30 minutes in the dark. To

stop the reaction, 100µl of 1M NaOH was added and the absorbance of each plate was read at 405nm with an ELISA plate reader.

2.11 Triton X-100 induced autolysis assay

Triton X-100 induced autolysis assays were performed essentially as described previously with the following modifications [272,273]. Cultures of *S. pneumoniae* were cultured overnight in BHI broth at 37°C until an approximate A_{600} of one was obtained. Cultures were centrifuged for ten minutes at 3600g. Pellets were re-suspended in 1 ml of fresh PBS and the A_{600} was adjusted to one in one ml of PBS containing either 0.01% of Triton X-100 (Sigma) in a cuvette. Cuvettes were covered in parafilm and samples vortexed for 5 seconds. The A_{600} of the culture at time zero was noted and samples were incubated at 37^0C and 180 rpm for 1 – 3 hours. The A_{600} was recorded every 15 minutes. Triton X-100 induced autolysis was presented as a percentage of the initial A600 at time zero. Each experiment was performed in triplicate.

2.12 LDH Cytotoxicity Assay

An LDH cytotoxicity kit (Thermo Scientific Pierce) was used to measure Lactate dehydrogenase (LDH) which is a cytosolic enzyme present in many cell types. When the plasma membrane of a cell is damaged, LDH is released into the cell culture media and is therefore an indicator of cellular toxicity.

50µl of cell supernatant was transferred into a 96 well plate and incubated with 50µl of reaction mixture (LDH cytotoxicity kit, Thermo Scientific) and incubated at room temperature for 30 minutes before the addition of 50µl/well of stop

solution (LDH cytotoxicity kit, Thermo Scientific). Absorbance was measured at 490nm and 680nm on a Spectrophotometer.

2.13 Transepithelial Electrical Resistance measurements (TEER)

2.13.1 A549 Human alveolar epithelial cell culture

Human lung adenocarcinoma epithelial cell line (A549) was used to mimic bacterial lung infections *in vitro.* A549 cells were maintained with DMEM supplemented with 10% FBS. Cells were detached from tissue culture flask using Trypsin-EDTA and 500μl of cells ($2x10^5$cells/ml) were seeded onto Greiner Inserts (0.94cm^2 surface area, 3μm pore diameter). Inserts were placed into a 12 well tissue culture plate (Greiner) with 1.5ml of media and incubated at 37^0C 5% CO_2 for three days before infection with bacteria.

2.13.2 Bacterial infection

Frozen bacterial stocks were thawed, washed with PBS and re-suspended to a desired final concentration in DMEM+10% FBS. 500μl of bacterial suspension was added to the tissue culture inserts. For control wells, media was removed and replaced with 500μl of fresh media.

2.13.4 Transepithelial Electrical Resistance measurement (TEER)

TEER was measured in Ohms using an EVOM2 Epithelial Voltohmeter (WPI). The mean of three readings per insert was calculated. Wells with media only (no cells) used as blank measurement which was subtracted from each insert.

Blank corrected resistance values were then multiplied by 0.94 (the surface area of insert) to give a TEER reading in Ohms x cm^2.

2.13.5 Liposome treatment

Cholesterol:sphingomyelin (66mol/%cholesterol) and sphingomyelin-only liposomes were provided by Lascco (Geneva Switzerland – product name CAL02). In all experiments a concentration of 2μg/ml of liposomes was used. In the supernatant swap experiments, $2x10^7$ CFU of bacteria were prepared in 1ml of PBS. After 45 minutes of incubation at room temperature, the bacteria were pelleted and the supernatants removed to a fresh Eppendorf tube. 2ug/ml of Cholesterol:sphingomyelin (66mol/%cholesterol) and sphingomyelin-only liposomes were added to the supernatants for 30 minutes. After 30 minutes, Eppendorfs of bacterial supernatant were centrifuged at 400g for 5 minutes to pellet liposomes. Liposome-treated bacterial supernatant was then removed and used to re-suspend the pelleted bacteria prior to intranasal infection of mice.

2.14 Haemolytic Assays

10mls of Sheep Blood (Sigma) was centrifuged at 3000rpm for 14 minutes at 4^0C and supernatant was removed. 400μl of blood pellet was added to 10mls of PBS to make a 4% RBC solution. Bacterial stocks were thawed, centrifuged at 10,000g for ten minutes and re-suspended in PBS to give a concentration of $1x10^7$CFU/ML. Bacteria were then lysed using Penicillin and Streptomycin antibiotics for 30 minutes at room temperature. 50μl of lysed bacteria was taken and placed in the first row of wells in a round bottom microplate with

50μl of 4% RBC solution. Bacteria was then diluted two-fold with 4% RBC solution before culture at 37^0C for 30 minutes. After incubation, plate was centrifuged at 1000rpm for 1 minutes and supernatant was removed from each well (without disturbing pellet) and transferred into a fresh flat-bottomed 96 well microplate. The OD was measured at 540nm on a spectrometer to determine the levels of haemoglobin released upon culture with lysates from different serotypes of *S. pneumoniae*.

2.15 Statistical analysis

GraphPad Prism 6 software was used to create all the graphs shown in this thesis. All statistical analysis was carried out using the GraphPad Prism statistical package.

One and two way ANOVAs were used and statistical significance was categorized as $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, or $^{****}P < 0.001$.

Investigating the role of Streptococcus pneumoniae African Serotype 1 capsule in nasopharyngeal carriage and invasive disease

Chapter 3

Investigating the role of *Streptococcus pneumoniae* African Serotype 1 capsule in nasopharyngeal carriage and invasive disease.

3.1 Introduction

Serotype 1 pneumococci are among the most common serotypes isolated from invasive disease cases in sub-Saharan Africa but are rarely found causing asymptomatic nasopharyngeal carriage [44]. We can understand more about serotype-1 disease pathogenesis by focusing on the role of the polysaccharide capsule - a major bacterial virulence factor - in carriage, invasive disease and interaction with the host immune response.

The aim of this work was to elucidate the role of polysaccharide capsule in both carriage and invasive serotype 1 pneumococcal disease. Firstly, growth rates, C3b complement deposition, resistance to opsonophagocytosis killing, and quantification of capsule thickness assays were performed (see Materials and Methods sections 2.2, 2.3 and 2.4). The aim of these assays was to compare *in vitro* characteristics of a serotype 1 nasopharyngeal carriage isolate (W000168) to serotype 1 isolates from blood (D25796) and cerebral spinal fluid (C9471). Further details of each isolate can be found in Table 5.

Having obtained these results, pathogenesis of the three isolates was compared in murine models of pneumococcal infection (Materials and Methods section 2.7). Intranasal infections were performed to establish nasopharyngeal carriage and pneumonia infection models. For the murine

sepsis models, bacteria were intravenously injected into the tail veins of mice. Mice were monitored for signs of disease, survival rates recorded and bacteria densities monitored over time.

3.2 Results

3.2.1 Growth Curves

As bacterial growth rates can be an indicator of their invasive potential in the host [274], the growth rates of carriage (W000168), sepsis (D25796) and meningitis (C9471) ST217 serotype 1 isolates were compared *in vitro* (Figure 7).

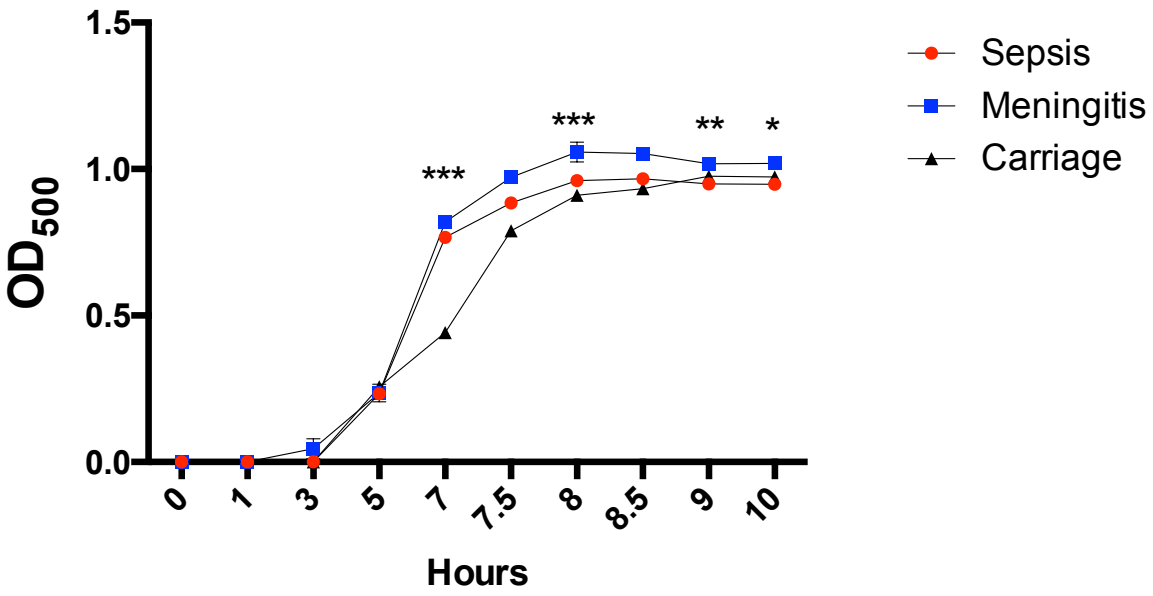


Figure 7* Growth curves of sepsis (D25796), meningitis (C9471) and carriage (W000168) ST217 serotype 1 isolates in BHI over time**. Data shown as mean Optical Density (OD_{500}) of bacterial cultures determined 60 minutes until 7 hours and then every 30 minutes until stationary phase at 9 hours. Growth of each isolate was measures in duplicate. Data presented as mean and SEM. Statistical analysis was performed by Two-way ANOVAs (*P < 0.05, **P < 0.01,P < 0.005, or ****P < 0.001*)*

As shown in figure 7, all isolates show a similar overall pattern of growth with all remaining in the lag phase for the first three hours. This phase is

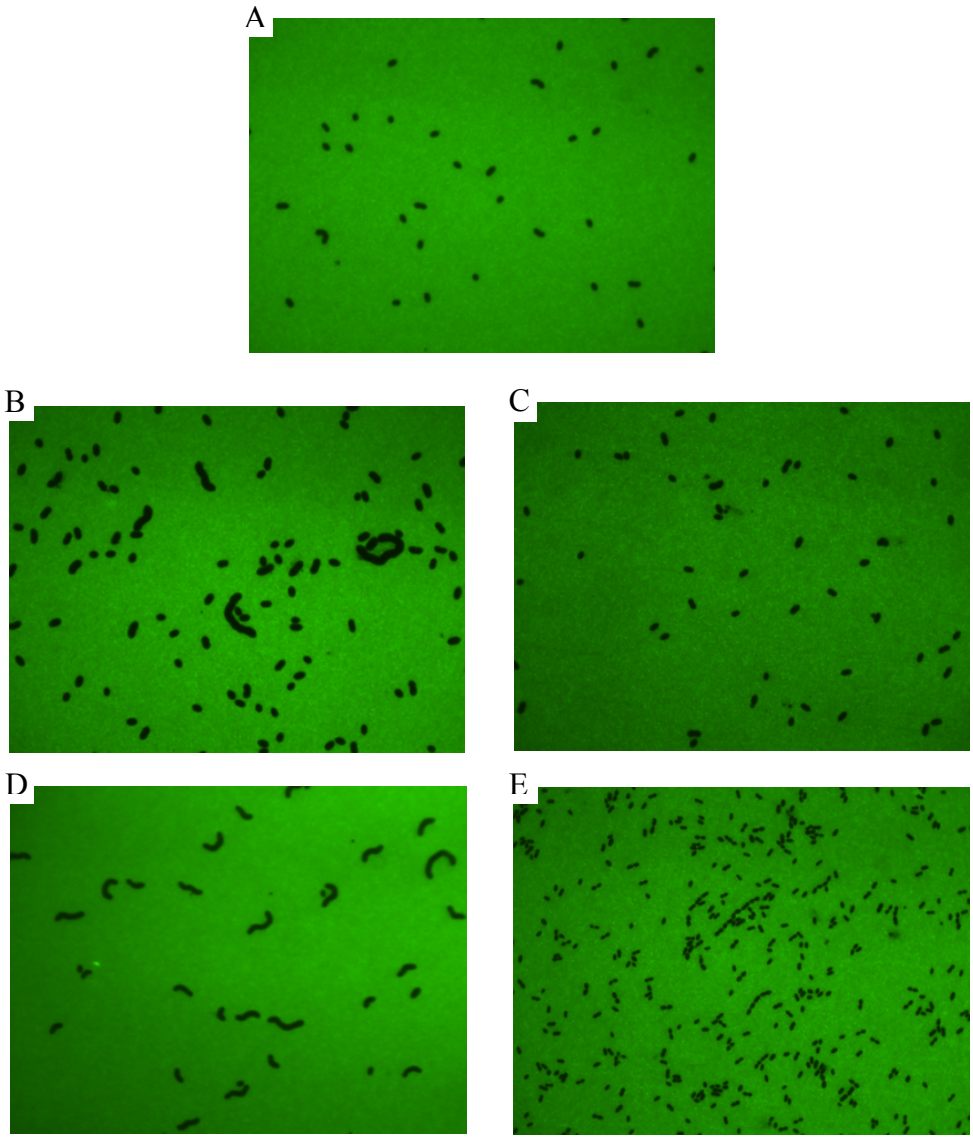
characterized by little to no growth as bacteria are adjusting to the environment[275].

Growth rates appear to be similar in both the meningitis and sepsis isolates while carriage isolates follow a similar trend but have significantly slower growth during the second phase, known as the log or exponential phase. This phase is characterized by a rapid increase in bacterial numbers. In pathogenic species, it is usually in the log phase that disease symptoms occur due to expanding bacterial populations which cause the most tissue damage in the host [275]. At the 7 hour timepoint, Meningitis and Sepsis isolates enter the late exponential phase with a significantly higher OD reading ($P<0.005$) compared to the Carriage isolates which enter late exponential phase at 7.5 hours.

The final stage is the stationary phase, when population growth levels off as the rate of cell death begins to equal the rate of cell division. Bacteria stop replicating due to exhaustion of nutrients available and buildup of toxic waste products detrimental to bacterial growth[275]. At 8 hours, Meningitis and Sepsis isolates reach stationary phase. Meningitis isolates appear to reach stationary phase at a significantly higher OD compared to sepsis isolates ($P<0.005$). An hour later carriage isolates reached stationary phase and at ten hours, both carriage and sepsis isolates remain in stationary phase at a significantly lower OD compared to meningitis isolates. These results suggest that sepsis and meningitis invasive isolates could have greater invasive potential due to faster growth during exponential phase compared to a nasopharyngeal carriage serotype 1 isolate. These results also indicate that meningitis may have a greater ability to utilize nutrients in BHI due to higher OD readings during stationary phase.

3.3.2 Polysaccharide capsule thickness

The polysaccharide capsule surrounding *S. pneumoniae* protects the bacteria from the host immune response and aids bacterial virulence. However, the role of the capsule in nasopharyngeal carriage is still not fully understood. FITC-dextran staining (Section 2.2) was used to quantify the size of the bacterial capsule and compare capsule sizes of carriage (W000168) and invasive isolates (D25796 and C9471) when cultured in BHI + 10% FCS medium (Figure 8).



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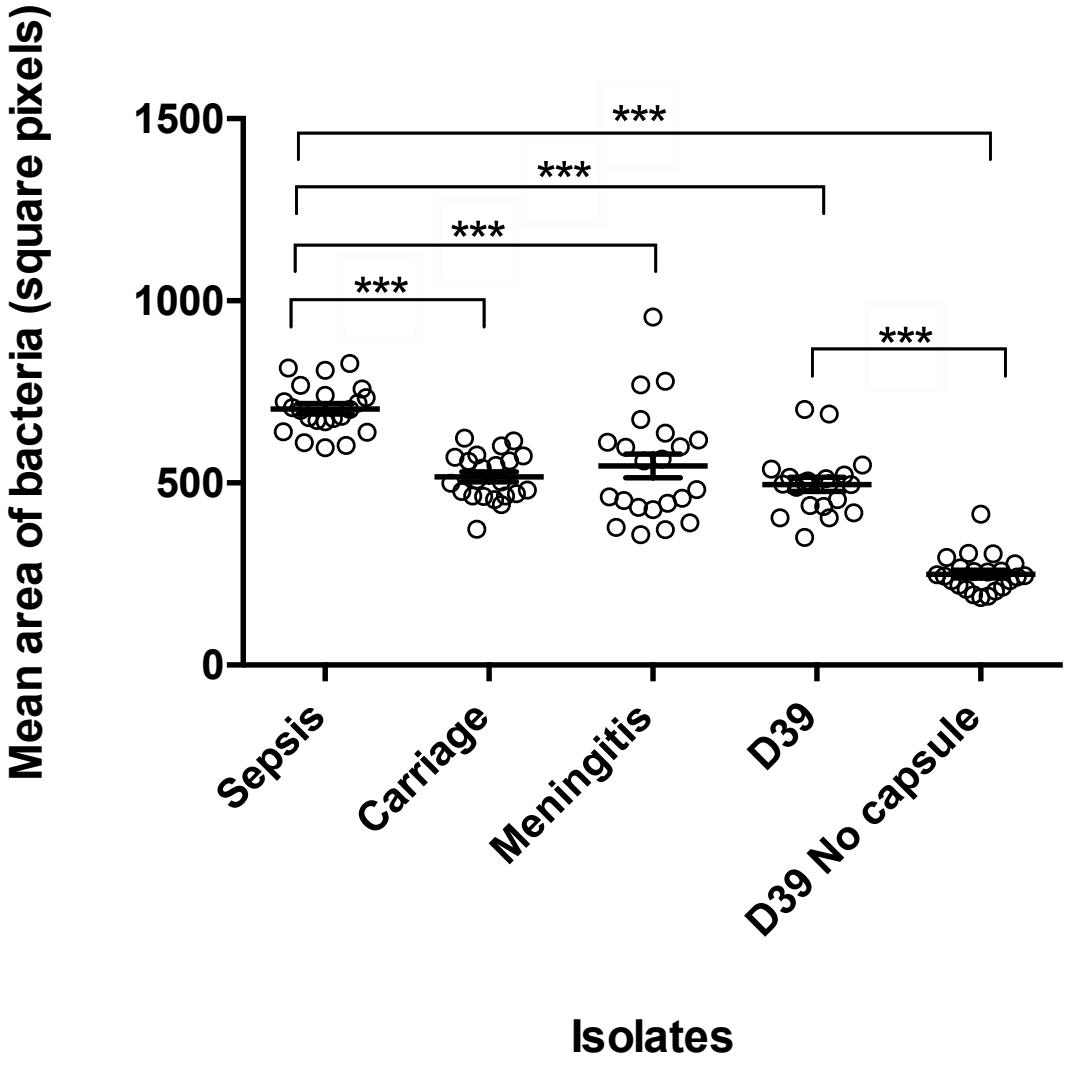


Figure 8 Capsule thickness of serotype 1 ST217 isolates. 100x magnification images of (A) Carriage W000168, (B) Sepsis D25796, (C) Meningitis C9471, serotype 1 ST217 isolates and comparison with (D) serotype 2 strain D39 and (E) its unencapsulated mutant. (F) Mean area of sepsis, carriage and meningitis isolates plus D39 and unencapsulated D39 mutant. Error bars represent Mean and SEM. Statistical analysis was performed using One-way ANOVAs. (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001*)*

FITC-Dextran assays are a method of providing a physiologically relevant measure of capsule thickness [75]. Results obtained demonstrate differences in capsule thickness of serotype 1 isolates taken from difference tissues (blood, nasopharynx and CSF). D25796 sepsis isolate has a significantly thicker capsule compared to carriage and meningitis isolates.

3.3.3 Differences in resistance to complement deposition

Significant differences in capsule thickness were observed between three ST217 isolates (Figure 8). As capsule thickness has been reported to correlate with resistance to complement deposition on the bacterial surface,[70] complement deposition assays measuring the amount of C3b deposition on the surface of bacteria were performed (Figure 9).

Resistance to complement deposition correlated with capsule thickness when comparing sepsis and carriage isolates. ST217 sepsis isolate has a significantly thicker capsule compared to the carriage isolate and had significantly lower levels of C3b deposited on the bacterial surface ($P<0.05$). The meningitis isolate however, has a thinner capsule than the sepsis isolate but demonstrates significantly lower levels of complement deposition.

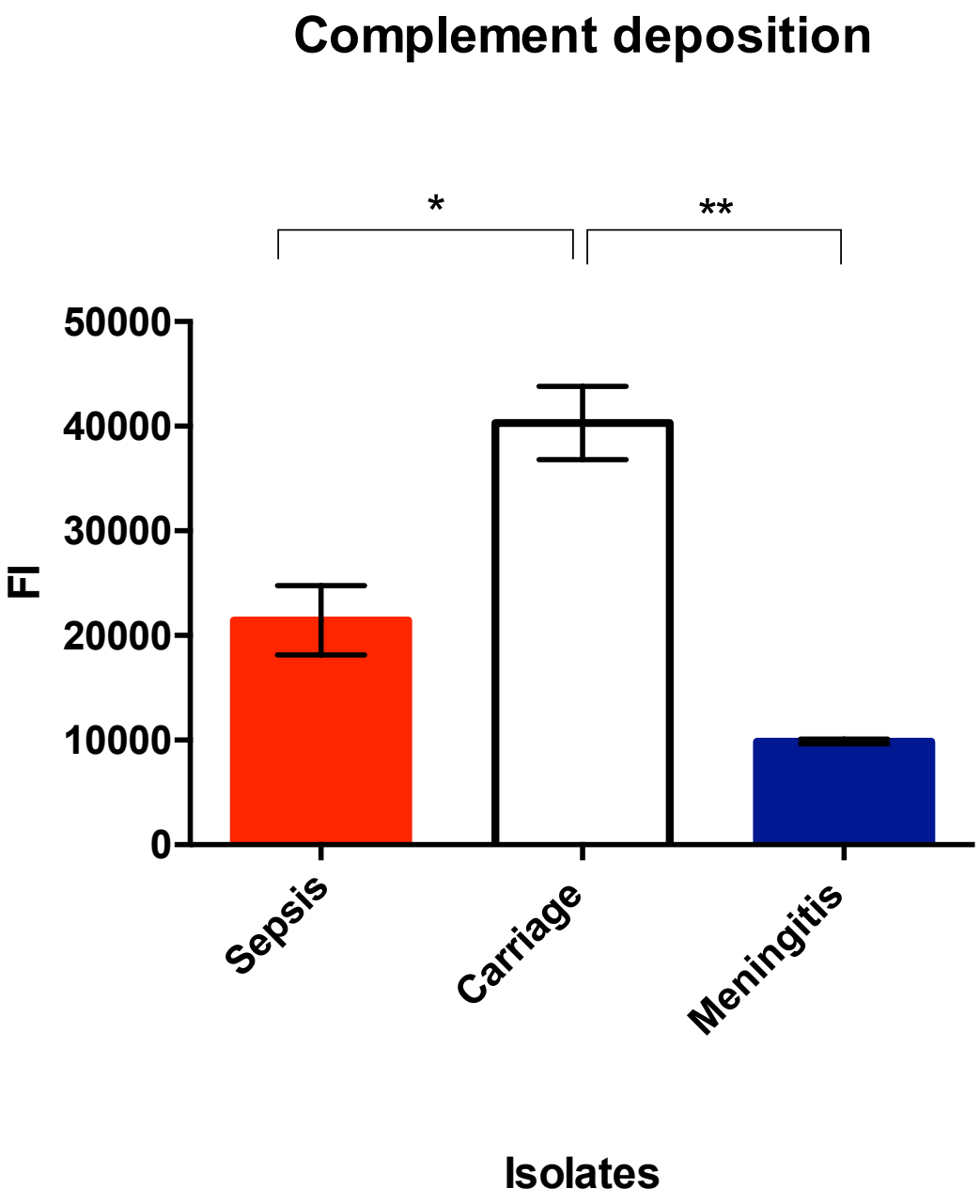


Figure 9. C3b deposition on the surface of serotype 1 isolates. Data presented as mean fluorescent intensity ±SEM. Experiments were performed in triplicate as described in Materials and Methods section 2.4). C3b deposition is expressed as Fluorescence Index (FI) = mean fluorescence intensity X % of $C3b^{+}$ bacteria). Statistical analysis was performed using one-way ANOVA. (* $P<0.05$, ** $P<0.01$).

3.3.4 Opsonophagocytosis assays:

Phagocytosis of *S. pneumoniae* by neutrophils is a vital part of the host immune defence and is largely dependent on complement activation[270]. A human neutrophil cell line (HL-60) was incubated with *S. pneumoniae* and human complement, to examine the phagocytic capability of neutrophils against the three ST217 serotype 1 isolates (Figure 10).

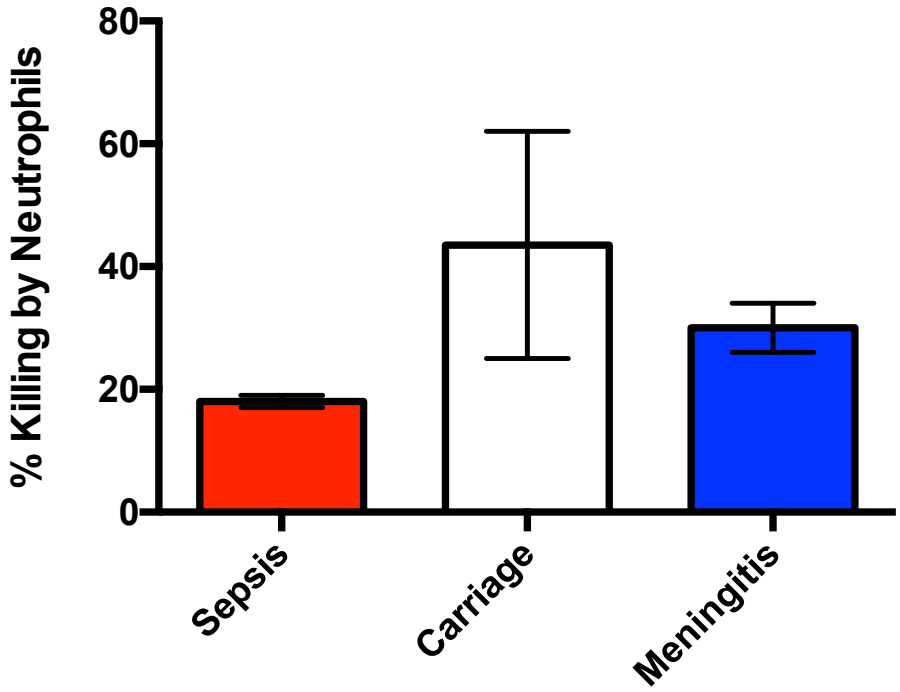


Figure 10 Opsonophagocytosis of African Serotype 1 isolates by neutrophil cell line, HL-60. Results are shown from one experiment performed in duplicate (Materials and Methods section 2.3). The isolates used were three ST217 isolates: Sepsis (D25796), Carriage (W000168) and Meningitis (C9471). Data represented as mean ± SEM. Human serum was used as a complement source and diluted 1:4 ratio in PBS.

Results in figure 10 show that serotype 1 carriage (W000168) isolate appears to be highly susceptible to phagocytosis as we observe over 40% killing of the total bacterial population, although no significant differences were observed between isolates. This is compared with a sepsis (D25796) serotype 1 isolate where less than 20% of bacteria are killed. This data correlates with capsule thickness, suggesting that a thicker capsule can protect from phagocytosis. The meningitis isolate appears to not fit with the current dogma as it shows significantly lower levels of surface complement deposition but this does not correlate with resistance to phagocytosis when compared to the sepsis isolate.

3.3.5 Murine model of nasopharyngeal carriage

Animal models have been widely used to study the pathogenesis of *Streptococcus pneumoniae* [276]. There have been many reports published on virulence of different serotypes but relatively little is known about the pathogenesis of African serotype 1 *in vivo*. Since serotype 1 is known for having a high attack rate despite rarely being detected in the nasopharynx [277], murine carriage models were used to study the density and duration of serotype 1 colonisation of the nasopharynx of mice. Mice were intranasally infected with 10^5 CFU of either ST217 sepsis (D25796), carriage (W000168) or meningitis (C9471) to establish nasopharyngeal colonization (Materials and Methods 2.7.2). Mice were culled at pre-determined timepoints and bacterial density in the nasopharynx was recorded.

The *in vitro* characterisation of three ST217 isolates showed differences in capsule thickness, complement deposition and resistance to phagocytosis (figures 8-10). A well-established murine nasopharyngeal carriage model was

used to examine whether the phenotypic differences observed *in vitro* resulted in a difference in the ability of each isolate to colonise the nasopharynx (Figure 11).

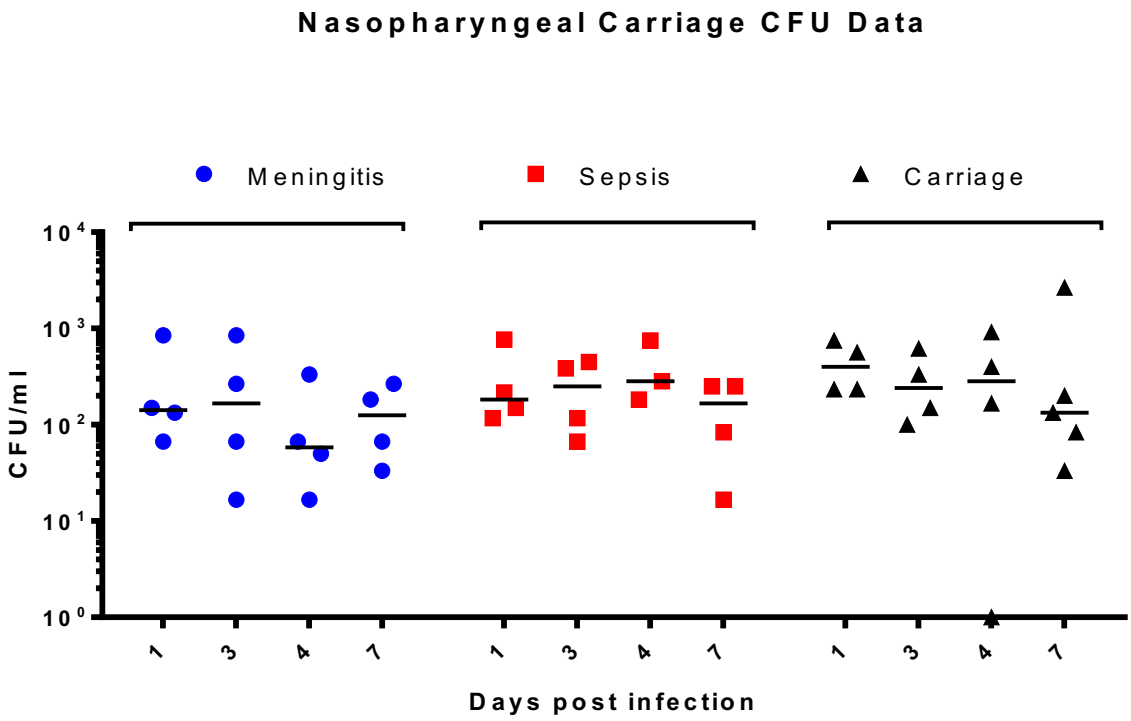


Figure 11 The establishment of murine nasopharyngeal carriage by different serotype 1 isolates. Results are shown as a time course of 7 days during which bacterial numbers in the nasopharynx of MF-1 mice were recorded at set time points. Results are shown as CFU/ml of individual mice. Statistical analysis was performed using Two way ANOVAs.

Figure 11 demonstrates that all isolates were able to establish asymptomatic nasopharyngeal carriage for 7 days at a density between 10^3 and10^4 CFU/ml. All three isolates were able to maintain stable density of bacteria over the course of seven days and no significant difference was seen in the bacterial numbers at day 7 which demonstrates that serotype 1 pneumococci isolated from both patients with invasive disease and asymptomatic carriage are able to successfully establish carriage in a naïve murine nasopharynx.

3.3.5.1 Serotype 1 colonisation of deeper tissues of the upper airway

Current literature suggests that serotype 1 is not able to colonise the nasopharynx with the same success as other serotypes of *S. pneumoniae*[238]. This theory is supported by rare detection of serotype 1 by nasopharyngeal swabs or washes both in areas of current disease outbreaks and routine nasopharyngeal swabbing around sub-Saharan Africa [44]. To further understand why serotype 1 carriage detection rates are so low, a more sophisticated murine model of nasopharyngeal carriage was established. In this experiment, colonisation patterns of serotype 1 in deeper tissues of the upper airway such as the olfactory epithelium, the olfactory bulb and the pharynx (Figure 12) were investigated as a potential reservoir for serotype 1 carriage that may explain why it is rarely isolated from nasopharyngeal swabs (see Materials and Methods section 2.7.2).

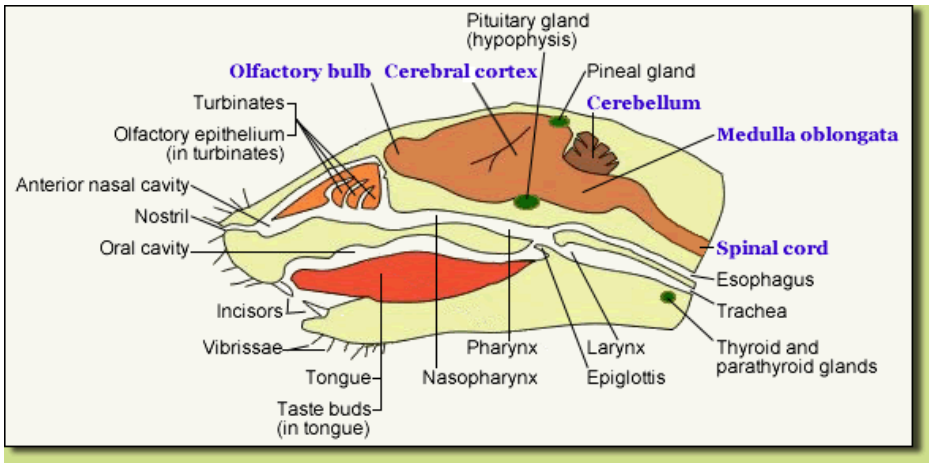


Figure 12 Diagram showing the areas of the upper airway that have the potential to be colonised by serotype 1. Areas of interest are Olfactory epithelium, Olfactory bulb, and Pharynx. Image taken from ©2004 Texas Histopages.

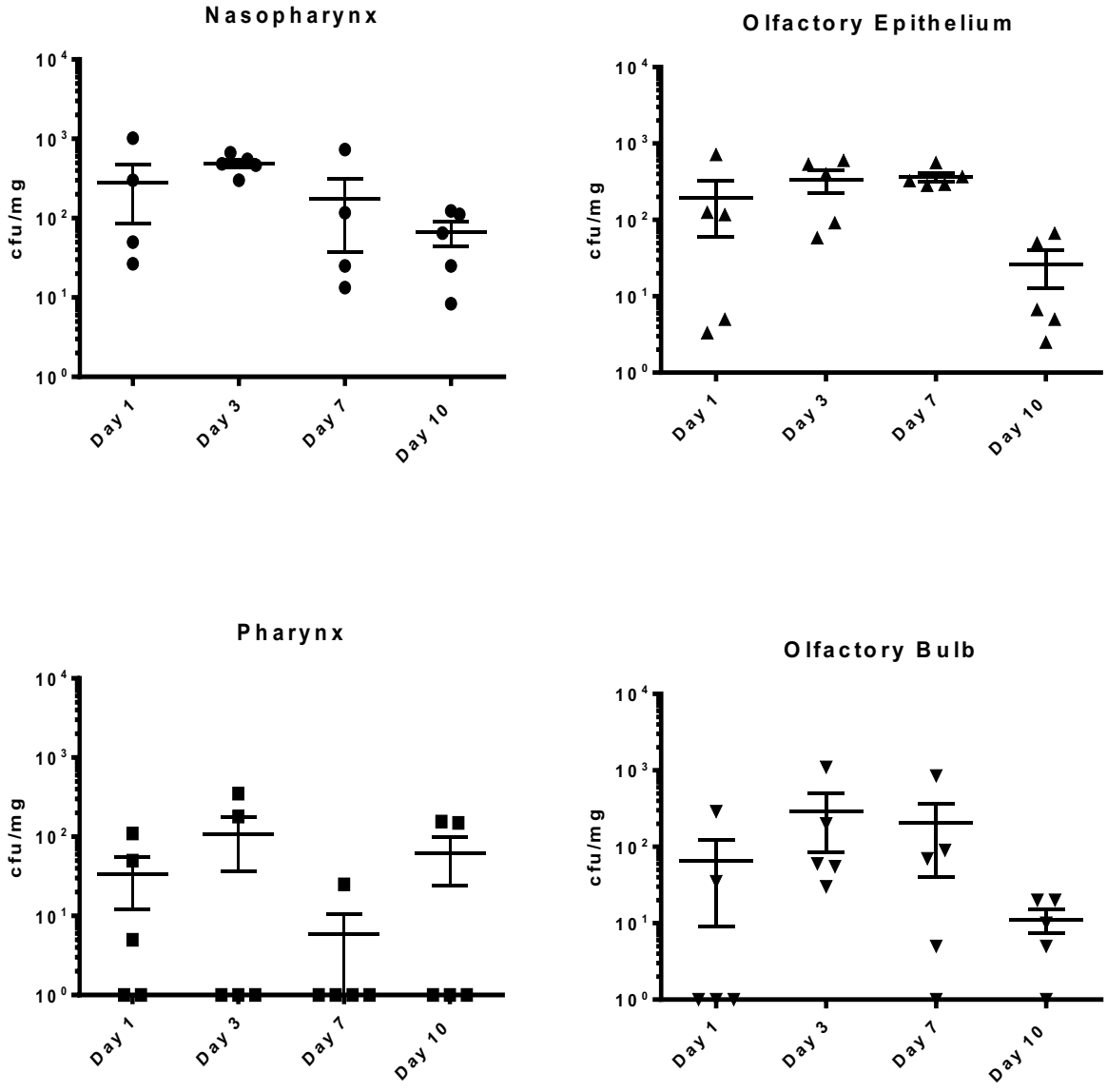


Figure 13 Streptococcus pneumoniae serotype 1 colonisation of deeper tissues of the upper airway. MF1 mice were intranasally infected with serotype 1 isolate (D25796, Sepsis) at a concentration of 10^5 CFU in 10µl of PBS. At days 1,3,7 and 10 post infection mice were culled and CFU counts performed on tissue from the nasopharynx, olfactory bulb (OB), olfactory epithelium (OE) and pharynx. 5 mice per timepoint. Data presented as mean ± SEM. No statistical analysis performed.

Figure 13 demonstrates that during a nasopharyngeal carriage episode, serotype 1 can invade local tissues and successfully colonise these areas for at least seven days. Invasion of the olfactory bulb and epithelium by bacteria is thought to increase chances of meningitis occurring due to the proximity to the brain [278]. It appears from these findings that African serotype 1 favours colonisation of the tissues above the nasopharynx compared to that of the pharynx where we observe poor colonisation rates with 3-4 mice per group not able to establish carriage. (Serotype 1 colonisation of OE and OB are compared to Serotype 2 strain D39 in chapter 5)

3.3.5.2 Changes in polysaccharide capsule thickness over the duration of nasopharyngeal carriage

Data from murine colonisation models suggest that serotype 1 can successfully colonise the nasopharynx and other tissues in the upper airway (Figure 13). To examine how isolate phenotype might change over the course of nasopharyngeal colonisation, capsule thickness measurements (Materials and Methods 2.2) were performed on serotype 1 (Sepsis D25796) isolated at days 2, 7 and 14 post-infection (Figure 14).

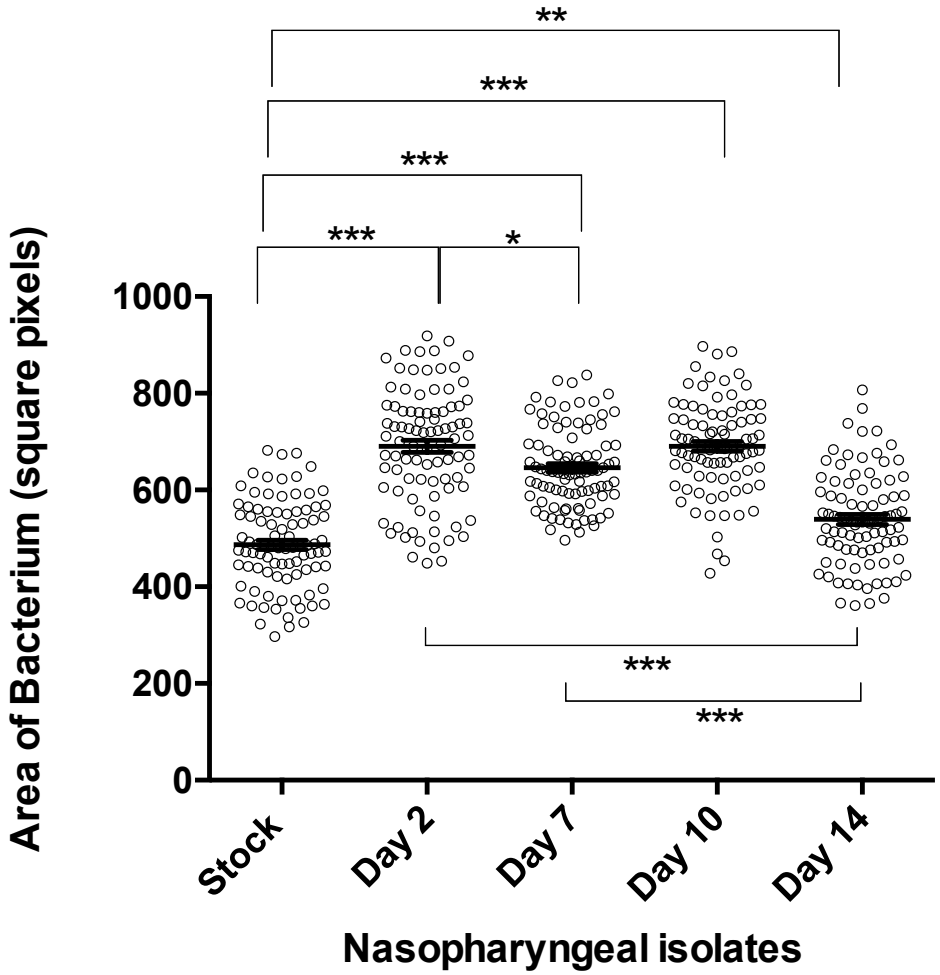


Figure 14. Changes in capsule thickness over the duration of nasopharyngeal carriage. Female MFI mice were intranasally inoculated with serotype 1 (D25976, Sepsis) *S. pneumoniae*. 5 mice were culled per time-point and bacteria collected, pooled and made into bead stocks for FITC-dextran analysis of capsule thickness. Statistical analysis was performed using one-way ANOVA. (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001*)*

Results presented here (Figure 14) show that serotype 1 capsule thickness increases significantly in the nasopharynx within the first 48 hours of infection. Serotype 1 capsule then decreases from day 2 to 7 and then further decreases by day 14 but remains significantly increased over all time points compared to the original stock isolates used for the infection.

3.3.5.3 Complement deposition resistance and association with changes in capsule thickness during serotype 1 colonisation of the nasopharynx.

It has been widely published that increased capsule thickness correlates with increased protection from C3b complement deposition [270]. With significant changes observed in capsule thickness over 14 days in the nasopharynx, we wanted to examine the effect this had on C3b complement deposition on isolates taken at different time-points during nasopharyngeal carriage.

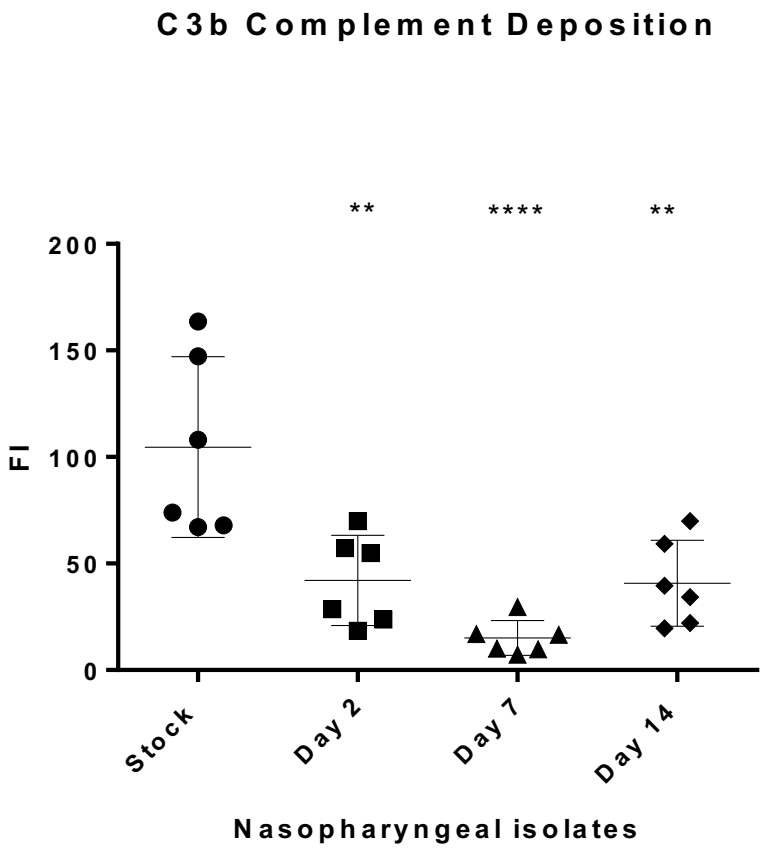


Figure 15. Changes in complement deposition over duration of nasopharyngeal carriage. Female MFI mice were intranasally inoculated with serotype 1 *S. pneumoniae* (D25796, Sepsis). 5 mice were culled per time-point and bacteria collected, pooled and made into bead stocks for C3b deposition on bacterial surface. C3b deposition was measured in triplicate on two independent days. C3b deposition is expressed as Fluorescence Index (FI) = mean fluorescence intensity X % of $C3b^{+}$ bacteria). One-way ANOVA test was used for statistical analysis whereby Day 2,7 and 14 isolates were compared to stock isolate. (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001*)*

The results in Figure 14 and 15 demonstrate an inverse correlation between serotype 1 capsule thickness and the amount of complement deposition on the surface of bacteria. C3b complement deposition of serotype 1 isolates in the nasopharynx is reduced in the first two days of infection as capsule thickness increases. The amount of C3b deposition on the bacterial surface increases on day 14 which correlates with the decrease in capsule thickness from day 7 to day 14 post infection.

These results suggest that serotype 1 can rapidly adapt to the environment of the nasopharynx by significantly increasing capsule thickness, and thus reducing complement deposition of the surface of the bacteria.

3.3.8 Host immune cell responses during nasopharyngeal carriage

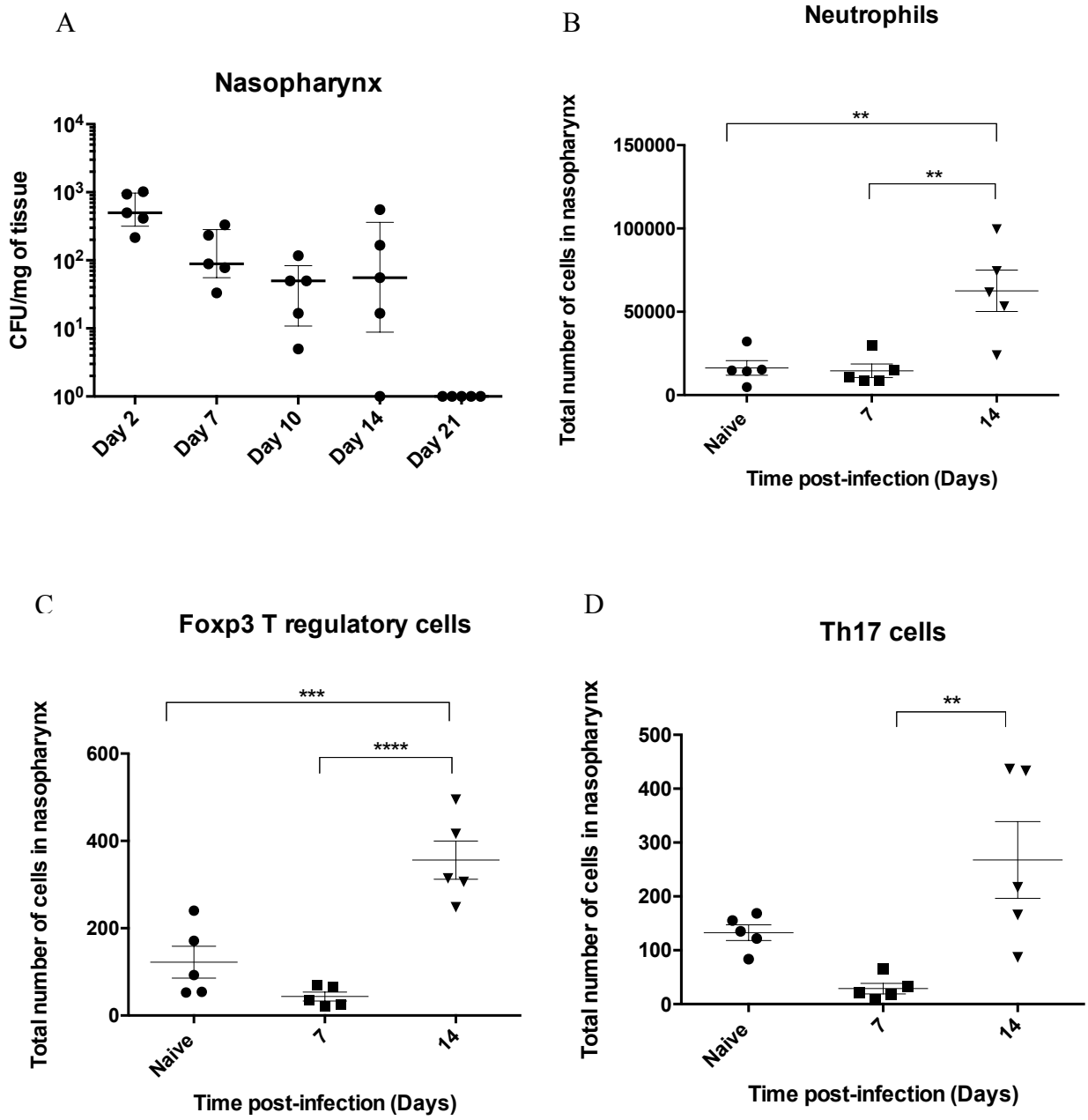


Figure 16 Changes in host immune response during serotype 1 nasopharyngeal colonisation. (A) bacterial load (CFU) per mg nasopharynx at time-points over 21 days of colonisation. 5 mice/group. Flow cytometric analysis of (B) Neutrophils ($CD45^+$ $CD11b^+$ and $Gr1^+$), (C) FoxP3 T regulatory cells ($CD45^+$, $CD4^+$, $Foxp3^+$) and (D) T_H17 cells ($CD45^+$, $CD4^+$, $ROR\gamma t^+$ and $IL\text{-}17^+$) in nasopharynx. One-way ANOVA test was used for statistical analysis (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001)

Changes in capsule thickness and resistance to complement deposition during serotype 1 colonisation of the nasopharynx may be in response to signals received from the host immune system. The immune cell profiles of cells associated with nasopharyngeal carriage was examined over 14 days (Figure 16). Serotype 1 (D25796) was able to successfully colonise for at least 14 days in the nasopharynx with all mice clearing the infection by 21 days post infection. Results demonstrate little difference in the innate immune response (neutrophils) at 7 days post-infection. However, an increase in neutrophil numbers is observed at day 14. As no bacterial colonies are detected at 21 days post infection in the nasopharynx, the increase of neutrophil numbers at day 14 may contribute to bacterial clearance.

At 14 days post infection increases in cells of the adaptive immune response such as Th17 cells was observed. These may also contribute to bacterial clearance. An increase in T regulatory cells at day 14 was noted and may represent a response to an increase in inflammation during bacterial clearance. Further analysis is needed to define all cell types involved in clearance of the bacteria in the nasopharynx. This is examined further in results chapter 5.

3.3.5 Murine models of invasive pneumococcal disease

Comparison of serotype 1 isolates in a murine pneumonia model

As no difference was seen in the ability of different serotype 1 ST217 isolates to establish nasopharyngeal carriage despite significant *in vitro* phenotypic differences, isolates were compared in an *in vivo* invasive pneumonia model (Figure 11). I aimed to assess whether the differences in capsule size, complement deposition and resistance to phagocytosis seen in the *in vitro* phenotyping assays correlated with differences in disease severity during lung infection. Previous work has shown that strains producing more capsule *in vitro* are more virulent *in vivo* [279], however, differences of capsule expression within the same serotype and the effect it has on virulence has not previously been examined.

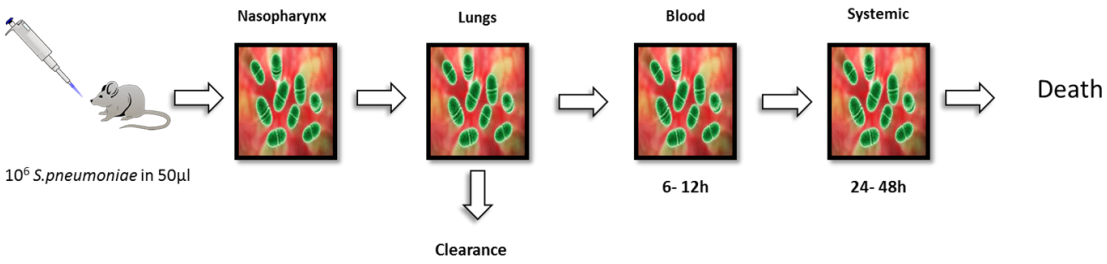


Figure 17. Schematic representation of in vivo murine pneumonia model, including disease progression from pneumonia to sepsis. Mice are intranasally infected with *S. pneumoniae*. Bacteria in 50µl move straight down into the lungs and establish pneumonia. Bacteria can be cleared from the lungs or disseminate into the bloodstream. If bacteria continue to multiply in the blood then a systemic infection occurs and leads to death.

Mice were intranasally infected with either meningitis, carriage or sepsis serotype 1 ST217 isolates. Mean survival times, pain scores and blood and lung bacterial load for pneumococcal pneumonia model were recorded to compare the virulence of the serotype 1 isolates (figure 18).

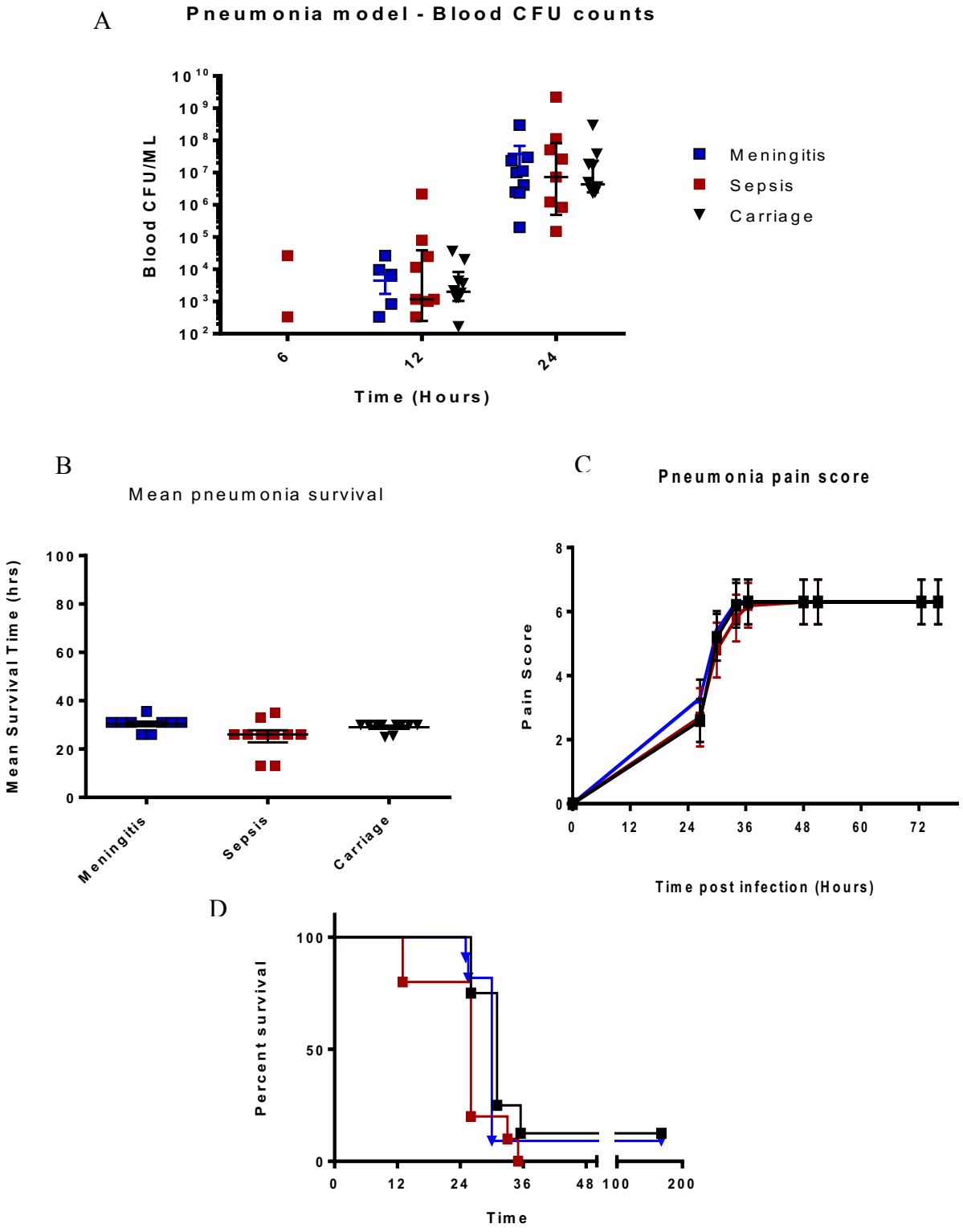


Figure 18 Comparison of Serotype 1 isolates in a murine pneumonia model. Mice were administered 10^6 *S. pneumoniae* intranasally on day 0. Mice were monitored closely for signs of ill health and once mice became lethargic, they were culled and time of death noted. A) Tail bleeds were performed at 6, 12 and 24 hours to examine blood CFU levels. B) Mean survival time for each mouse. C) Pain scores were determined for infected mice until time of death D) Kaplan Meier Survival Curve. Error bars represent the standard error of the mean.

Results in Figure 18 show no difference in virulence between carriage, meningitis and sepsis serotype 1 isolates in a pneumonia model of infection. There was no difference between the three groups in mean survival times or pain scores of infected mice. There was also no difference in bacterial loads in the blood at 12 and 24 hours post infection. The only difference seen was that 20% of sepsis isolate (D25796) infected mice had bacterial dissemination from lungs into blood at 6 hours post infection compared to 0% in carriage and meningitis isolate groups. However, at 12 and 24 hours post infection there was no difference in blood bacterial loads between three serotype 1 isolates.

3.3.6 Comparison of serotype 1 isolates in a murine model of sepsis

No differences in disease severity where observed between three serotype 1 isolates in a pneumonia murine model of infection (Figure 18). To check for differences in virulence during sepsis, bacteria were directly administered into in the bloodstream. Mice were intravenously infected with either carriage, sepsis or meningitis serotype 1 ST217 isolates and survival times, blood cfu counts and pain scores were recorded (Figure 19). At time of death, the bacterial load in the lungs was also noted.

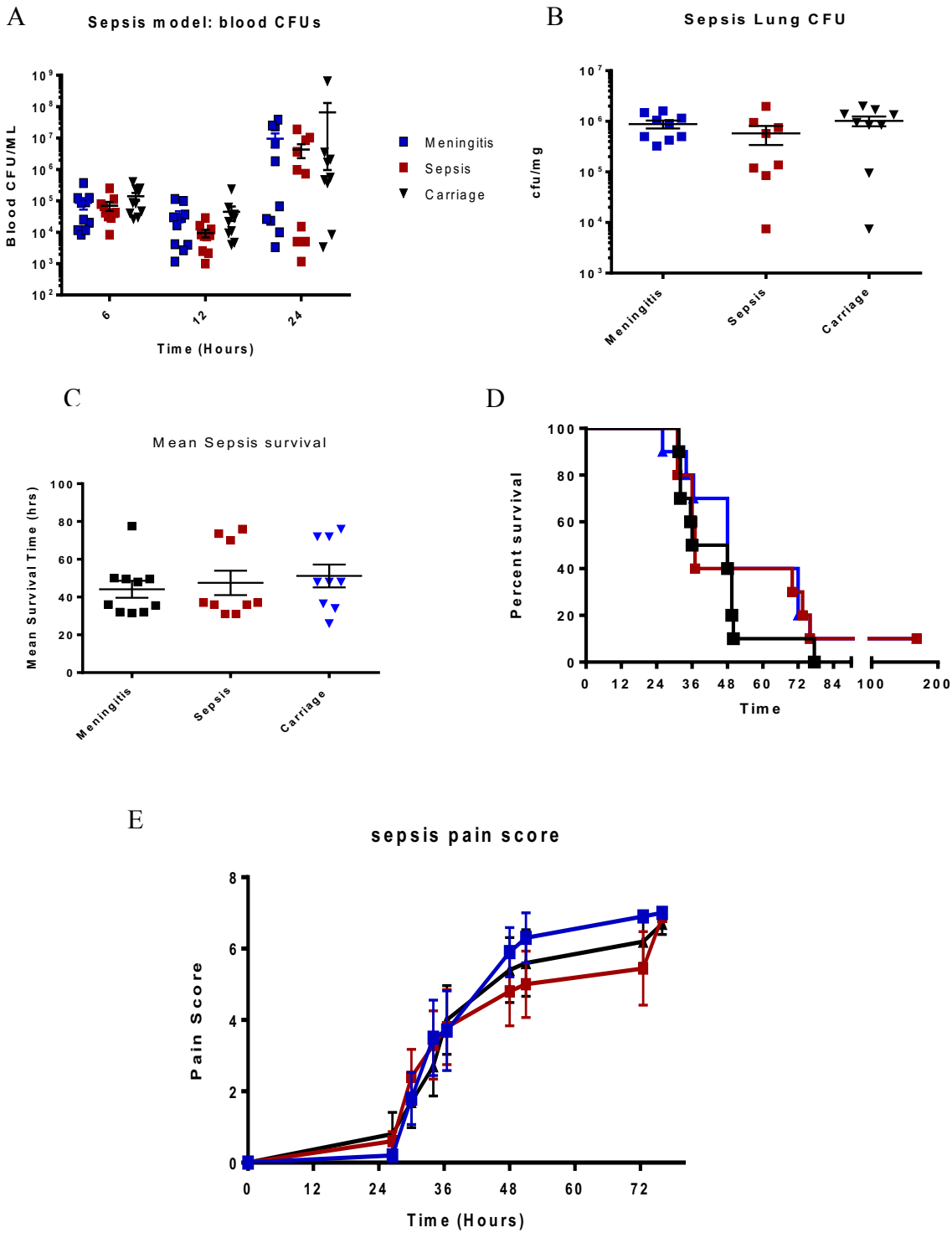


Figure 19. Comparison of Serotype 1 isolates in a murine sepsis model. Mice were administered 10^6 *S. pneumoniae* intravenously on day 0. A) Tail bleeds were performed at 6, 12 and 24 hours to examine blood CFU levels. B) At time of death, Lung CFU values were examined. C) Mean survival times and D) Kaplan-Meier survival curve. E) Pain scores were determined for infected mice until time of death Error bars represent the standard error of the mean.

These experiments demonstrated no differences in the lung or blood CFU counts, mean survival time or pain scores between three serotype 1 isolates in a murine model of sepsis (Figure 19). Thus, site of bacterial isolation, for example, lungs and nasopharynx can influence capsule thickness as seen *in vitro* but ultimately this does not lead to differences in virulence in murine models of infection.

3.3.7 Changes in capsule thickness during invasive pneumococcal disease.

Serotype 1 isolates from the nasopharynx, CSF and blood have significant phenotypic differences *in vitro* which did not translate to differences in virulence and ability to colonise in murine models. I hypothesised that this may be due to rapid compensatory changes in capsule thickness and therefore resistance to complement deposition in the host following infection. To test this hypothesis, a pneumonia infection model was performed in mice with carriage, sepsis and meningitis isolates (figure 20) to examine how capsule thickness changes over the course of infection and between different anatomical compartments of the host (figure 21A-C). Furthermore, the effect of these changes on susceptibility to complement deposition was examined (figure 21D-F).

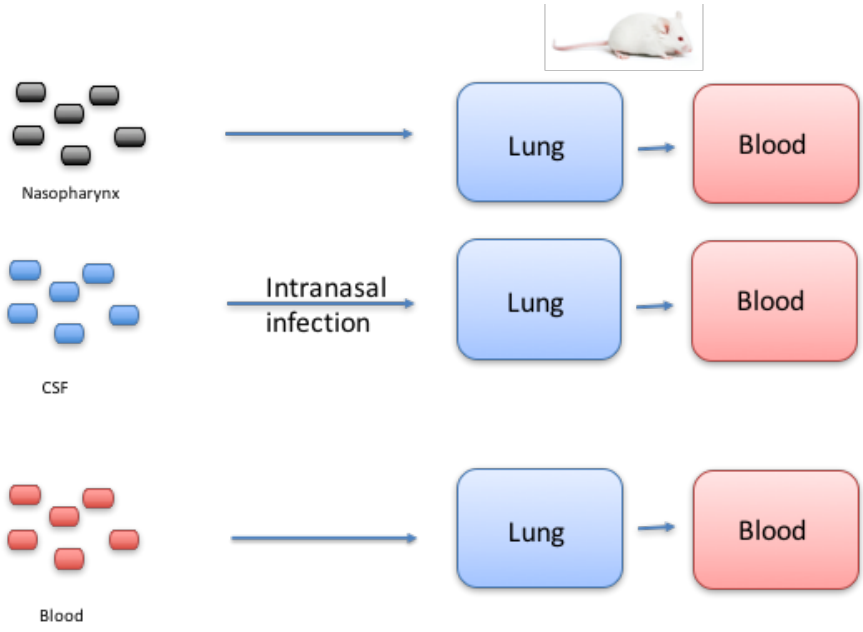


Figure 20. Schematic representation of pneumonia infection model to assess changes in capsule thickness in invasive serotype 1 infection.

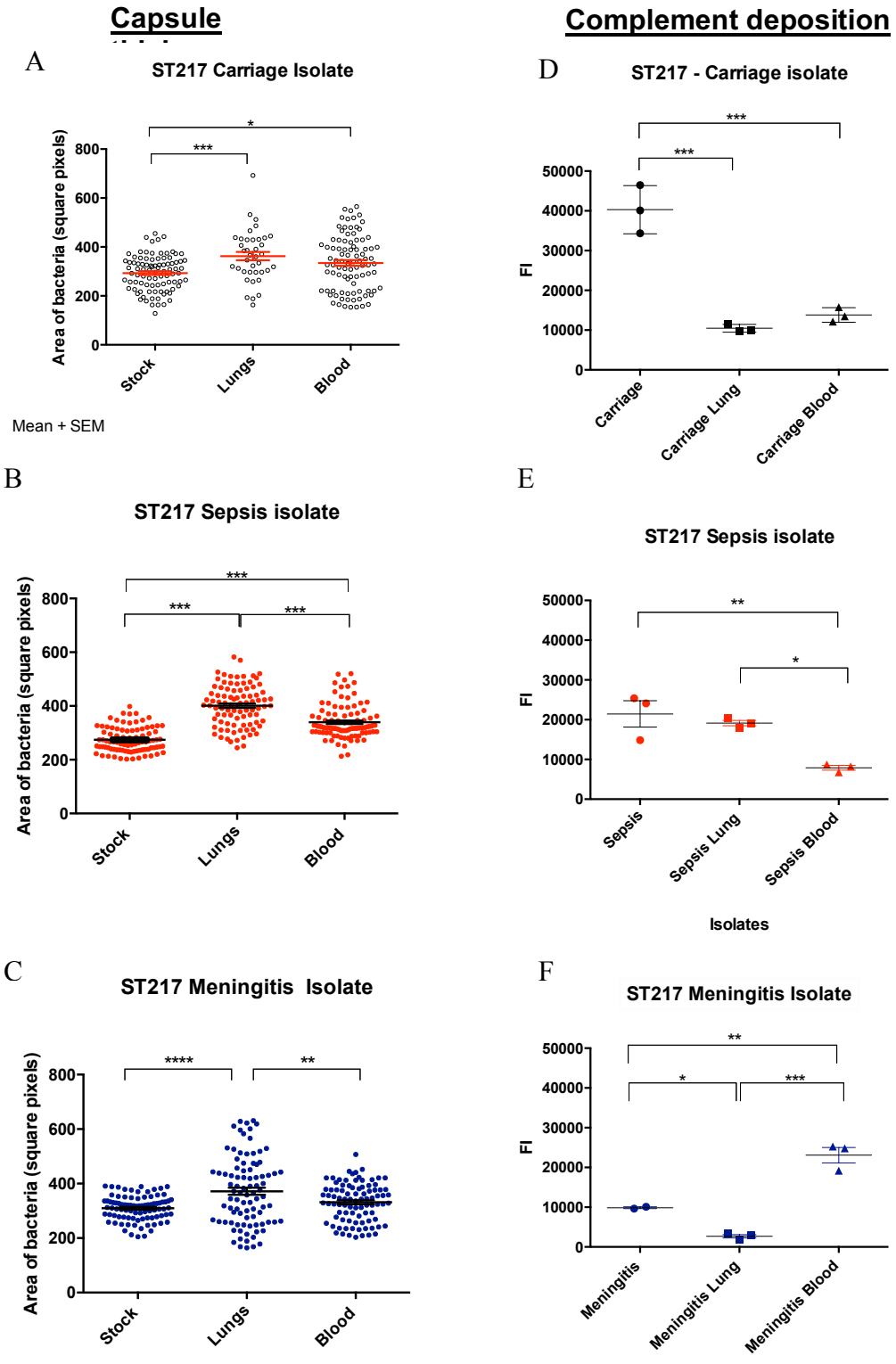


Figure 21 Changes in capsule thickness and susceptibility to complement deposition during pneumococcal pneumonia. The capsule thickness of (A) Carriage W000168, (B) Sepsis D25796, (C) Meningitis C9471 ST217 serotype 1 isolates was measured using FITC-dextran analysis. C3b deposition assays were performed (in triplicate) on (D)Carriage, (E) Sepsis, (F) Meningitis ST217 serotype 1 isolates Data presented as mean ± SEM. One-way ANOVA test was used for statistical analysis (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001).

Results demonstrate that changes in capsule thickness follow the same patterns for all three isolates. Capsule thickness increases significantly in the lungs but then decreases slightly in blood compared to lungs (figure 21A-C). In contrast to a clear pattern of capsule thickness changes, the changes in complement deposition do not appear to follow any pattern between the isolates (figure 21D-F).

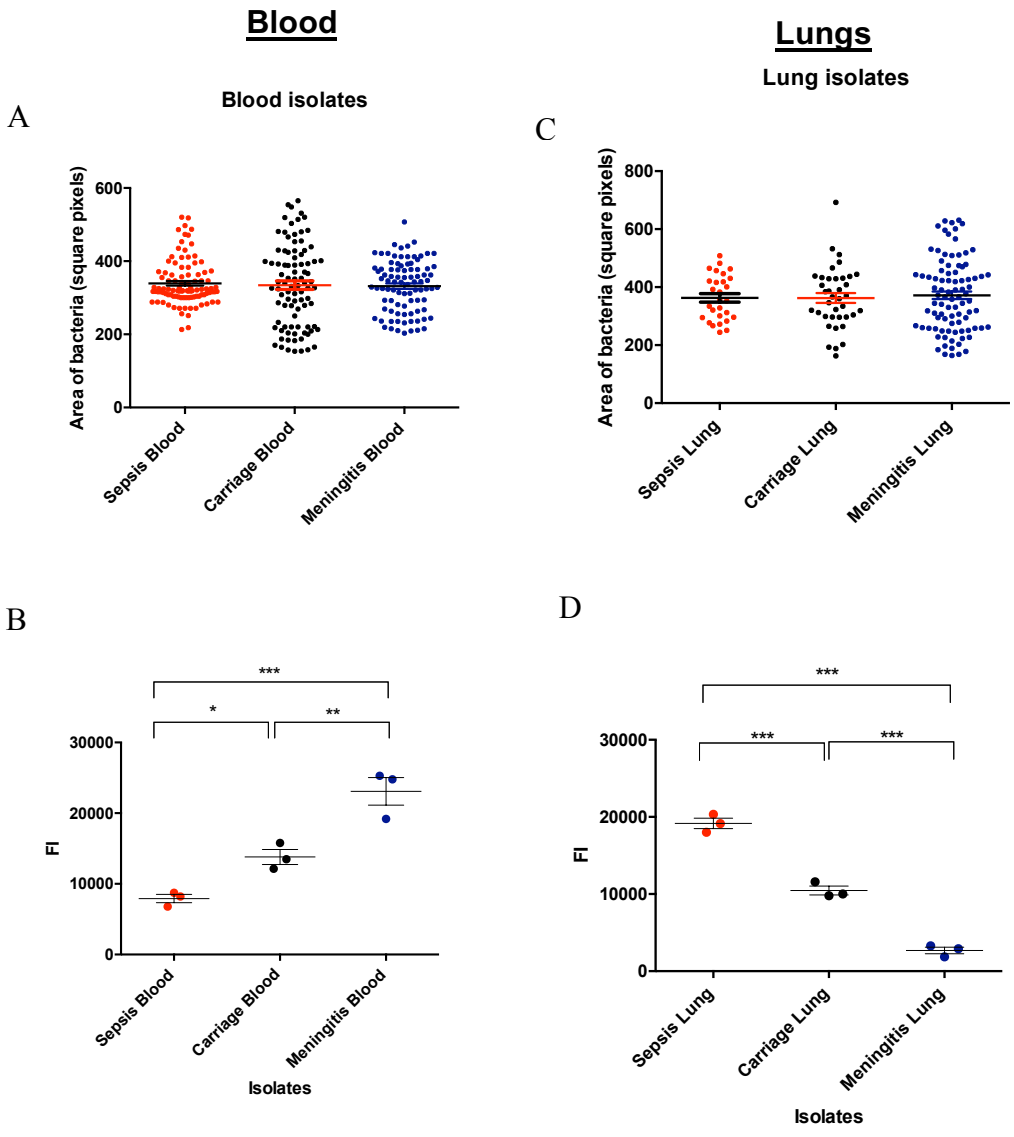


Figure 22. Resistance to complement deposition varies in serotype 1 isolates in the lungs and blood despite no differences in capsule thickness. Data from figure 21 has been re-arranged to compare C3b deposition on three serotype 1 isolates in the blood and also in the lungs. (A) Capsule thickness of sepsis, carriage, meningitis serotype 1 isolated from blood. (B) C3b deposition on sepsis, carriage and meningitis serotype 1 isolated from blood. (C) Capsule thickness of sepsis, carriage, meningitis serotype 1 isolated from lungs (D) C3b deposition on sepsis, carriage and meningitis serotype 1 isolated from lungs. One-way ANOVA test was used for statistical analysis (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001).

One interesting observation from these data is that when all ST217 isolates were recovered from the blood at 24 hours post infection, there were no differences in capsule thickness (Figure 22A) but there were significant differences in C3b surface deposition, with the sepsis isolate significantly more resistant to C3b deposition than either the carriage or meningitis isolate (Figure 22B). The reverse effect is seen for isolates taken from the lungs, where the meningitis isolate proved most resistant and the sepsis isolate most susceptible to C3b deposition (Figure 22D). Thus, when the sepsis isolate is in the blood, the complement deposition is low compared to the meningitis isolate which has high levels. However, when these bacteria are isolated from the lungs at the same time-point, the trend is reversed with sepsis isolate having high complement deposition and the meningitis isolate low. These results suggest that the meningitis isolate may not be as well adapted to survive in the blood as the sepsis isolate.

3.3.8 Sepsis survival experiment with murine *ex vivo* blood serotype 1 ST217 isolates

To follow on from the observations in Figure 22, I examined whether ST217 isolates with differences in resistance to complement deposition, that had been acquired in murine blood, resulted in differences in virulence in a sepsis infection model in naïve mice. Figure 23 shows a schematic representing the experimental set up.

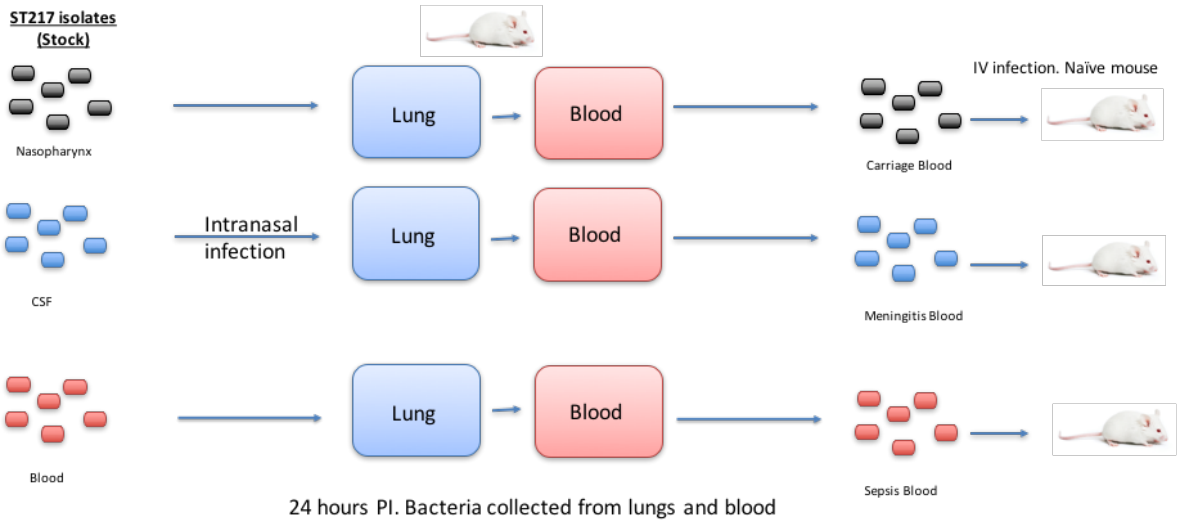


Figure 23. Schematic representation of experimental setup. Serotype 1 isolates from human NP(carriage W000168), CSF (meningitis C9471) and blood(sepsis D25796) were intranasally infected into naïve mice and isolated from blood 24 hours post infection. *Ex vivo* blood isolates were then IV infected into a naïve mouse and blood survival was compared.

Mice were infected intranasally with ST217 stocks from either nasopharynx, CSF or blood. After 24 hours, bacteria were isolated from the blood and renamed carriage blood, meningitis blood and sepsis blood, respectively. Previously, the meningitis blood and sepsis blood isolates had significantly different levels of complement deposition on the surface despite no difference in capsule thickness (Figure 22). To examine whether this equated to differences in virulence in the blood, naïve mice were intravenously injected

with the three ex vivo blood isolates and survival times of infected mice were recorded (Figure 24).

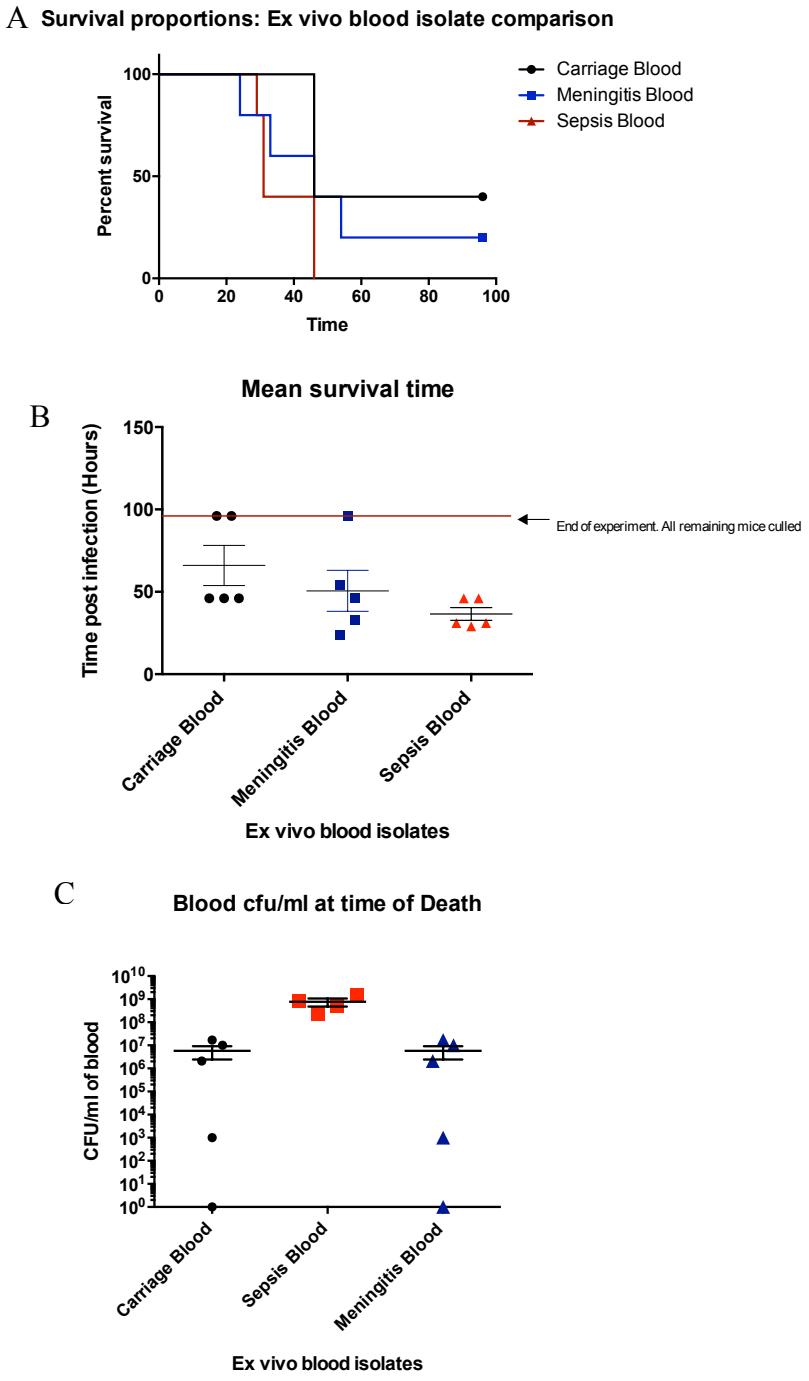


Figure 24 Comparison of ex vivo serotype 1 isolates in sepsis infection model. Mice were infected IV and monitored closely for signs of ill-heath. If infected mice became lethargic, they were culled, survival times noted and CFU from blood determined by Miles and Misra. A) Kaplan Meier survival curve showing survival times of mice infected with carriage blood, meningitis blood and sepsis blood ex vivo murine isolates. B) Mean survival times 5 mice/group C) Bacterial blood density at time of death. Data shown as Mean ± SEM.

Results demonstrate that the significant differences in complement deposition for the sepsis blood isolate compared to carriage and meningitis blood isolates correlate with differences in virulence in a sepsis model (Figure 24). Sepsis blood isolates have significantly lower C3b surface deposition and increased virulence and higher mortality rates are seen in infected mice compared those infected with meningitis and carriage blood isolates. Results show 100% mortality in the sepsis blood infected mice, however only 60% mortality in mice infected with carriage blood isolates and 80% mortality in meningitis blood infected mice (Figure 24A). Results also show that 20% of meningitis and carriage groups were able to clear bacteria from the blood by 96 hours post infection (Figure 24C).

3.3.9 Sepsis survival comparisons from niche-adapted ST217 isolates

I next determined whether isolates passaged through the nasopharynx, lungs or blood showed altered virulence in an invasive disease model in mice. An ST217 sepsis isolate was introduced into the nasopharynx and left to colonise for 14 days before removal (D14 Carriage isolates). In another group of mice, ST217 isolates were intranasally infected to induce pneumonia. After 24 hours post infection, bacteria were isolated from lungs and blood and renamed 24-hour lung isolates and blood isolates respectively. These ex vivo ST217 isolates were then intravenously infected into a naïve mouse and survival rates and times were compared (Figure 25).

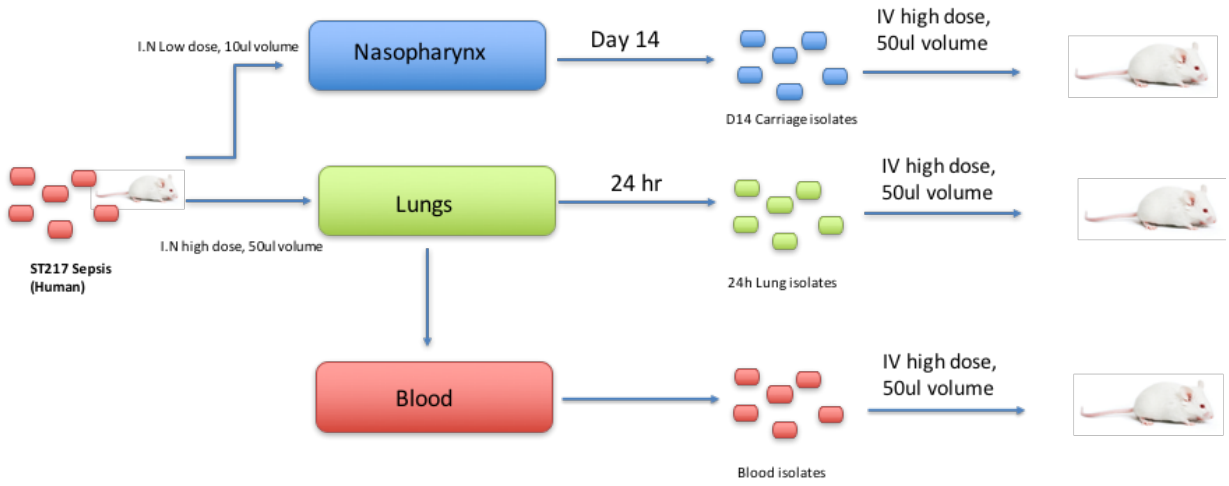


Figure 25. Schematic representation of experiment. 5 female MF1 mice were used per group.

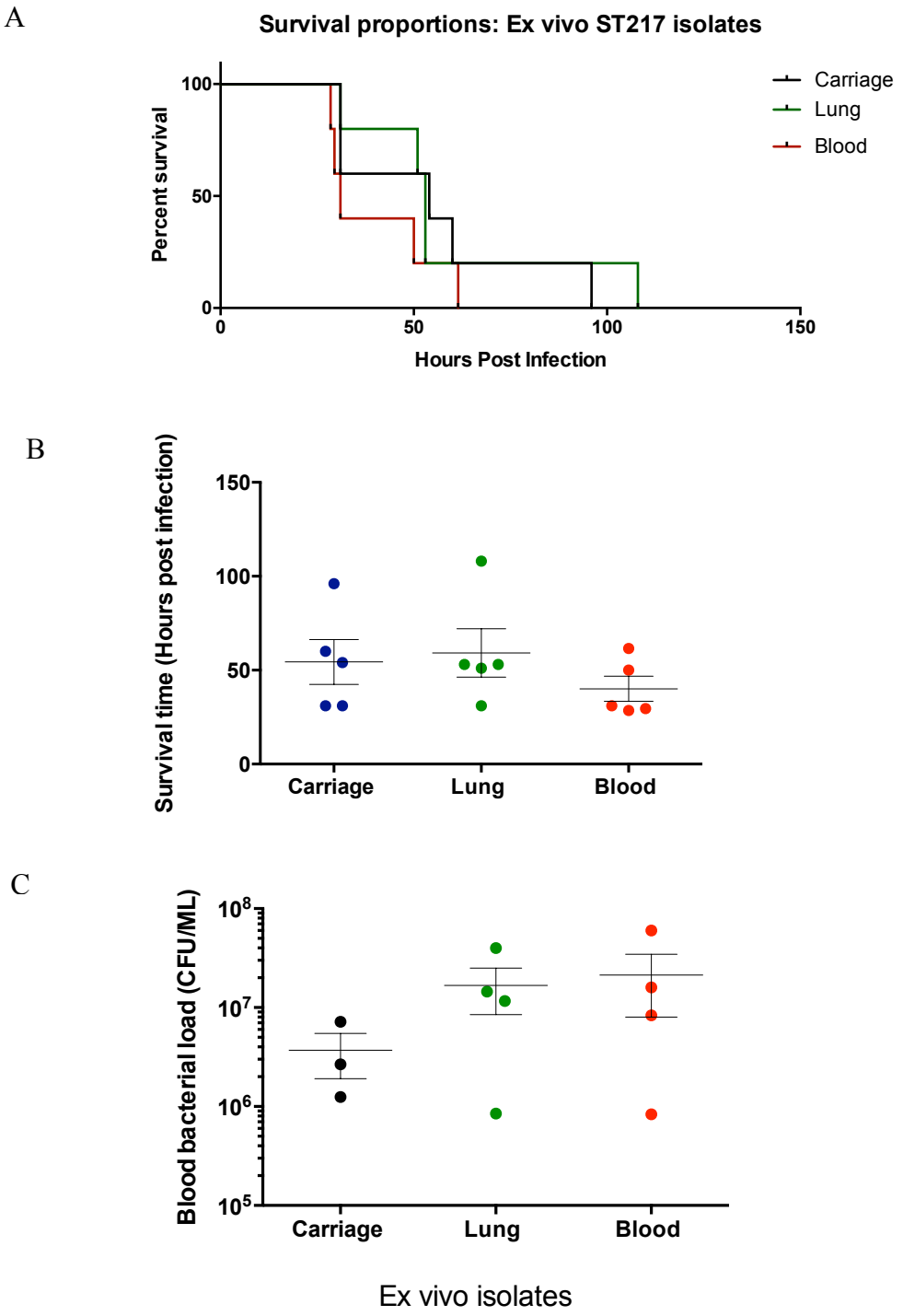


Figure 26. Comparison of tissue-passaged serotype 1 isolates in a sepsis infection model. Mice were infected IV and monitored closely for signs of ill-heath. If infected mice became lethargic, they were culled, survival times noted and CFU from blood determined by Miles and Misra dilution. A) Kaplan Meier survival curve showing survival times of mice infected with D14 carriage, 24hr lung and 24hr blood ex vivo murine isolates. B) Mean survival times 5 mice/group C) Bacterial blood density at time of death (CFU/ml)

Results show that ST217 isolates that have been passaged through lungs, blood and nasopharynx show no difference in overall mortality rates when compared in a sepsis model of infection (Figure 26A). However, there is a difference in the survival times between the groups, with 100% mortality in mice infected with blood isolates occurring by 58 hours post infection compared to carriage and lung isolates which take over 96 hours.

3.4 Discussion

The polysaccharide capsule forms the outermost layer of the pneumococcus and is attached to the surface of the cell wall peptidoglycan. [80] Due to the pioneering work by Griffith, who reported that unencapsulated pneumococci were virtually avirulent, the capsule is now recognised as a key virulence factor of the pneumococcus. [280] The capsule helps to protect the bacteria from the host's innate immune responses by forming an inert shield that appears to have a capacity to resist phagocytosis [73]. The virulence of *S. pneumoniae* seems to relate to capsule expression of a particular strain and serotype, with pneumococci from different capsular polysaccharide (CPS) serotypes differing in their capacity to cause disease, presumably reflecting their ability to resist complement deposition and subsequently phagocytosis by host immune cells[279]. Published work has suggested a link between capsule thickness and colonization preference, with Weinburger *et al* suggesting that increased carriage prevalence is associated with heavier encapsulation for protection from neutrophil mediated killing [79]. However, published data also states that thicker capsule inhibits bacterial adherence to respiratory cells and may therefore affect ability to colonise [77]. Hammerschmidt *et al* have shown intimate contact between bacteria and epithelial cells using electron

microscopy, suggesting thinner capsule is needed for adherence during colonisation [77].

For this chapter of work, experiments were designed to focus on studying the polysaccharide capsule, a major virulence factor of *S. pneumoniae*. The capsule is the current target of pneumococcal vaccines but little is understood about its role in both carriage and invasive disease in the context of serotype 1 infection.

In vitro experiments were performed to compare phenotypic characteristics between three ST217 serotype 1 isolates from different niches in the host. This work began by comparing growth rates, capsule thickness, complement deposition and resistance to phagocytosis in serotype 1 bacteria isolated from human nasopharynx with invasive isolates from blood and CSF. The *in vitro* assays performed showed significant differences in phenotypic characteristics between ST217 from nasopharynx (NP), cerebral spinal fluid (CFS) and blood. Capsule thickness assays demonstrated that a serotype 1 isolate taken from human blood (sepsis) had a significantly thicker capsule compared to D39, and carriage and meningitis serotype 1 isolates. There were no significant differences seen in the capsule size of carriage verses meningitis serotype 1 isolates. These results may reflect the need for a thicker capsule to protect from high complement levels in blood[270], however, when *S. pneumoniae* resides in the nasopharynx it is seen as a commensal bacterium that can horizontally transmit to other hosts with minimal activation of the host immune response. Therefore, a large, nutrient-demanding capsule is not required[75] and this is supported by the results presented here. The meningitis isolate was found to have a significantly smaller capsule compared to the sepsis

isolate which may help progression across the blood-brain barrier to cause meningitis. The thicker recorded diameter of D39 compared to its unencapsulated demonstrates that this method of capsule quantification is accurate and reliable.

Increased capsule thickness is known to correlate with increased protection from phagocytosis by blocking deposition and function of opsonins such as complement directed against cell surface antigens [263]. Levels of C3b complement deposition on the surface of serotype 1 isolates were measured. When comparing sepsis and carriage serotype 1 isolates, there was a correlation between increased capsule thickness and decreased complement deposition which resulted in lower levels of phagocytosis. Levels of C3b complement on the surface of the meningitis serotype 1 isolate was significantly lower than both sepsis and carriage isolates. However, this failed to correlate with increased protection from phagocytosis, thus suggesting other factors affecting phagocytosis resistance may be important.

In addition to capsule, *S. pneumoniae* has evolved a number of mechanisms to evade complement-mediated immunity. These include binding of the host inhibitor of the alternative pathway inhibitor factor H (FH) to cell wall proteins PspC and PhtD[281]. Capsule is known to affect FH binding, therefore differences in capsule thickness may affect FH binding[270] and may result in resistance to complement deposition as seen here between serotype 1 isolates.

These results suggest that capsule thickness could be the key to resistance to phagocytosis and amount of complement deposition on the surface of bacteria

plays a less important role in comparison. Further work is needed to elucidate the levels of FH binding on the surface of the ST217 serotypes tested and its significance in resistance to phagocytosis.

To investigate the role of serotype 1 capsule in carriage and invasive disease, *in vivo* models of asymptomatic carriage and invasive pneumococcal pneumonia and sepsis were used to compare ST217 isolates. Despite differences in capsule thickness, complement deposition and resistance to phagocytosis, ST217 isolates showed no difference in virulence in both sepsis and pneumonia models or establishment of nasopharyngeal colonisation. Further investigation into serotype 1 nasopharyngeal carriage demonstrated that serotype 1 pneumococci was able to successfully colonise a murine nasopharynx for over 14 days. Therefore, the idea that serotype 1 is a poor coloniser may be unjust as it is able to colonise the murine nasopharynx and in addition, olfactory areas of the upper airway, which may not be readily detected using current wash or swabbing techniques in humans. However, our murine carriage models do not completely represent the human nasopharynx which would have many species of bacteria, potentially including other pneumococci, which serotype 1 would need to compete with. It is worth considering that serotype 1 may be a poor competitor in the nasopharynx when other pneumococci and bacterial species are present, resulting in low carriage detection.

As bacterial capsule is a target in the current PCV13 vaccination, changes in capsule expression over duration of colonisation and immune cell mediated

clearance in the nasopharynx were investigated. Close examination of changes in capsule thickness over the course of serotype 1 murine nasopharyngeal colonisation showed fast and significant changes occurring within two days. Current understanding of the role of capsule during colonisation varies from needing a large, thick capsule to protect from phagocytosis to a thin capsule being advantageous for bacterial adhesion to local epithelial cells [70] [270] [77]. My data have shown that serotype 1 capsule increased significantly in the nasopharynx compared to the original infection stock. Presence of a thick capsule during serotype 1 carriage will likely offer protection to the bacteria from the host immune response. This may allow serotype 1 not only to colonise the nasopharynx but also deeper tissues such as olfactory epithelium and olfactory bulb. It is not clear whether the ability of serotype 1 to invade these tissues is directly linked to capsule thickness but these results do demonstrate the ability of serotype 1 to readily invade local tissues and may point to a potential mechanism for the high rates of invasive disease seen with serotype 1.

Changes in capsule expression during invasive pneumonia was also studied. Mice were intranasally infected with either carriage, meningitis or sepsis ST217 isolates. After 24 hours, bacteria were isolated from the lungs and blood and the capsule thickness and C3b deposition of these isolates was compared to the original stock. Results showed that all ST217 isolates showed the same pattern of capsule change over the course of infection; capsule thickness increased significantly in the lungs and then decreased in the blood compared to the lungs. When comparing all isolates in the lungs, complement

deposition on sepsis isolates were significantly higher than carriage and meningitis isolates despite no differences in capsule thickness. This effect was reversed when comparing all isolates in the blood with meningitis having high levels of surface bound complement compared to carriage and sepsis isolates. The survival of mice infected with *ex vivo* blood isolates was examined further in an *in vivo* sepsis model. Isolates were intravenously injected into a naïve mouse and survival times monitored. 100% mortality was observed in mice infected with sepsis blood isolates (low complement deposition) while mice infected with meningitis blood (high complement) resulted in 80% mortality which one mouse fully clearing bacteria from the bloodstream.

Results up to this point have demonstrated that serotype 1 is able to significantly alter capsule thickness in response to the local environment. It is likely that these changes occur in response to oxygen/nutrient availability, host immune response or competition from other colonising bacteria.

Our next aim was to examine whether serotype 1 passaged for 14 days in a murine nasopharynx would differ in virulence when used to induce sepsis in a naïve mouse compared to isolates passaged through lungs and blood. Results suggested serotype 1 isolated from murine nasopharynx after 14 days had no increased virulence in blood compared to isolates passaged through lungs. However, isolates passaged through blood induced 100% mortality around 36 hours faster than nasopharynx and lung passaged bacteria, suggesting passage through blood may have increased niche-specific virulence. Ideally multiple passages through either nasopharynx, lungs or blood are needed to investigate niche-specific virulence adaptations of pneumococci.

Final comments

The aim of this chapter was to understand the role of one of the key pneumococcal virulence factors; the capsule, in serotype 1 disease pathogenesis. By comparing African ST217 serotype 1 isolates from nasopharynx, CSF and blood I was able to compare capsule thickness, resistance to both complement deposition and phagocytosis. Despite significant differences in the phenotypes *in vitro*, no differences in virulence or ability to colonise in murine models of infection were observed. This is likely due to fast-occurring changes in capsule thickness during carriage and invasive disease that contribute to pathogenesis. Despite current literature describing serotype 1 as a poor coloniser, I observed carriage for over 14 days and successful colonisation of deeper tissues of the upper airway.

The main finding of this work is observation of significant changes in capsule thickness that occurs during infection. Capsule size does not appear to be defined by serotype as it can significantly vary within one sequence type between not only sites of isolation but also by the duration of infection and status of the host immune response.

Identification of pneumolysin as a key virulence factor associated with *Streptococcus pneumoniae* African serotype 1 invasive disease.

Chapter 4

Identification of pneumolysin as a key virulence factor associated with *Streptococcus pneumoniae* African serotype 1 invasive disease.

4.1 Introduction

Pneumolysin is expressed by virtually all clinical pneumococcal isolates and has been described as a key virulence factor contributing to high morbidity and mortality rates in invasive disease. Pneumolysin is a cholesterol dependant hemolysin [282]. It lyses cells with cholesterol-containing membranes, induces cellular cytotoxicity via DNA damage and activates host complement and induces pro-inflammatory reactions in immune cells [283]. At higher lytic concentrations it has also been shown to cause widespread cellular and tissue damage allowing for increased bacterial replication and tissue invasion [284]. As pneumolysin is an intracellular toxin, release is thought to be dependent on autolysin-dependant autolysis [88]. This process is characterised by cell wall degradation by a peptidoglycan hydrolase (autolysin), the most common of which is known as LytA.

To investigate the role of pneumolysin and other potential virulence factors in serotype 1 disease pathogenesis, murine models of experimental pneumococcal pneumonia were used (Materials and Methods 2.7). Briefly, mice were intranasally infected with either serotype 1 or serotype 2 and monitored for signs of clinical disease (2.7.4). In addition, blood cfu titres and survival times were recorded. Pneumonia experiments were performed in BALB/c mice, and disease severity with African serotype 1 (D25796) was compared to serotype 2 (D39) infection.

4.2 Results

4.2.1 Comparison of serotype 1 and serotype 2 in BALB/c pneumococcal pneumonia model.

BALB/c mice are highly resistant to respiratory challenge by a wide range of invasive pneumococci, confine infection to the lung and eliminate bacteria entirely within 7 days [208]. At no point do bacteria disseminate into the bloodstream to cause sepsis [173]. To identify key virulence factors associated with serotype 1 pathogenesis, BALB/c mice were intranasally infected with serotype 1 to establish an acute lung infection (2.7.2). Serotype 2 D39-infected BALB/c mice were used as a comparison, as this is a well characterized laboratory strain in these infection models and does not cause any mortality [173]. Once infected, mice were monitored for signs of disease over seven days.

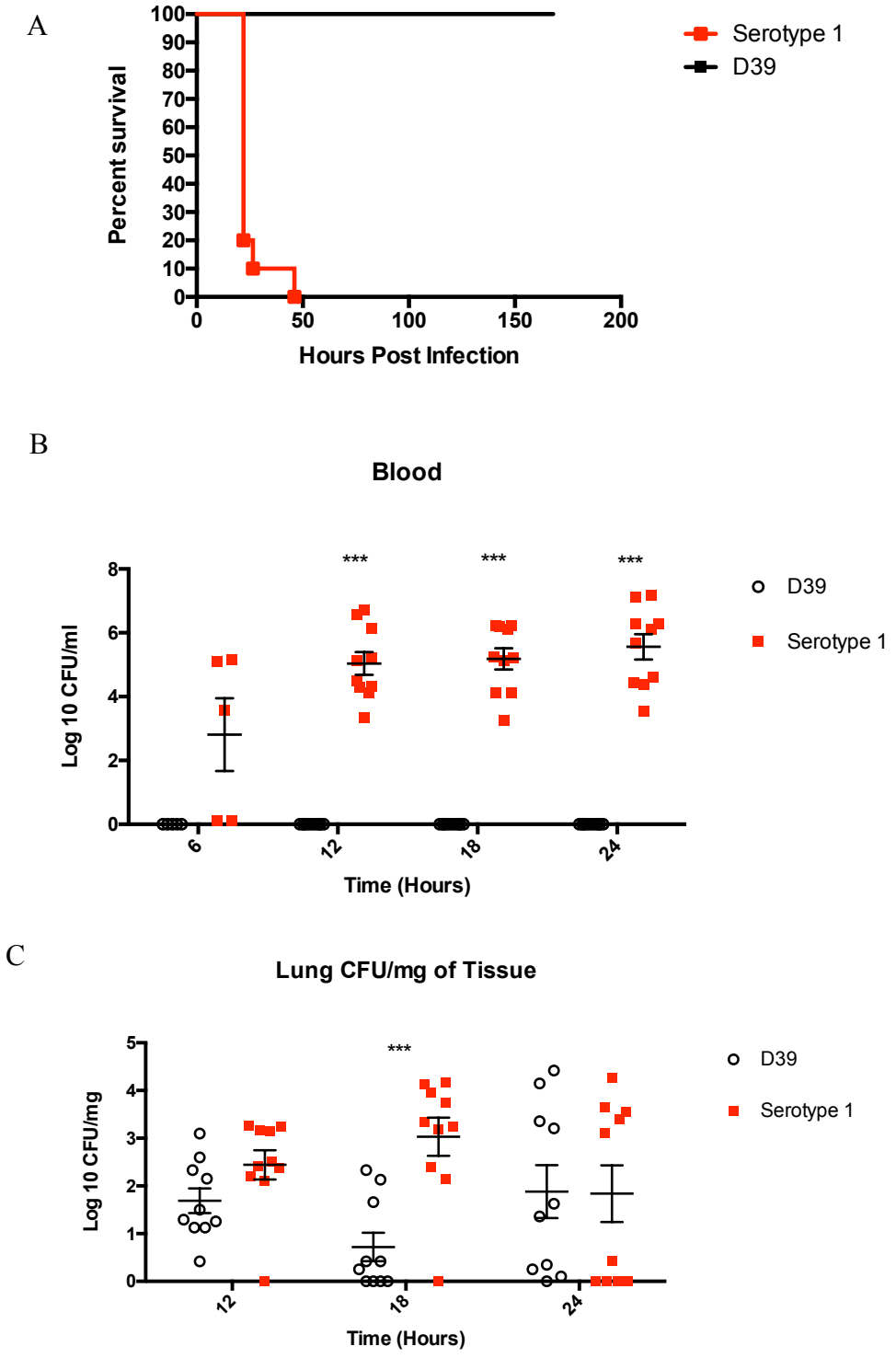


Figure 27. Comparison of Serotype 1 (ST217) and Serotype 2 (D39) strains in BALB/c pneumonia model. Mice were infected intranasally (IN) and monitored closely for signs of ill-heath. If infected mice became lethargic, they were culled, survival times noted and CFU from blood determined by Miles and Misra dilution. A) Kaplan Meier survival curve showing survival times of mice infected with ST217 sepsis (D25796) or D39. B) Blood bacterial load at 6, 12, 18 and 24 hours post infection from three experiments (CFU/ml) (C) Lung bacterial load (CFU/mg) at 12, 18 and 24 hours post infection. Statistical analysis was performed using Two-way ANOVA (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001)

Striking differences were observed in disease severity between serotype 1 and serotype 2 infected mice (Figure 27A). Mice infected with serotype 1 exhibited 100% mortality within 48 hours post infection. In addition, by 12 hours post infection, all serotype 1 infected mice had high levels of bacteraemia which remained consistent until death. In some mice, serotype 1 was able to disseminate into the bloodstream as early as 6 hours post infection (Figure 27B). Serotype 2 infected mice, as predicted, showed no mortality over 7 days with no detectable colonies found in the blood throughout the experiment. Development of bacteraemia appeared to be independent of bacterial numbers in lungs as there were no significant differences in lung bacterial loads until 18 hours, and development of bacteraemia was observed as early as 6 hours post infection (Figure 27C).

4.2.2 Comparison of clinical invasive isolates in BALB/c pneumococcal pneumonia model

To assess whether high mortality rates in BALB/c mice are limited to ST217 infection, the pneumonia survival model was repeated with other clinical *S. pneumoniae* isolates (Figure 28). Of the serotypes tested, serotype 1, 5, 7F and 6B are all invasive clinical isolates that are included in the current PCV13 vaccine [264]. An additional ST217 isolated from the nasopharynx of a Malawian child (ST217 carriage) and an ST3018 serotype 1 isolate from The Gambia were included in the experiment. A European serotype 1 isolate (ST306) expressing a non-haemolytic pneumolysin was also included. Of all the serotypes tested, mortality was only observed in BALB/c mice infected with African serotype 1 (ST217 and ST3018 sequence types) (Figure 28). This

mouse strain maintained resistance to invasive pneumococcal pneumonia when infected with a range of clinically relevant serotypes such as 5 and 6B.

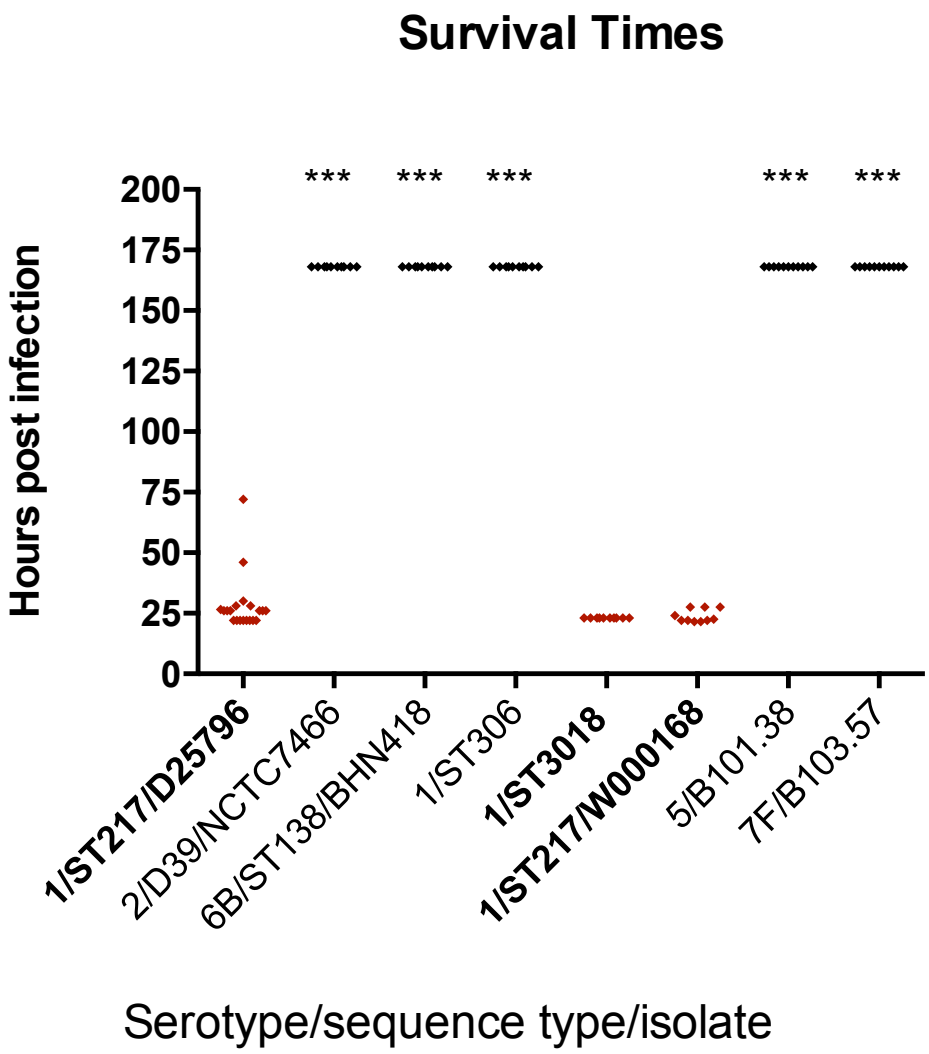


Figure 28. Comparison of survival rates of clinical isolates of *S. pneumoniae* in BALB/c pneumonia model. Mice were infected intranasally and monitored closely for signs of ill-health. If infected mice became lethargic, they were culled, survival times noted and CFU from blood determined by Miles and Misra dilution. Survival times of mice infected with different African serotype 1 strains (red) and other clinically relevant isolates (black) (10 mice/group). Statistical analysis was performed using One-way (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001).

4.2.3. Comparison of progression of disease symptoms in BALB/c mice infected with clinically invasive isolates of *S. pneumoniae*.

Differences in clinical signs of disease progression were compared between mice infected with either Serotype 1 (ST217 W000168 Carriage), Serotype 5 or 7F.

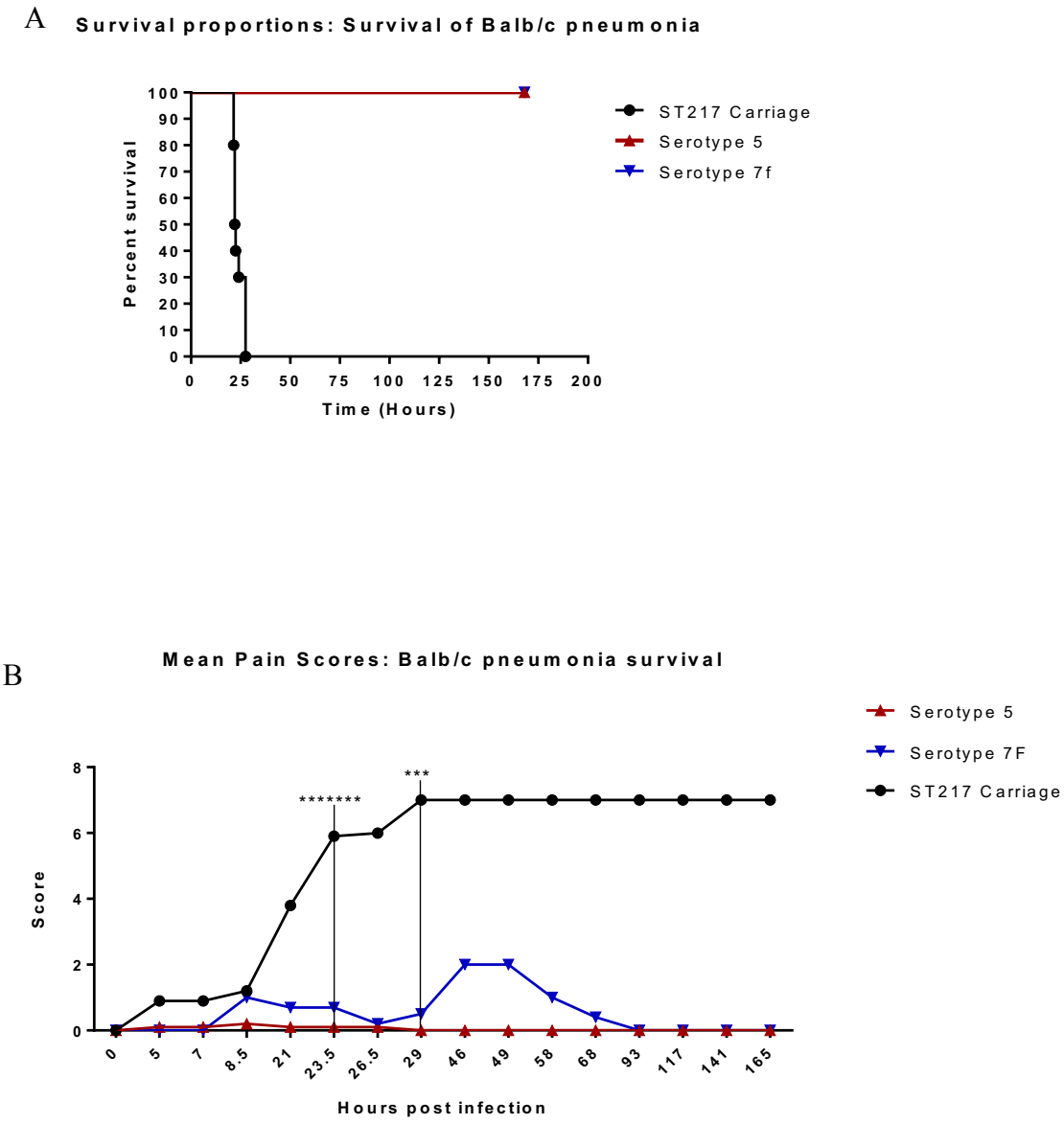


Figure 29. Comparison of disease progression through pain scoring system in infected BALB/c mice. 10 mice per group were IN infected with either serotype 5, 1(ST217 W000168 Carriage) or 7F and monitored for signs of disease using a pain score system (see methods section 2.7.4) When mice reached lethargic status they were culled. (A) Kaplan Meier survival curve showing survival times of mice. (B) Mean pain scores. * represents 1 mouse culled due to reaching lethargic stage of infection.

A pain/disease scoring system was used to monitor disease progression of a pneumococcal pneumonia infection in BALB/c mice (Figure 29). Details of the scoring system can be found in methods section 2.7.4. Mice infected with serotype 5 showed no clinical symptoms such as hunching or piloerect coat throughout the experiment. Mice infected with serotype 7F showed some signs of disease throughout the experiment but did not score above 2 and no mice appeared lethargic, thus resulting in 100% survival rate. However, mice infected with serotype 1 strain ST217 showed early signs of disease (starry coat and hunching) within five hours post infection. Symptoms of disease continued with seven mice reaching the lethargic end-point at 23.5 hours post infection. These mice were culled. At 29 hours post infection, the remaining three serotype 1 infected mice became lethargic and were also culled. By the end of the experiment, mortality (100%) was only observed in ST217-infected mice.

4.2.4 Examination of haemolytic activity of pneumolysin

I hypothesised that differences in virulence seen in Figure 28, could be attributable to pneumolysin as ST306, with a non-hemolytic pneumolysin, was the only serotype 1 strain not to cause 100% mortality. An ELISA based method (Materials and Methods section 2.10) was used to quantify the amount of pneumolysin released upon lysis of bacteria of different serotypes and haemolytic assays used to assess the haemolytic activity of pneumolysin produced by different serotypes (Figure 30A+B). Two isolates of each serotype were tested where possible to compare variation within serotypes.

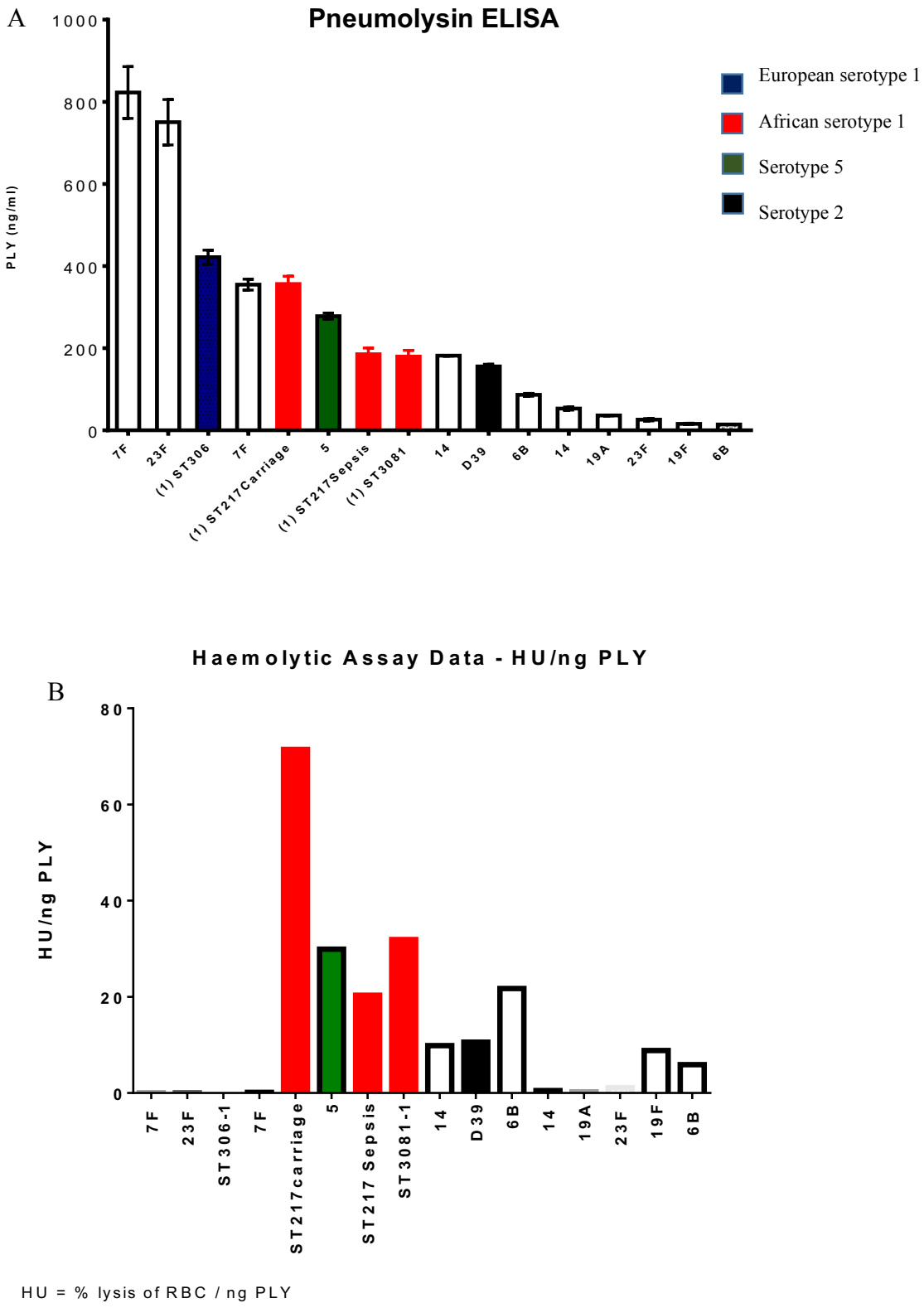


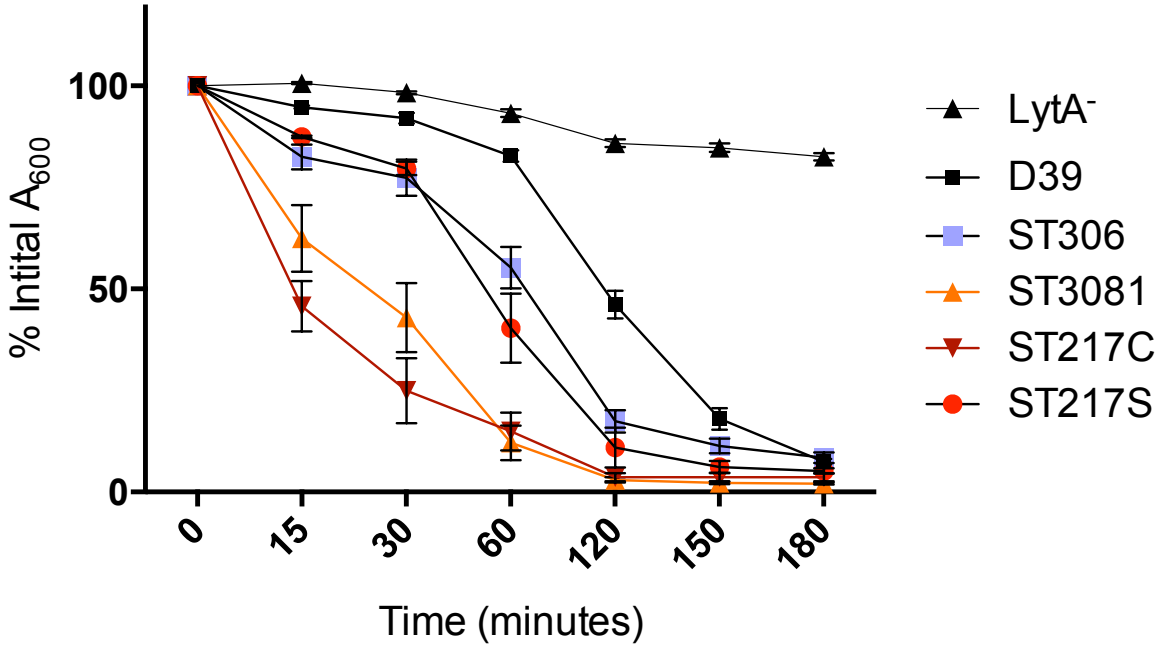
Figure 30 Comparison of quantity of pneumolysin and hemolytic activity of pneumolysin produced by different serotypes of *S. pneumoniae*. (A) ELISA-based detection method to quantifiy the amount of pneumolysin released by different serotypes (10^7 cfu) upon lysis by penicillin/streptomycin antibiotic treatment. (B) Hemolytic assay was performed on 10^7 cfu of lysed bacteria to asses the amount of sheep red blood cell lysis occurring within 45 minutes. Data presented as Hemolytic units (HU), calculated by % lysis of RBC/ng of pneumolysin (detected by ELISA).

Serotype 1 isolates (ST217 and ST3018) produced between 200 and 400ng of pneumolysin/ml upon lysis. The serotype producing the largest amount was 7F with 800ng/ml and the lowest was a 6B isolate with 10ng/ml (Figure 30A)
The ability of pneumolysin from different serotypes (10^7 CFU) to lyse sheep red blood cells was used to calculate the hemolytic activity (Figure 30B). The results identified African serotype 1 ST217 and ST3018 isolates as producing highly hemolytic pneumolysin compared to other clinical serotypes such as 6B, 14, 23f and 7F.

4.2.5 Serotype 1 rate of autolysis is significantly higher than other invasive serotypes

Triton-X induced autolysis assays were performed to assess the rates of autolysis and how this correlates with pneumolysin release (Figure 31). See appendix for rates of autolysis for serotypes 1, 2, 5, 6b, 7F, 14 19A, 19F and 23F. See Materials and Methods section 2.11 for further details.
Results from Triton-X-100-induced autolysis assays suggest rates of autolysis in serotype 1 are higher significantly higher than serotype 2 (D39) at 60 and 120 minutes of culture. After 15 minutes of incubation with Triton-X, the percentage decrease of OD_{600} of serotype 1 (ST3081 and ST217 Carriage) was significantly higher than that of D39 (Figure 31B). These differences in rates of autolysis continue until 150 minutes incubation. A D39 LytA knockout mutant was used as a control to show that Triton-X autolysis was dependant on LytA activation.

A



B

0 minutes		60 minutes	
D39 vs. ST306	ns	D39 vs. ST306	****
D39 vs. ST3081	ns	D39 vs. ST3081	****
D39 vs. ST217C	ns	D39 vs. ST217C	****
D39 vs. ST217S	ns	D39 vs. ST217S	****
15 minutes		120 minutes	
D39 vs. ST306	ns	D39 vs. ST306	****
D39 vs. ST3081	****	D39 vs. ST3081	****
D39 vs. ST217C	****	D39 vs. ST217C	****
D39 vs. ST217S	ns	D39 vs. ST217S	****
30 minutes		150 minutes	
D39 vs. ST306	*	D39 vs. ST306	ns
D39 vs. ST3081	****	D39 vs. ST3081	*
D39 vs. ST217C	****	D39 vs. ST217C	*
D39 vs. ST217S	ns	D39 vs. ST217S	ns

Figure 31. Triton X-100-induced autolysis assays comparing rates of autolysis in serotype 1 and serotype 2 isolates. Bacteria (OD_{600} 1.0) were incubated at 37^{0}C and 175rpm with 0.01% Triton X. At 15 - 30 minute intervals, OD_{600} was measured and converted to a percentage of the original OD_{600} reading. (A) Comparison of rates of autolysis between serotype 1 sequence types and serotype 2 (D39). (B) Statistical analysis was performed by Two-way ANOVA. Rates of autolysis were measured in triplicates; data is presented as mean ± SEM. (ns=non-significant. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

As the polysaccharide capsule is designed to protect the bacteria from external factors such as complement deposition and other aspects of the host immune response, I was interested in examining whether capsule thickness affected rates of autolysis. FITC-dextran analysis was performed on Serotype 1(ST217 Sepsis), 2 and 5 (Figure 32). Results demonstrated that serotype 1, which had the highest rates of autolysis had a significantly thicker polysaccharide capsule compared to both D39 and serotype 5.

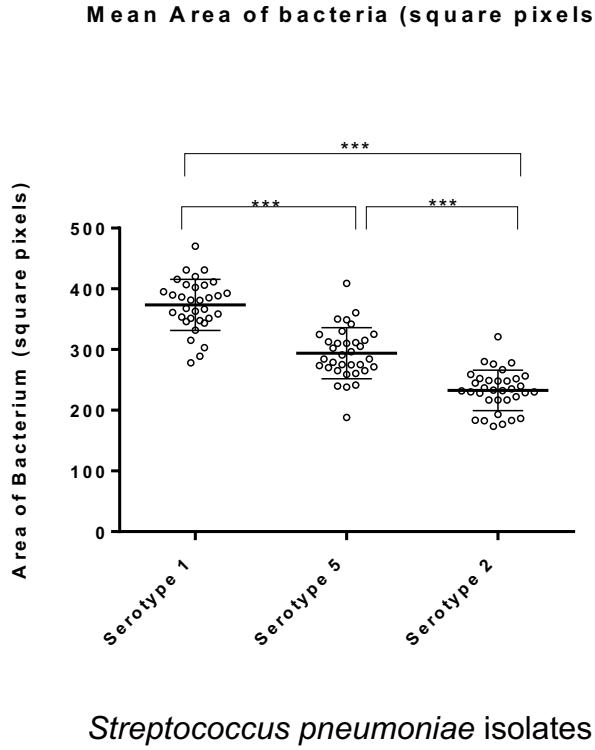
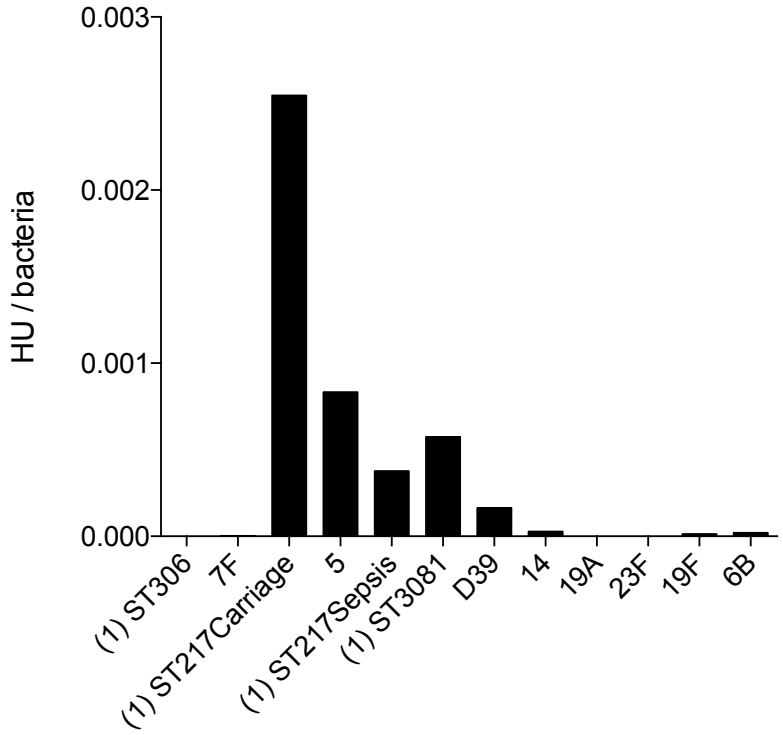


Figure 32. Fitc-dextran exclusion assays showing differences in capsule thickness between three *S. pneumoniae* serotypes. Each data point represents an individual bacterium. Error bars show the mean±SEM. Statistical analysis was perfromed using One-way ANOVA (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001).

4.2.6 Effect of autolysis on the release of pneumolysin: a comparison of serotypes of *S. pneumoniae*.

Comparison of rates of autolysis, production of pneumolysin and the haemolytic ability of pneumolysin from different serotypes were performed separately. Results presented here incorporate together the haemolytic unit (HU) production per bacteria (Figure 33A) and the maximum HU released per minute when bacteria undergo autolysis with Triton X treatment (Figure 33B).

A



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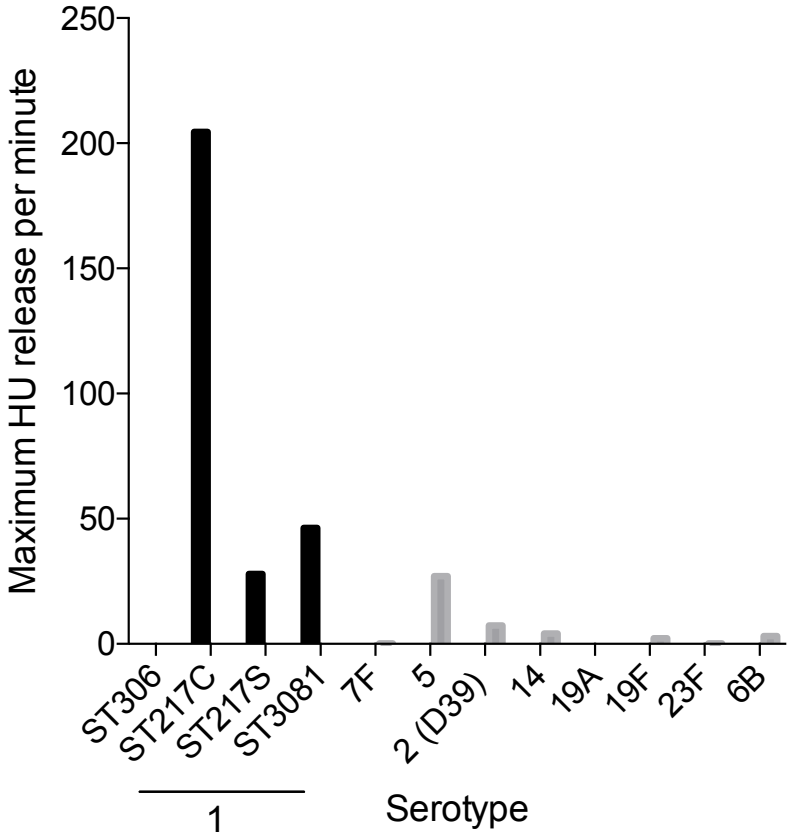


Figure 33. Rates of autolysis, pneumolysin production and haemolytic activity combined to show the HU per bacteria and HU released per minute by autolysis. (A) HU per bacteria. Calculated by (Ply conc (ng/ml)*HU per ng of pneumolysin) / number of bacteria. (B) Maximum HU of pneumolysin released per minute. Calculated from number of lysed bacteria/minute * HU/ bacteria. ST217C = W000168. ST217S = D25796.

Figure 33A compares the haemolytic activity (HU) per bacteria of different serotypes of *S. pneumoniae*. Of the serotypes tested in the BALB/c model, ST217 carriage has the highest haemolytic activity per bacteria and ST306 and 7F have the lowest. Figure 33B incorporates the autolysis data for all serotypes tested to show the amount of haemolytic pneumolysin released per minute when bacteria undergo autolysis. Results show that ST217(carriage) has the highest HU released per minute whilst serotype 19A has the lowest. Levels of HU released per minute are almost identical when comparing serotype 1 (ST217Sepsis) and 5. However, when these serotypes are

compared in a pneumonia infection model in BALB/c mice, serotype 5 causes no mortality whereas both ST217 isolates and ST3081 Gambian serotype 1 cause 100% mortality (Figure 28).

4.2.7 Pneumolysin-containing supernatant from serotype 1 ST217 increases virulence of D39 in BALB/c pneumonia model

Significantly higher rates of autolysis were observed in Serotype 1 isolates compared with D39 (Figure 31A). To see if high natural rate of autolysis might contribute to *in vivo* virulence and that extracellular pneumolysin within the supernatant of the infection inoculum might impact disease progression, a supernatant switch experiment was designed. All experiments from this figure were performed with serotype 1 (ST217 Sepsis D25796) isolates. Serotype 1 (ST217sepsis) and serotype 2 (D39) doses ($2x10^7$ CFU each) were prepared in 1 ml of PBS. The doses were left for 45 minutes at room temperature to mimic the time taken between preparation of dose to infection of mice. An ELISA-based detection method was then used to measure the concentration of pneumolysin released into the infection dose (Figure 34). Method detailed further in Materials and Methods section 2.10.

Figure 34. Pneumolysin concentrations released by natural rates of autolysis.. In separate tubes, $2x10^7$ CFU of ST217 (sepsis, D25796) and D39 were prepared in PBS. Dose preparations were incubated at room temperature for 45 minutes before a sample of supernatant was removed and a pneumolysin detection ELISA used to quantify the amount of pneumolysin released into the supernatant. Experiment was performed in triplicate and error bars represent mean±SEM. Statistical analysis was performed using a one-way ANOVA (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001).

Figure 34 demonstrates that when doses of either serotype 1 or serotype 2 are prepared and used to infect mice, mice receiving a serotype 1 dose will be given significantly higher concentrations of pneumolysin in the supernatant compared to D39-infected mice (P<0.01). To investigate the effect of high concentrations of pneumolysin in the serotype 1 infection dose, the supernatants were swapped before infection of mice. D39 and serotype 1 infection doses were prepared, and then immediately prior to infection, bacteria were pelleted by centrifugation and the supernatants of the two doses were swapped. After infection, mice were closely monitored for signs of disease and survival rates recorded (Figure 35).

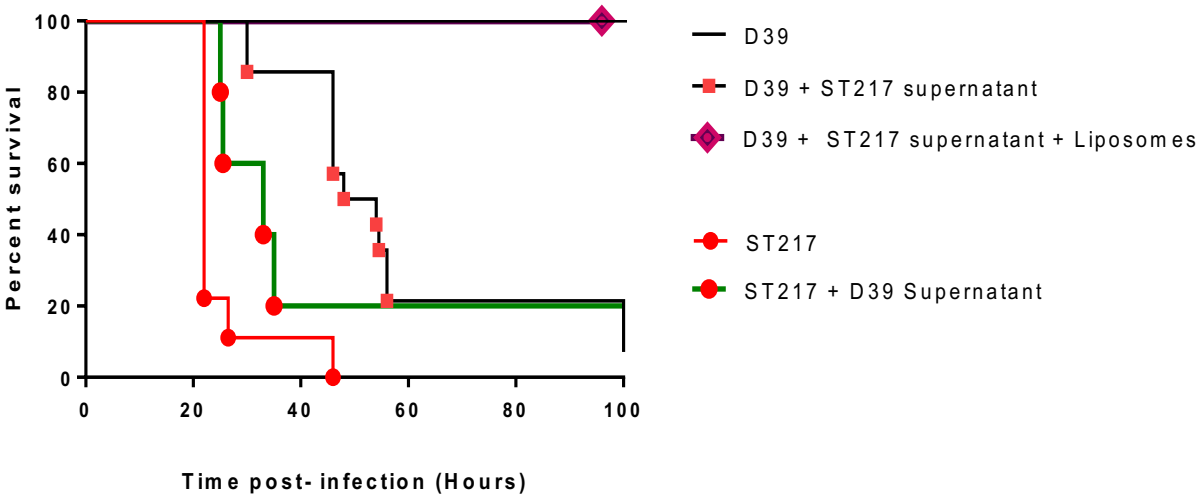


Figure 35. Murine pneumococcal pneumonia survival rates after supernatant swap. Kaplan Meier survival curve showing survival times of mice infected with different combinations of 10^6 cfu in 50μl of either serotype 1 (ST217 D25796) or serotype 2 (D39) bacteria. For one group of mice, the supernatant from ST217 was treated with Cholesterol:sphingomyelin (66mol/%cholesterol) and sphingomyelin-only liposomes for 30 minutes.

When mice were infected with D39 resuspended in ST217 supernatant containing high concentrations of pneumolysin, mortality rates increased from 0 to 80%. In addition, when ST217 was administered to mice with supernatant from D39 (containing signifcantly less pneumolysin), 20% survival rates were observed, compared to 0% without D39 supernatant. When ST217 supernatant was pre-treated with cholesterol-containing liposomes (engineered lipid vesicles able to sequester cholesterol binding toxins) [285] and then administered into the mice with D39 bacteria, survival rates returned to 100% (Figure 35), confirming that pneumolysin is responsible for the observed effects of supernatant swap.

4.2.8 Examination of the cytotoxic effects of Serotype 1 (ST217) pneumolysin on lung epithelial cells.

Excess concentrations of pneumolysin in the lung have previously been shown to aid bacterial replication and increase rates of tissue invasion and dissemination into the bloodstream [96,284]. *In vitro* assays were set up to measure cytotoxicity in A549 human lung epithelial cells upon infection with a range of serotypes of *S. pneumoniae* (materials and methods 2.12). Different pneumolysin concentrations and levels of haemolytic ability were previously observed with different serotypes of *S. pneumoniae* (Figure 30).

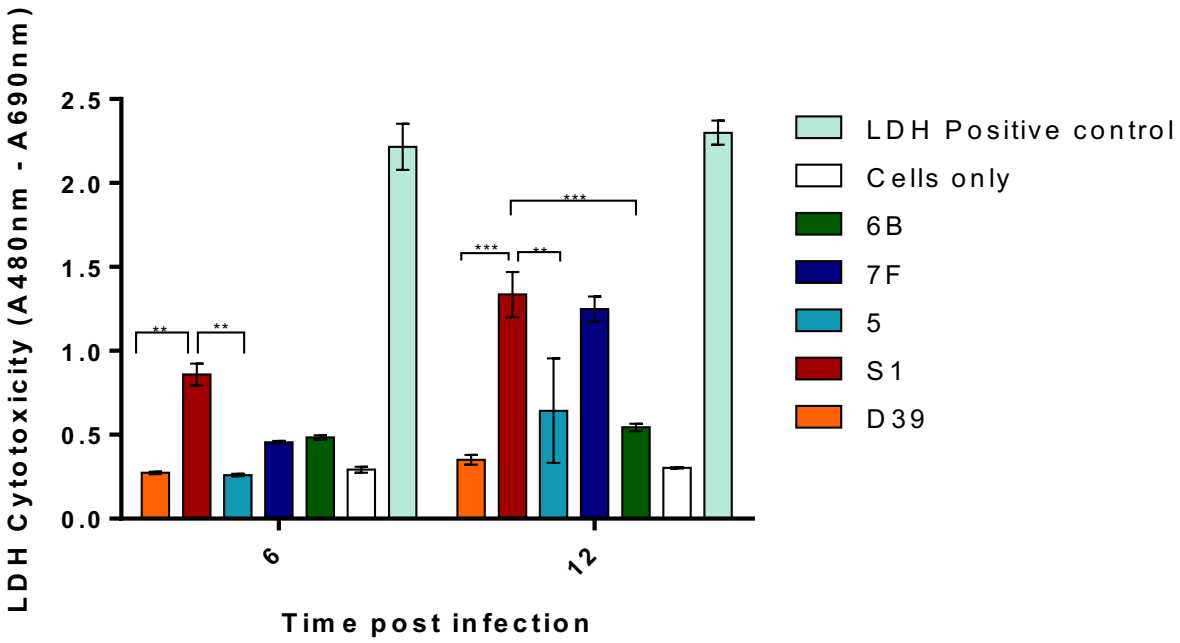


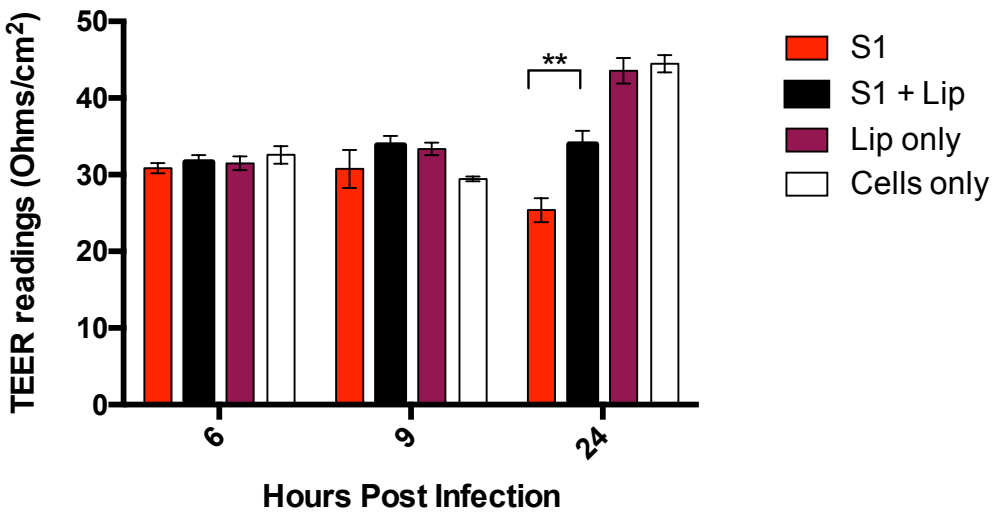
Figure 36. Lung epithelial cell cytotoxicity during infection with *S. pneumoniae* differ with different serotypes. A549 lung epithelial cells were infected with $6x10^5$ cfu of *S. pneumoniae* and, at 6 and 12 hours post infection, samples of cell culture supernatant were removed and amount of LDH (an indicator of cytotoxic damage) was measured. Infections were performed in triplicate wells and error bars represent the mean±SEM. Statistical analysis was performed using two-way ANOVA (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001).

Infection with serotype 1 (ST217) caused more cytotoxic damage to lung epithelial cells compared to other *S. pneumoniae* serotypes (Figure 36). At 6 hours post infection, cells cultured with ST217 had significantly higher levels of LDH compared to infection with D39 and serotype 5. Levels were also higher, but not statistically significant, compared to infection with 6B and 7F clinical isolates at 6 hours post infection. At 12 hours post infection, levels of LDH released from cells infected with ST217 was significantly higher than those infected with D39, 5, and 6B. Thus, these data strongly suggests that high rates of autolysis, observed with ST217, lead to release of highly haemolytic pneumolysin which contributes to significantly increases in cellular cytotoxicity of lung epithelial cells.

4.2.9 Pneumolysin produced by African serotype 1 damages tight junctions between lung epithelial cells during infection.

As ST217 is able to disseminate from the lungs into blood as early as 6 hours post infection in murine models of acute pneumonia (Figure 27). To further investigate this, *in vitro* assays were set up to measure the barrier function of lung epithelial cells during infection with a range of serotypes. It was hypothesised that bacteria rapidly disseminate through the lung into the blood during ST217 infection due to lung damage caused by rapid release of pneumolysin. The next stage was to examine the effect of ST217 infection on barrier function, using a monolayer of epithelial cells and performing trans-epithelial electrical resistance (TEER) measurements as an indicator of barrier integrity.

A


TEER readings (Ohms/cm²)
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Cells only

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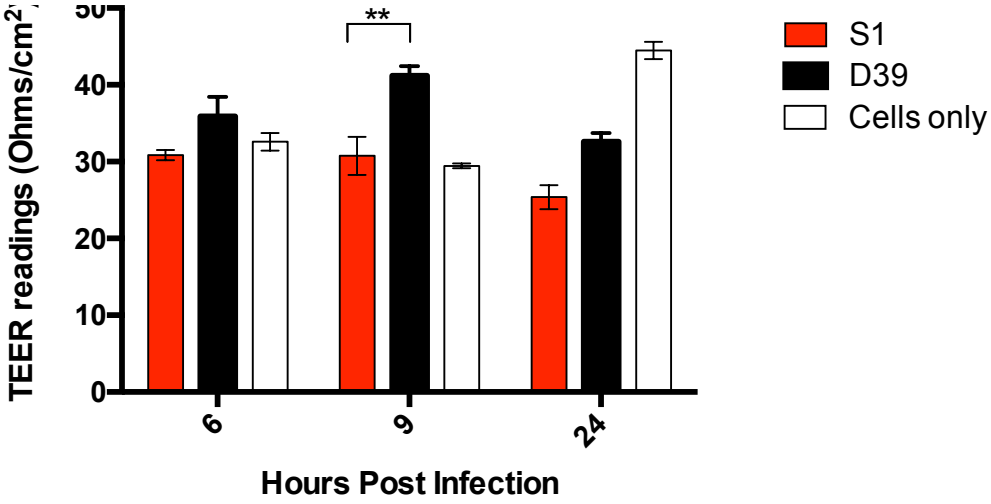
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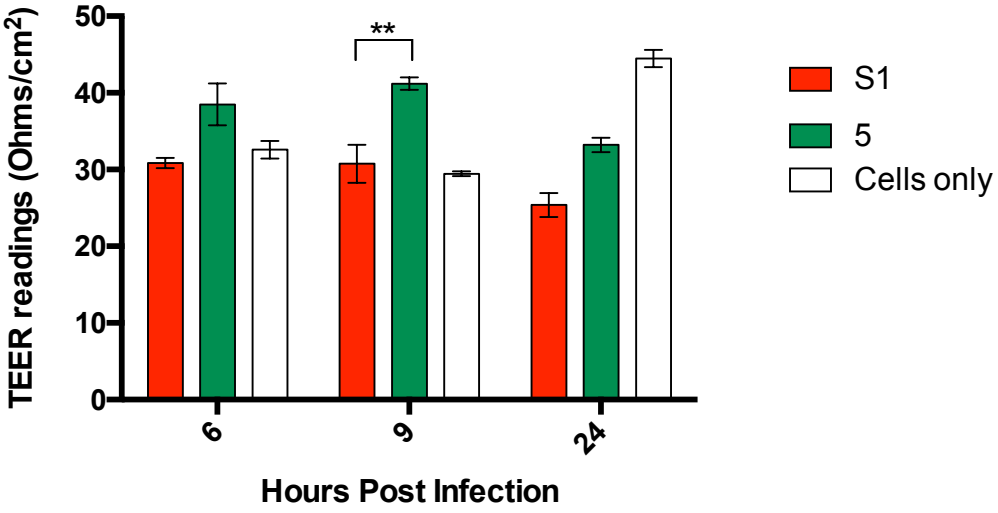
TEER readings (Ohms/cm²)
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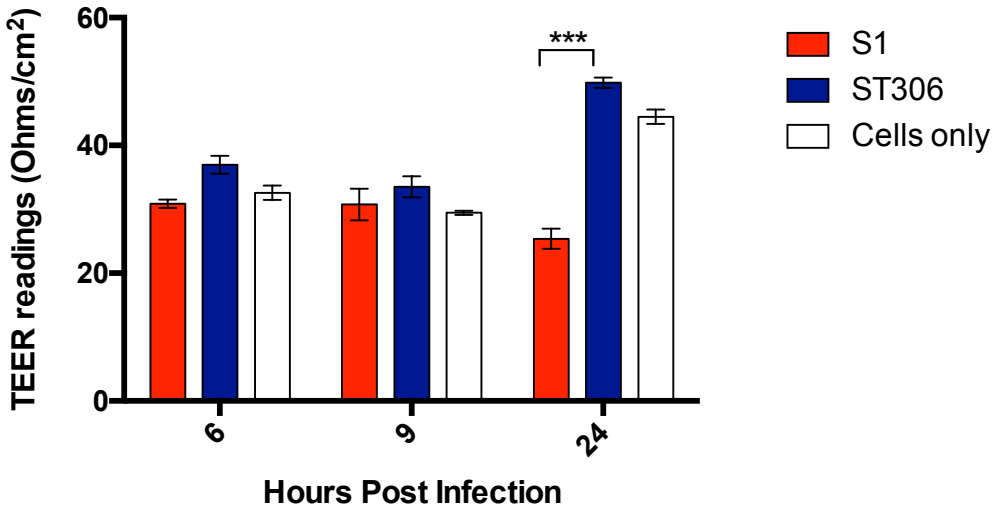

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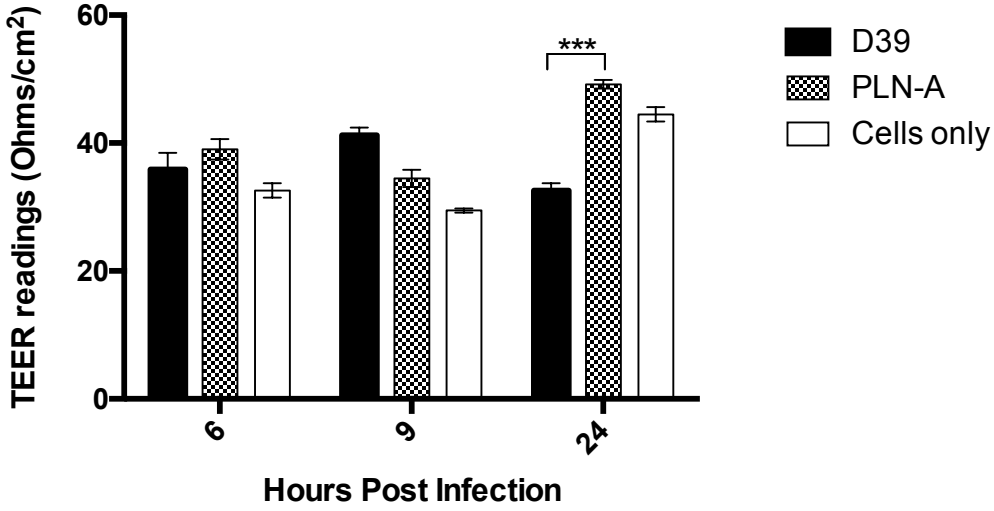


Figure 37. Measurements of damage to tight junctions of lung epithelial cells during *S. pneumoniae* infection. A549 human lung epithelial cells were cultured on trans-well inserts for three days to establish a monolayer. 10^6 cfu of *S. pneumoniae* were added and trans-epithelial electrical resistance readings (TEER) were taken at 6, 9 and 24 hours post infection to assess the damage to tight junctions between epithelial cells. (A) Experiment 1: S1 (ST217Sepsis) with and without liposomes comparison. (B) Experiment 2: Comparison between S1 (ST217Sepsis) and D39. (C) Experiment 3: Comparison between S1 (ST217Sepsis) and serotype 5 infection. (D) Experiment 3: Comparison between S1 (ST217Sepsis) and ST306 infection. (E) Experiment 3: Comparison between D39 and PLN-A infection. TEER readings were performed in triplicate from duplicate wells. Statistical analysis was performed by two-way ANOVA (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001). Error bars represents the mean±SEM.

TEER readings are used as an indicator of the permeability of tight junctions between cells grown in culture [286]. Decreases in the TEER value is an indicator of disruption or damage of tight junctions. Results demonstrate that S1 (ST217Sepsis*)* infection causes significant disruption to the permeability of tight junctions compared to other serotypes tested (D39, 5, ST306). Addition of liposomes to bind and neutralise pneumolysin resulted in significantly less damage to tight junctions at 24 hours post infection (Figure 37A). A liposome only control was added to test the effect of liposomes on the TEER readings of A549 cells in the absence of bacteria. At 9 and 24 hours post infection, ST217 readings were significantly lower than during infection with serotype 5 and D39 (Figure 37B&C). Comparisons of the TEER readings between D39 and PLN-A infected cells demonstrates that pneumolysin has a damaging effect on the tight junctions between lung epithelial cells as TEER readings are significantly lower for D39 infected cells compared to PLN-A (pneumolysin negative D39) at 24 hours post infection (Figure 37E).

4.3 Discussion

Serotype 1 was one of the first pneumococcal serotypes to be described and has some unusual clinical, epidemiological and microbiological characteristics, making it the most prevalent of the highly invasive pneumococci in sub-Saharan Africa [44]. Serotype 1 appears to behave more like a primary pathogen compared to other pneumococci as disease rates in the young and otherwise healthy adults are high whilst carriage rates in the population are low even when symptomatic disease is prevalent in the population [240]. In this chapter of work, murine models were used to identify key virulence factors associated with serotype 1 invasive disease pathogenesis.

Previous published work from our group and others report that mouse strains differ in their susceptibility to the pneumococcus. Gingles *et al* have reported that BALB/c mice are highly resistant to intranasal infection with *S. pneumoniae* serotype 2 (strain D39), with no bacterial dissemination from lungs into the bloodstream observed, resulting in 100% survival rates [173 208]. However, our findings show that when BALB/c mice were intranasally inoculated with the same CFU of serotype 1 (ST217 sepsis), 100% mortality was observed as a result of high levels of bacteraemia occurring within 24 hours post infection, a dramatic change in phenotype. To test if this case was specific to serotype 1, the resistance of BALB/c mice to other invasive clinical serotypes of *S. pneumoniae*, was assessed and the experiment repeated with clinical serotypes such as 6B, 7F, 23F and 5. Resistance to pneumonia and the onset of bacteraemia was maintained during infection with these clinical isolates, exactly as we observed for D39, hence mortality was only observed in mice infected with African serotype 1 isolates (ST217 and ST3081). The

serotype 1 isolate from Europe (ST306) was the only serotype 1 strain not to cause mortality in BALB/c mice. ST306 expresses a non-haemolytic pneumolysin, which prompted further experiments to examine the role of pneumolysin in serotype 1 disease pathogenesis.

ST217(sepsis) bacterial dissemination from the lungs into the bloodstream occurred as early as 6 hours post infection. Previous work has shown that the initial phase of pneumonia is marked by flooding of the alveoli with bacteria but very few inflammatory cells [287]. Subsequent breaching of endothelial barriers is thought to facilitate pneumococcal bacteraemia and is commonly associated with high mortality rates [288]. This often occurs in the absence of a significant inflammatory response suggesting that this rapid dissemination is likely to be driven by bacteria toxin-induced damage of the lung epithelial barriers. However, serotype 1 virulence is unlikely to be due to toxin mediated damage alone as previous publications have shown the importance of the host immune responses in controlling disease severity[215 208 209 173]. Further work needs to be done to assess the host immune responses during pneumonia infections with serotype 1 compared to serotype 2.

A pneumolysin ELISA was developed to detect the amount of pneumolysin produced by a range of different serotypes. Results suggested that the amount of pneumolysin produced by serotype 1 (ST217) was not significantly higher than other serotypes. However, when the ability of pneumolysin to lyse sheep red blood cells was compared across serotypes, both serotype 1 (ST217, ST3081) and serotype 5 pneumolysin was found to be highly haemolytic, causing high levels of red blood cell lysis. Thus, African serotype 1 does not appear to produce significantly higher concentrations of pneumolysin

compared to other serotypes, but the pneumolysin it produces is significantly more haemolytic.

Previous work has suggested that there is some diversity in the amount of pneumolysin produced by different serotypes of *S. pneumoniae*. However, no relationship between the amount of pneumolysin produced and virulence has been found [234]. For example, when pneumolysin titres were measured from a range of serotypes upon lysis, serotype 1 and 5 were shown to have the highest titres whereas serotype 8 had the lowest. Interestingly, serotype 8 was later shown to be more virulent in murine models of infection compared to both serotype 1 and 5 [234]. In another study, growth rates of different serotypes in blood and subsequent survival times of infected mice were examined to test whether pneumolysin production was related to growth *in vivo*. Again, results concluded that there was no obvious association between extracellular pneumolysin titres and virulence in blood [123]. However, these findings did not take into account rates of autolysis between serotypes. Release of pneumolysin has been shown to be dependent on the activation of autolysin which causes rapid cell wall breakdown and cell lysis [119]. Pneumolysin-deficient (PLY-) and autolysin (LytA-) deficient mutant pneumococcal strains have been shown to be less virulent after intranasal administration into mice [125 124 98]. Data from the pneumolysin ELISA and haemolytic assay suggest that serotype 5 produces similar concentrations of pneumolysin and has similar haemolytic ability compared to ST217 sepsis. However, this serotype is not able to cause mortality or dissemination into the bloodstream during pneumonia models in BALB/c mice. Thus, this prompted experiments to compare rates of autolysis in serotype 1, 2, 5, 6B, 7F, 14, 19 and 23F. Results

showed that rates of autolysis *in vitro* were significantly faster in serotype 1 strain, ST217 compared to both serotype 5 and D39 and this led to significantly higher concentrations of pneumolysin being released from ST217 compared to D39.

One limitation to the autolysis studies presented here is that rates of induced autolysis *in vitro* may not be the same as in the context of infection with the pressures of the host immune system and different environmental factors. Recent studies have found that in areas such as sub-Saharan Africa, high temperatures can increase rates of autolysis, thus increasing the release of pneumolysin by *S. pneumoniae* [246]*.* Given the inherently high rates of autolysis I described here for serotype 1 and the highly haemolytic activity of the pneumolysin produced, high temperatures may make a significant contribution to invasive serotype 1 disease in sub-Saharan Africa.

I have also shown that having a thick capsule does not appear to protect bacteria from autolysis. Previous work has suggested that a thick capsule may have a stabilizing effect on the bacteria and reduce rates of autolysis [288] but this was not found to be the case with serotype 1. Finding a definitive link between capsule thickness and rates of autolysis will always be challenging as demonstrated by significant capsule thickness changes significantly over the course of infection in the host and the inability to measure rates of autolysis *in vivo*.

When examining the combined results from haemolytic assays, pneumolysin ELISA and autolysis assays from a range of clinical serotypes, the haemolytic

units released per minute were similar for ST217 sepsis and serotype 5. However, when these bacteria were compared in a murine lung infection model, it was found that serotype 5 did not cause any mortality in a BALB/c pneumonia infection model. Furthermore, the calculations used to determine haemolytic unit release per minute are based on the assumption that rates of autolysis induced by 0.01% Triton-X are comparable to those observed *in vivo*, which may not be the case.

After establishing that supernatants of ST217 sepsis contained significantly higher concentrations of pneumolysin compared to D39, these supernatants were swapped between ST217 and D39 to assess the effect of high pneumolysin concentrations on disease progression in the lungs of BALB/c mice. When D39 bacteria were re-suspended in ST217 supernatant, excess pneumolysin concentrations present allowed D39 to disseminate from the lungs into blood and subsequently cause 80% mortality. When ST217 was infected with supernatant from D39 bacteria, mortality rates decreased by 20% due to lower concentrations of pneumolysin present in the infection dose. These results suggest that early release of pneumolysin following infection is key in aiding bacterial dissemination from the lungs into the blood resulting in high mortality rates. In addition, D39 which has been shown to produce small titres of pneumolysin, is capable increased virulence if aided across the lung epithelial barrier by serotype 1 pneumolysin.

In order to pinpoint pneumolysin as the key virulence factor that leads to dissemination into the bloodstream, liposomes were added to ST217 supernatant to bind and neutralise pneumolysin. The liposomes used were a mixture of cholesterol and sphingomyelin and were designed to be used as

decoy targets to sequester bacterial toxins such as pneumolysin. Liposome-bound toxins are unable to lyse mammalian cells and have been shown to protect mice from invasive pneumococcal pneumonia by minimising toxin-induced tissue damage in the lungs [285].

After treatment with liposomes, ST217 supernatant was used in combination with D39 live bacteria to infect mice in a pneumonia model. Sequestration of pneumolysin by liposomes resulted in no dissemination of D39 across the lung endothelial barriers and the return of 100% survival rates in infected mice. Liposomes have been shown to be beneficial in the treatment of severe infections through their ability to neutralise damaging cholesterol-binding toxins such as pneumolysin [285]. The results from the supernatant swap experiments suggest that liposomes may have great potential in cases of severe pneumococcal pneumonia where risk of bacterial dissemination into the blood is high. If bacterial toxins are key virulence factors that allow for tissue invasion and bacterial dissemination into the bloodstream, as these results strongly suggest, they should be major targets of potential new therapeutics, whether that be through the administration of liposomes or re-design of vaccines to target pneumolysin in the initial stages of infection. Furthermore, liposome therapy is likely to show variable efficacy depending upon the serotype and sequence type of pneumococcus responsible for the infection. For example, disease caused by the serotype 7F lineage studied in this thesis is unlikely to benefit significantly from liposome therapy as pneumolysin is produced in small amounts, is not highly haemolytic and is thus not likely to be a significant contributor to virulence. By contrast, patients infected with African serotype 1 lineages producing large amounts of highly

haemolytic pneumolysin would stand to benefit considerably from liposome administration.

As pneumolysin has been shown to contribute to bacterial dissemination from the lungs into the bloodstream [96], *in vitro* assays were set up to assess the damage ST217 pneumolysin causes at the cellular level in the lungs. Firstly, human lung epithelial cell line A549, were infected with a range of different serotypes (D39, 6B,5, 7F, ST217) and cellular cytotoxic damage was measured through release of LDH. At both 6 and 12 hours post infection, cells infected with ST217 had significantly higher LDH levels compared to D39 and serotype 5 suggesting that ST217 causes more damage to lung epithelial cells compared to other serotypes. In addition, the permeability of membrane barriers (TEER) between lung epithelial cells was also measured. Results suggested that ST217 causes more damage to tight junctions between epithelial cells compared to other serotypes such as 2 and 5. Addition of liposomes preserved barrier integrity during ST217 infection suggesting that pneumolysin is the main driver of cell damage and causes damage to tight junctions between cells.

These findings are in line with previous work which has shown that the presence of cytotoxic pneumolysin in the lung in the initial phase of pneumonia contributes to the development of bacteraemia through facilitating penetration of bacteria from the alveoli into the interstitium of the lung, and dissemination of pneumococci into the bloodstream during experimental models of pneumonia. [96]

These results have implications for furthering our understanding of serotype 1 disease pathogenesis and highlight the importance of other virulence factors

aside from capsule for consideration in future vaccine design and targets for new therapeutics. Results have revealed the damage highly haemolytic pneumolysin has on host cells both *in vitro* and *in vivo* in the context of IPD. One issue that has not been addressed here is the role of serotype 1 pneumolysin in nasopharyngeal carriage. Further work is needed to examine whether high rates of autolysis, resulting in the release of highly haemolytic pneumolysin are the reason carriage rates of serotype 1 have been described as rare [238]. If high titres of pneumolysin are present in the nasopharynx, this could lead to greater tissue invasion of serotype 1 and is likely to cause dissemination into areas such as the lungs and blood. Data from the supernatant swap experiments show that D39 can become virulent in an experimental pneumonia model with the addition of serotype 1 pneumolysin, a finding that could have important implications during multi-serotype nasopharyngeal colonisation. For example, serotype 1 pneumolysin released during autolysis in the nasopharynx could result in onset of IPD with other serotypes that are colonising the nasopharynx through damage to the epithelial cell barrier, facilitating bacterial dissemination into the lung or other areas.

Further work needs to be done to examine the host immune response during serotype 1 infection in the context of both nasopharyngeal colonisation and invasive disease. Resistance to pneumococcal pneumonia in BALB/c mice is a result of high influx of T-regulatory cells into the lung during D39 infection [173]. These anti-inflammatory immune cells, along with high levels of TGFβ, reduce inflammation which prevents damage to epithelial cell barriers and prevents bacterial dissemination into the bloodstream [174] [173]. With bacteria

confined to the lung, the host immune system can clear the infection within 7 days. In mice such as CBA/Ca, high numbers of T regulatory cells are absent during infection with D39. This results in high levels of inflammation, damage to lung epithelial cell linings and bacteraemia occurs, resulting in high mortality rates [173]. This is similar to what is seen with ST217 infection in BALB/c mice and suggests that perhaps during infection with African serotype 1 there is little or no infiltration of T regulatory cells into the lungs.

Conclusion:

My findings show that when BALB/c mice were intranasally administered with serotype 1 (ST217), 100% mortality was observed as a result of high levels of bacteraemia occurring within 24 hours post infection. Tissue invasion and rapid dissemination from lungs to blood resulted in severe bacteraemic pneumonia seen in serotype 1 but not D39 infection. Based on these findings, it is plausible that high mortality rates in serotype 1 infection are a result of rapid autolysis of pneumococci in the lungs, leading to the release of highly haemolytic pneumolysin. This subsequently leads to epithelial and endothelial damage in the lungs, and allows for bacterial dissemination into the bloodstream. These studies have highlighted pneumolysin as an essential factor contributing to serotype 1 virulence.

Investigating the host immune responses during *Streptococcus pneumoniae* serotype 1 nasopharyngeal carriage

Chapter 5

Investigating the host immune responses during *Streptococcus pneumoniae* serotype 1 nasopharyngeal carriage

5.1 Introduction

Streptococcus pneumoniae serotype 1 has previously been described as a poor coloniser of the nasopharynx as it is rarely found causing asymptomatic carriage despite causing high rates of disease in sub-Saharan Africa [11]. As nasopharyngeal carriage is an essential pre-requisite for invasive disease it is an important area to investigate to broaden understanding of serotype 1 pathogenesis.

Previous reports have suggested that host immune responses can be key in controlling nasopharyngeal colonisation through a careful balance between the induction of pro-inflammatory arms of the immune system such as Th17 cells and immunomodulatory cells such as T regulatory cells [100,289]. Th17 cells have been implicated in bacterial clearance in the nasopharynx, whilst T regulatory cells, together with TGF-β are important in reducing inflammation, limiting tissue damage and preventing bacterial dissemination within the host [172] [100]. Dysregulation of this balance is thought to contribute to the onset of invasive pneumococcal disease.

The aim of this work was to examine the ability of African serotype 1 (ST217) to colonise murine nasopharynx in a nasopharynx colonization model (Materials and methods 2.7) relative to that of the laboratory serotype 2 strain, D39. In addition, miles and misra techniques on murine tissue allowed

bacterial load to be assessed in lungs and along potential routes of bacterial dissemination to the brain, such as the olfactory epithelium and olfactory bulb. Finally, flow cytometry was used to compare host immune responses during colonisation of serotype 1 to serotype 2 (Materials and Methods 2.8).

5.2 Results

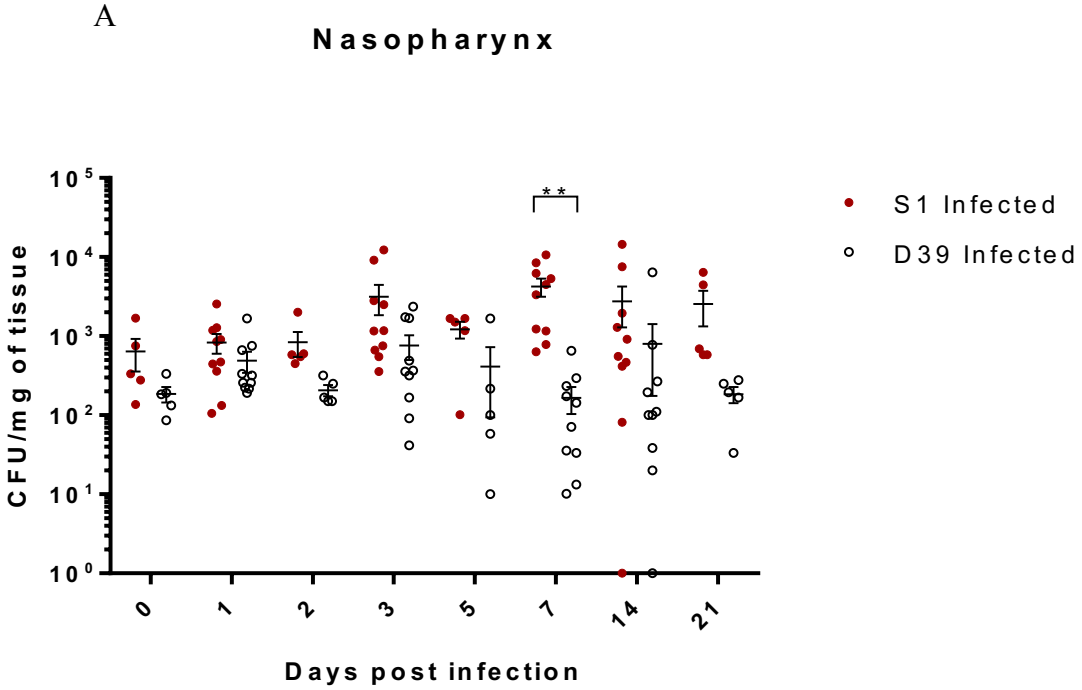
5.2.1 Comparison of nasopharyngeal carriage dynamics in African-serotype 1 and serotype 2 (D39) in BALB/c mouse strain.

BALB/c mice infected with African serotype 1 (ST217 Sepsis) resulted in 100% mortality in a pneumonia model of infection (Figure 27&28). In comparison, infection with serotype 2 strain D39 did not cause mortality due to a lack of dissemination of bacteria from the lungs into the bloodstream. As nasopharyngeal carriage is a prerequisite for IPD, density and duration of nasopharyngeal colonisation were compared in D39-infected and ST217-infected BALB/c mice using an infection model in which prolonged nasopharyngeal carriage without bacterial dissemination has previously been observed [101]. Bacterial load in the lungs, olfactory bulb (OB), olfactory epithelium (OE), blood and brain were examined over 14 days to determine whether bacterial dissemination occurred (Materials and Methods 2.7.5).

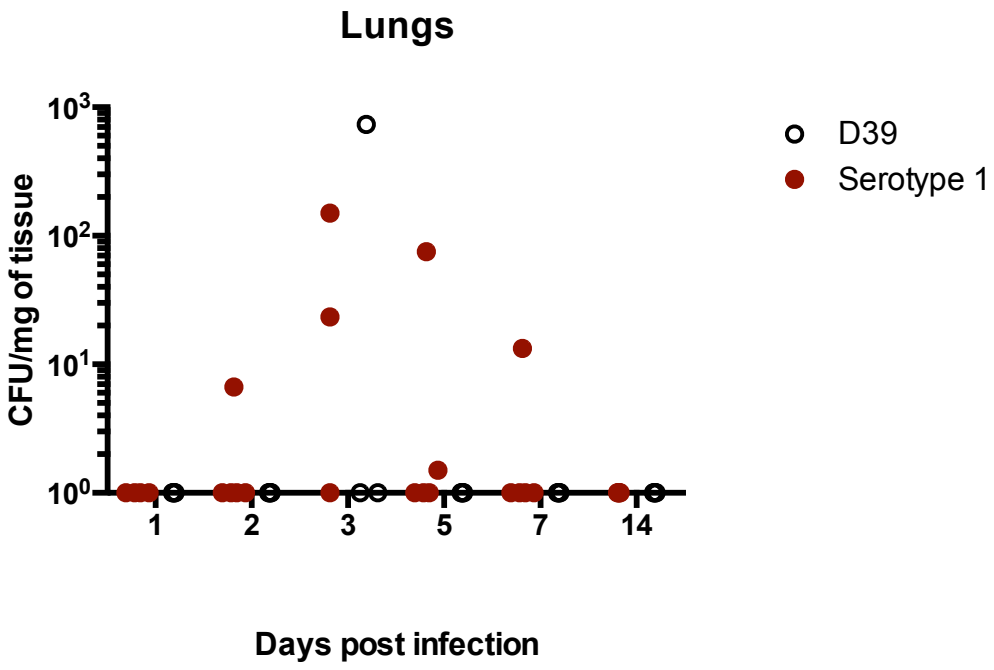
Results showed that both ST217 and D39 colonise the nasopharynx for at least 21 days (Figure 38). Carriage of ST217 was higher than that of D39 throughout and significantly so at day 7 post infection. Two-way ANOVA comparison showed significant differences ($p=0.0004$) in the overall carriage density of serotype 1 compared to serotype 2 over time. Bacterial dissemination into lungs peaked at day 3 post infection for both D39 and ST217-infected mice but was only observed in one or two mice per group. Interestingly, serotype 1 appeared to colonise the OB for 14 days and bacterial load was significantly higher than in D39-infected mice at day 7 post infection. Similar patterns of colonisation were observed in the OE with serotype 1 bacterial loads significantly higher compared to D39 at day 5 post infection. When examining bacterial loads in the brain, only serotype 1 colonies were

detected, whilst the D39-infected mice remained uninfected throughout the course of the experiment. Serotype 1 colonies were present in the brain as early as day 1 post infection but all colonies were cleared by day 14 post infection. Neither ST217 or D39 colonies were found when examining the blood, suggesting that entry into the brain was via the olfactory route and not across the blood-brain barrier.

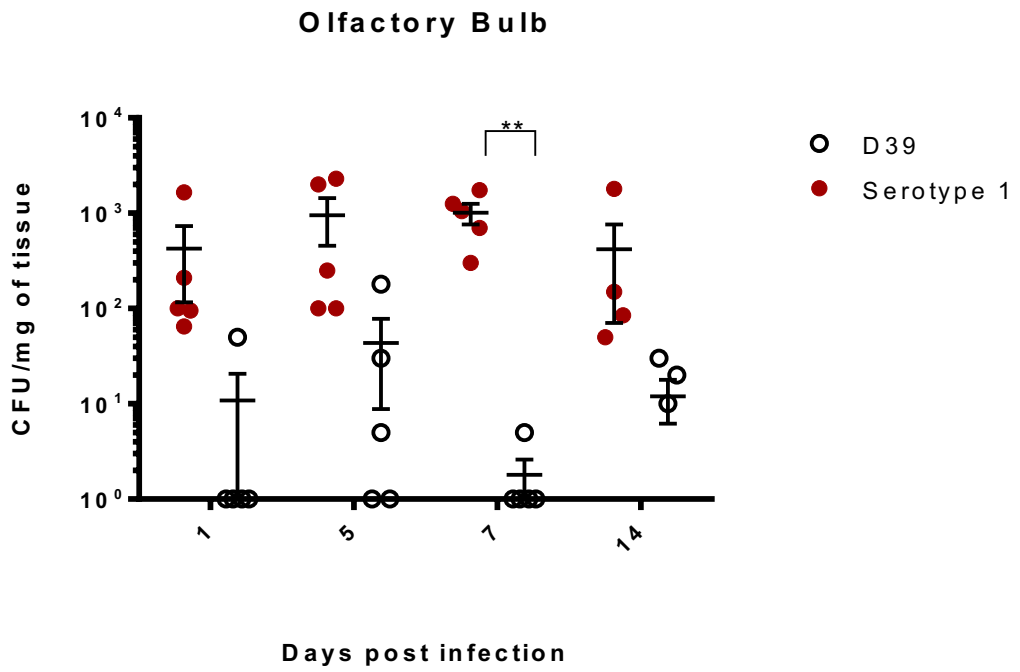
A


Nasopharynx
CFU/mg of tissue
10^5
10^4
10^3
10^2
10^1
10^0
**
0
1
2
3
5
7
14
21
Days post infection
S1 Infected
D39 Infected

B


Lungs
CFU/mg of tissue
10^3
10^2
10^1
10^0
1
2
3
5
7
14
Days post infection
D39
Serotype 1

C


Olfactory Bulb
CFU/mg of tissue
10 4
10 3
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10 0
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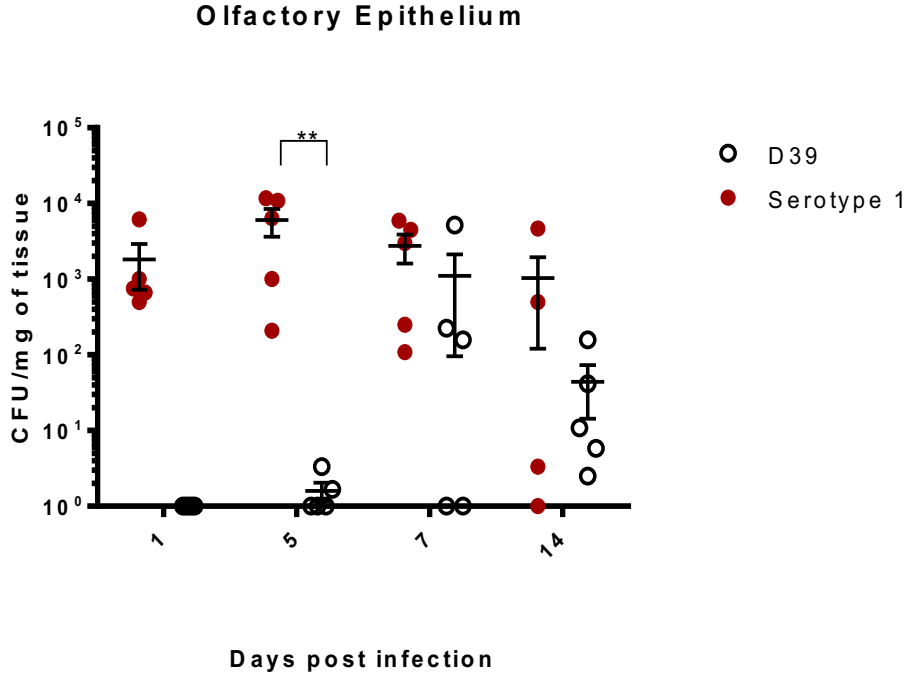
1
5
7
14
Days post infection
D39
Serotype 1

D


Olfactory Epithelium
CFU/mg of tissue
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Days post infection
D39
Serotype 1

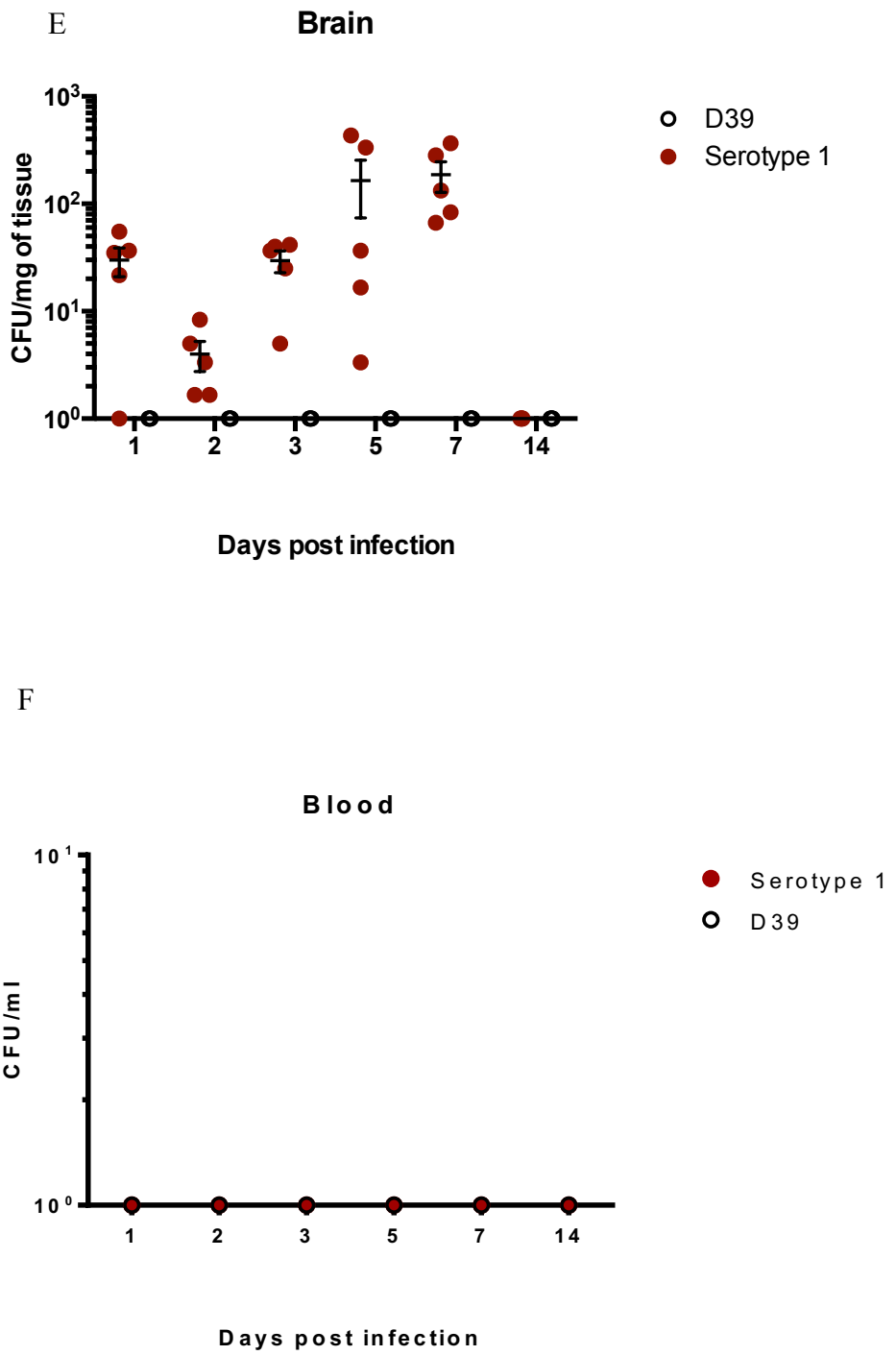


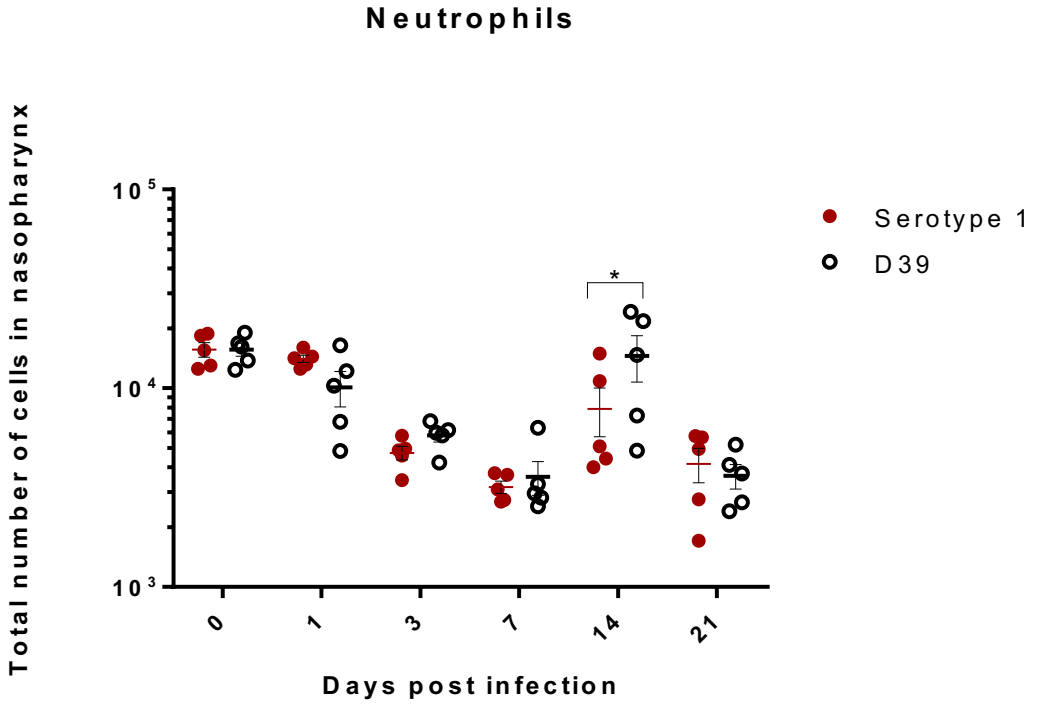
Figure 38. Comparison of Serotype 1 (ST217) and Serotype 2 (D39) in a BALB/c nasopharyngeal carriage model. Mice were IN infected with either 10^5 CFU of ST217 sepsis or D39 and culled at 1,2,3,5,7, 14 or 21 days post infection. 5-10 mice/group were culled and tissues were taken for analysis of bacterial load. (A) CFU counts in the nasopharynx (two experiments combined). (B) CFU counts in the lungs. (C) CFU counts in the Olfactory Bulb. (D) CFU counts in the Olfactory epithelium. (E) CFU counts in the brain. (F) CFU counts in the blood. Data is presented as follows; each symbol represents an individual mouse and errors bars show the mean ± SEM. Statistical analysis was performed by two-way ANOVA (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001).

5.2.2 Host immune response during nasopharyngeal carriage.

I next examined the numbers of different immune cell types that have previously been described as important in either maintenance or clearance of nasopharyngeal carriage. Firstly, the numbers of neutrophils in the nasopharynx in D39 and ST217-infected mice were compared over 21 days of colonisation. These cells are rapidly recruited into the nasopharynx after pneumococcal infection but reports have suggested that their presence does not correlate with clearance of pneumococci [176].

Figure 39 shows the fluctuation of neutrophil numbers over 21 days of colonisation in ST217-infected mice compared to D39-infected. No neutrophil infiltration relative to uninfected mice was observed in either ST217- or D39-infected mice and both groups showed a similar trend in neutrophil numbers over time. A significant difference was seen at day 14 post infection when D39-infected mice showed higher numbers of neutrophils compared to ST217-infected mice.

A



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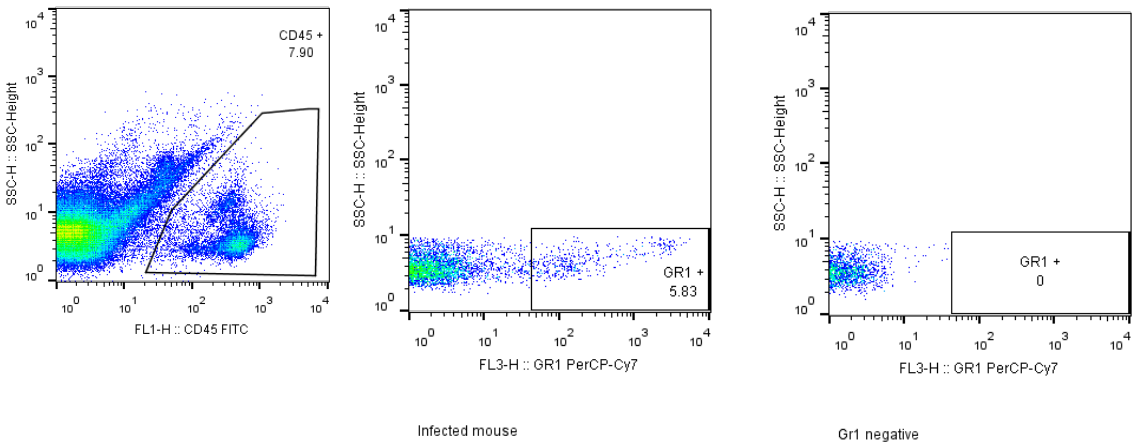


Figure 39 Changes in neutrophil numbers in the nasopharynx during Serotype 1 (ST217) and Serotype 2 (D39) colonisation. Infection described previously in figure 37. At pre-determined timepoints, mice were culled and the nasopharynx was processed to create a single cell suspension. Cells were stained for CD45 and GR1 markers to isolate neutrophils. (A) Number of neutrophils present in the nasopharynx over 21 days of colonisation. Each circle represents a single mouse and error bars shown the mean ± SEM. Two-way ANOVAs were performed to find any statistical differences between ST217 and D39-infected mice (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001).. (B) Gating strategy for isolation of $CD45^+$ $GR1^+$ Neutrophils. GR1 negative control was performed using GR1 fluorescence minus one (FMO).

In addition to staining for neutrophil markers, the macrophage marker F4/80 was used to identify macrophage numbers in the nasopharynx over time. Previous work has shown macrophages have potential involvement in carriage due to accumulation of macrophage numbers in the nasopharynx during colonisation [100].

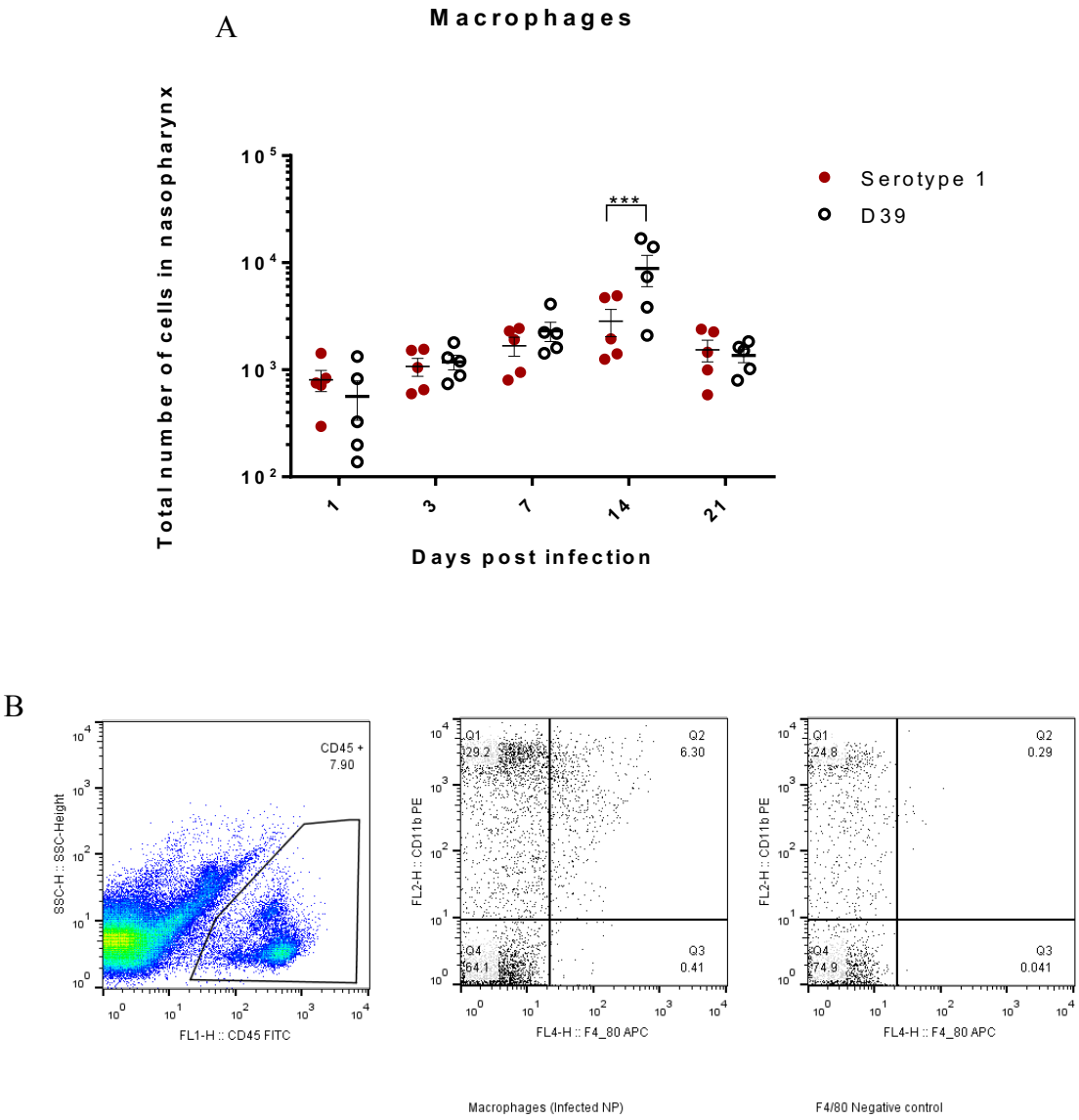


Figure 40 Macrophage numbers in the nasopharynx over 21 days of colonisation with either serotype 1 (ST217) or serotype 2 (D39). Infections described previously (figure 37). Nasopharyngeal tissue was prepared as a single cell suspension. Cells were stained with CD45 and F4/80 markers to identify macrophage populations present at each time point. (A) Number of macrophages present in the nasopharynx over 21 days of colonisation. Each circle represents a single mouse and error bars shown the mean ± SEM. Two-way ANOVAs were performed to find any statistical differences between ST217 and D39-infected mice (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001). (B) Gating strategy for isolation of $CD45^{+}$ $F4/80^{+}$ macrophages. F4/80 negative control was performed using F4/80 fluorescence minus one (FMO).

Colonisation of ST217 and D39 induce similar patterns of macrophage numbers in the nasopharynx over 21 days (Figure 38). Numbers of macrophages increases gradually until day 14 when we observe significantly higher numbers in D39-infected mice compared to ST217. Analysis using a two-way ANOVA shows firstly, numbers of macrophages change significantly over the course of carriage (21 days) for both serotype 1 and serotype 2 colonisation ($P<0.005$). Secondly, that there is a significant difference in macrophage numbers during colonisation between the two serotypes over 21 days of experimental carriage ($P<0.05$).

In addition to monitoring changes in innate immune responses (macrophages and neutrophils), cells associated with the adaptive immune response were also monitored over 21 days in the nasopharynx. It has been reported that immune responses initiated at mucosal surfaces need to be carefully balanced between Th17-mediated inflammation and immune-modulating T regulatory cells [183]. Th17 cells have been shown to be important in the clearance of pneumococci from the nasopharynx through activation and recruitment of neutrophils [290] [172]. As ST217 has been shown to colonise the nasopharynx at significantly higher densities and successfully invade local tissues such as the OB, OE and brain during experimental carriage experiments (Figure 38), it was hypothesised that the balance between induction of Th17 and $Foxp3^+$ T regulatory cells may be different between ST217 and D39-infected mice. Firstly, Th17 ($CD4^+$ $ROR\gamma t^+$) cell numbers were determined over the duration of carriage (Figure 41). No significant differences in the numbers of Th17 cells in the nasopharynx were observed between ST217 and D39-infected mice. There was a significant increase in the numbers of Th17 cells between day 0

and 14 post infection in serotype 1 – infected mice ($p<0.005$). Likewise, significant increases in the number of Th17 cells in D39-infected mice was seen between 0 and 14 days post infection ($P<0.05$).

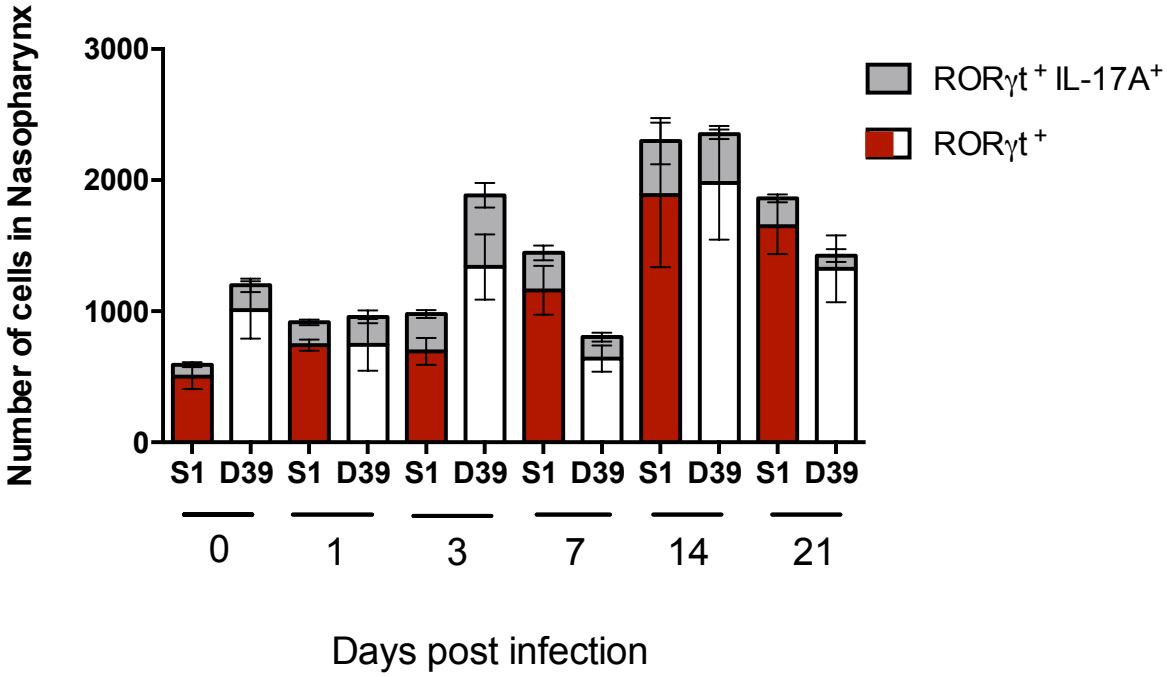


Figure 41. T helper 17 cell (Th17) numbers in the nasopharynx over 21 days of colonisation with either serotype 1 (ST217) or serotype 2 (D39). Mice were IN infected with either 10^5 CFU of ST217 sepsis or D39 and culled at 0,1,2,3,5,7, 14 or 21 days post infection. 5-10 mice/group were culled and tissues were taken for analysis of bacterial load. Nasopharyngeal tissue was prepared as a single cell suspension. Cells were stained for CD45, CD4 and RORγT (Th17 transcription factor) to identify Th17 populations present in each time point. Number of Th17 cells present in the nasopharynx over 21 days of colonisation (Red/white bars) Each bar represents the mean numbers of Th17 cells detected in 5 mice/timepoint. Grey bars represent the proportion of Th17 cells expressing IL-17A. Error bars represent the mean ±SEM. Two-way ANOVAs were performed to find any statistical differences between ST217 and D39-infected mice (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001).

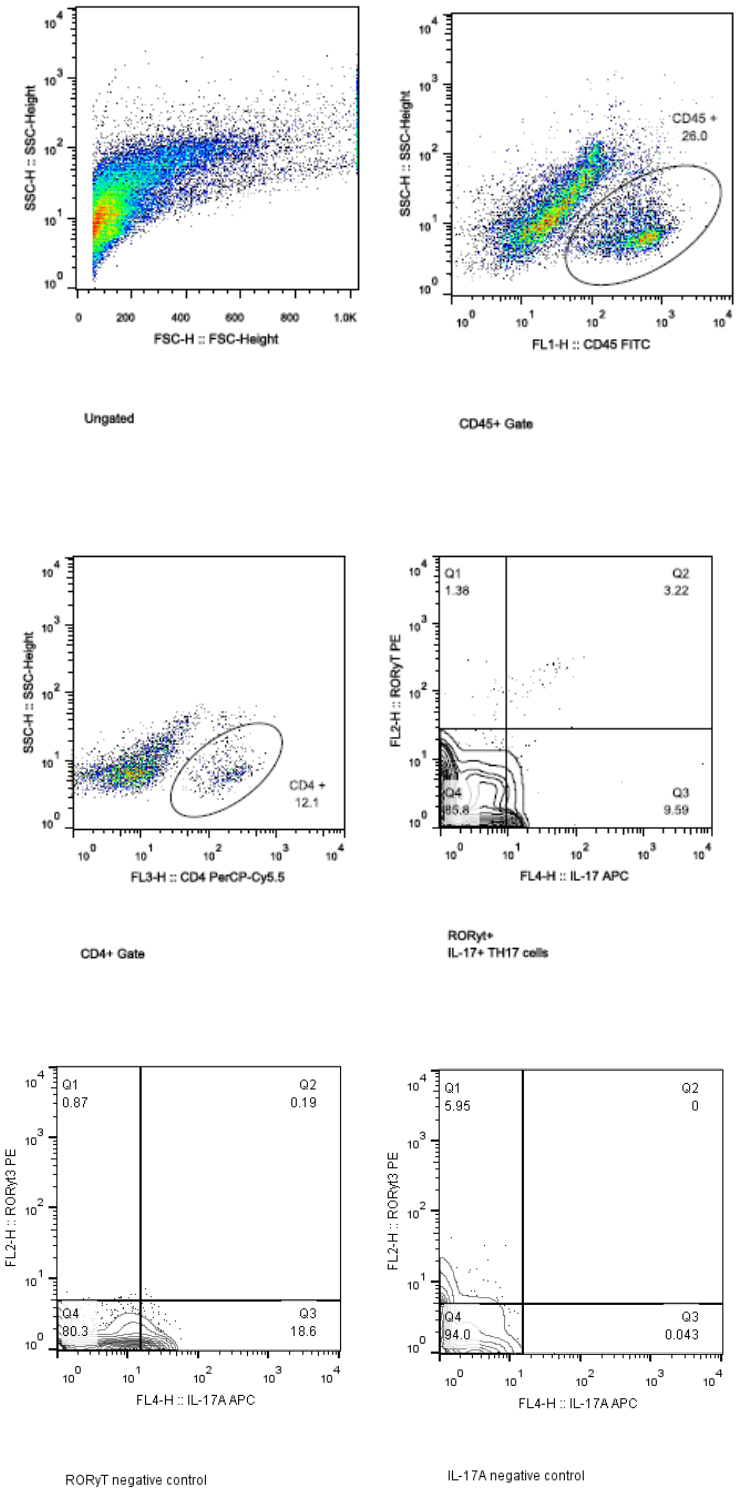


Figure 42. Gating strategy for isolation of CD45$^+$ CD4$^+$ RORγT$^+$ and IL-17A$^+$ T cells. Cells were first gated using CD45 staining. CD45$^+$ cells that were also positive for CD4 were gated next. Cells that were CD45$^+$ CD4$^+$ and RORγt$^+$ were classes as Th17 cells. The number of Th17 cells expressing IL-17A were also quantified for comparison between the two serotypes. Negative controls for IL-17A and RORγt were performed by fluorescence minus one of IL-17A and RORγt.

Th1 ($CD4^+Tbet^+$) T cells have also been shown to be important in clearance of bacteria from the nasopharynx and control multiplication of bacteria following dissemination [291] [184]. When numbers of Th1 cells in the nasopharynx were monitored over 21 days, no difference in cellular numbers were seen in serotype 1 compared to serotype 2 infected mice. However, colonisation did induce significant increases in Th1 cells in both serotype 1 and serotype 2 infected mice. Significant increases ($P<0.05$) in Th1 cells were observed between day 0 and 14 post infection in serotype 1 – infected mice. In addition, D39-infected mice showed a significant increase in Th1 cells between day 1 and 14 post infection ($P<0.001$). No significant differences in the proportion of Th1 cells producing IFNγ was observed between serotype 1 and serotype 2 infected mice (Figure 43).

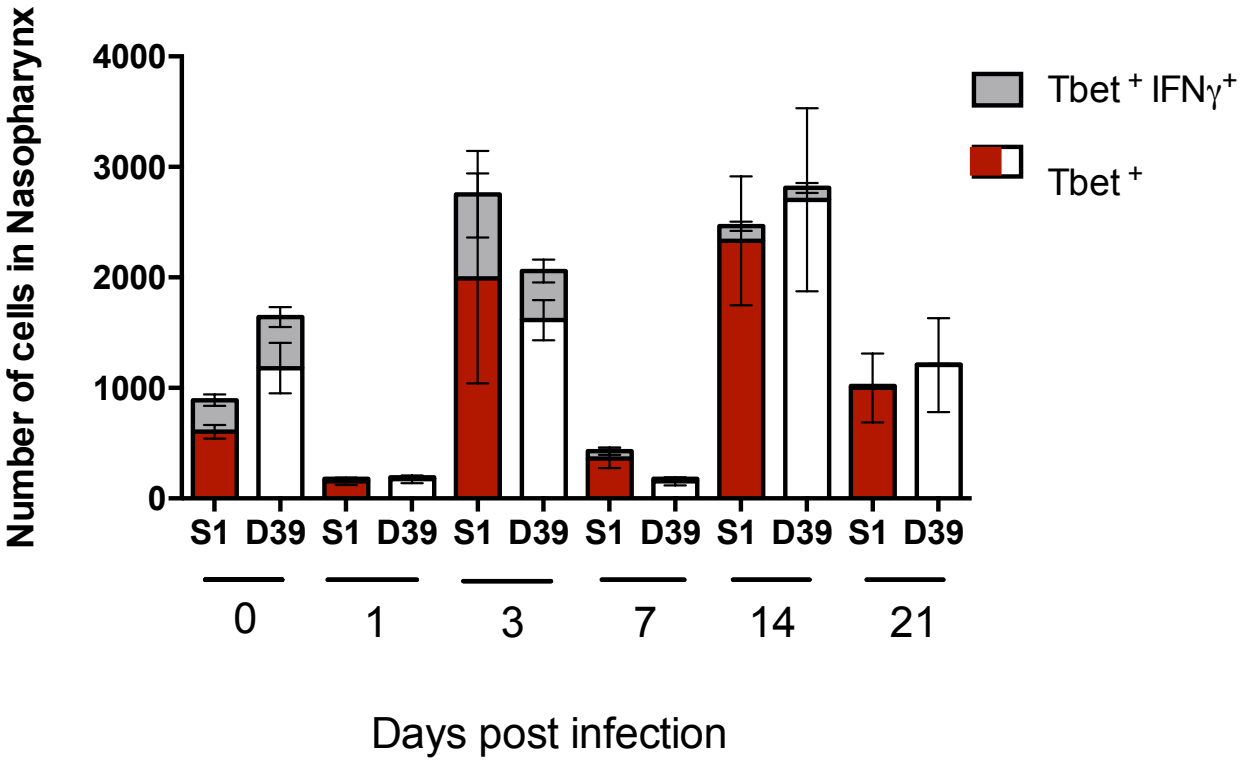


Figure 43. T helper 1 cell (Th1) numbers in the nasopharynx over 21 days of colonisation with either serotype 1 (ST217) or serotype 2 (D39). Mice were IN infected with either 10^5 CFU of ST217 sepsis or D39 and culled at 0,1,2,3,5,7, 14 or 21 days post infection. 5-10 mice/group were culled and tissues were taken for analysis of bacterial load. Nasopharyngeal tissue was prepared as a single cell suspension. Cells were stained for CD45, CD4 and Tbet (Th1 transcription factor) to identify Th1 populations present in each time point. Number of Th1 cells present in the nasopharynx over 21 days of colonisation (Red/white bars) Each bar represents the mean numbers of Th1 cells detected in 5 mice/timepoint. Grey bars represent the proportion of Th1 cells expressing IFN-γ Error bars represent the mean ±SEM. Two-way ANOVAs were performed to find any statistical differences between ST217 and D39-infected mice (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001).

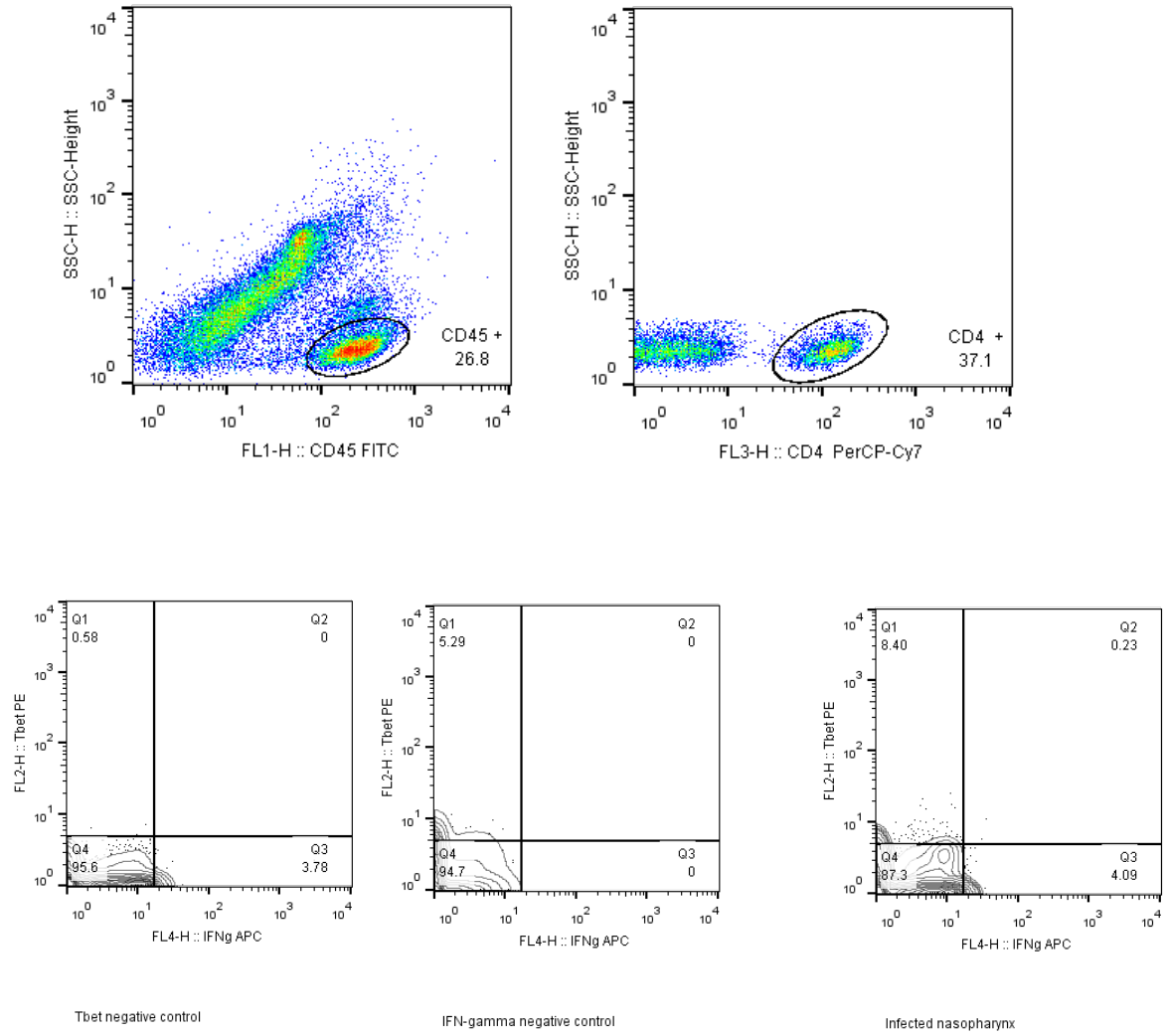


Figure 44. Gating strategy for isolation of CD45$^+$ CD4$^+$ Tbet$^+$ and IFN-γ$^+$ T cells. Cells were first gated using CD45 staining. CD45$^+$ cells that were also positive for CD4 were gated next. Cells that were CD45$^+$ CD4$^+$ and Tber$^+$ were classes as Th1 cells. The number of Th1 cells expressing IFN-γ$^+$ were also quantified for comparison between the two serotypes. Negative controls for IFN-γ$^+$ and Tbet were performed by fluorescence minus one of IFN-γ$^+$ and Tbet.

Pneumococcal nasopharyngeal carriage density and duration have previously been shown to be controlled by T-regulatory cells [100]. Carriage density is an important factor in inducing immune regulatory cells, with low numbers of pneumococci inducing high T-regulatory cell numbers in the nasopharynx, maintaining carriage, and reducing inflammation and bacterial dissemination into lungs [100]. However, little is known about the induction of host immune modulatory cells in response to clinically invasive serotypes during nasopharyngeal colonisation.

$Foxp3^+$ $CD4^+$ T regulatory cell numbers were monitored over 21 days of nasopharyngeal colonisation with D39 and ST217 infection (Figure 45). No significant differences were seen in the numbers of $Foxp3^+$ $CD4^+$ cells in the nasopharynx between ST217 and D39 colonisation. Mice infected with serotype 1 showed a significant increase in T regulatory cell numbers between day 3 and 7 post infection, however, no significant increases were seen in D39 infected mice. In addition, no significant differences were observed in the number of $Foxp3^+$ cells expressing TGFβ between infection with the different serotypes.

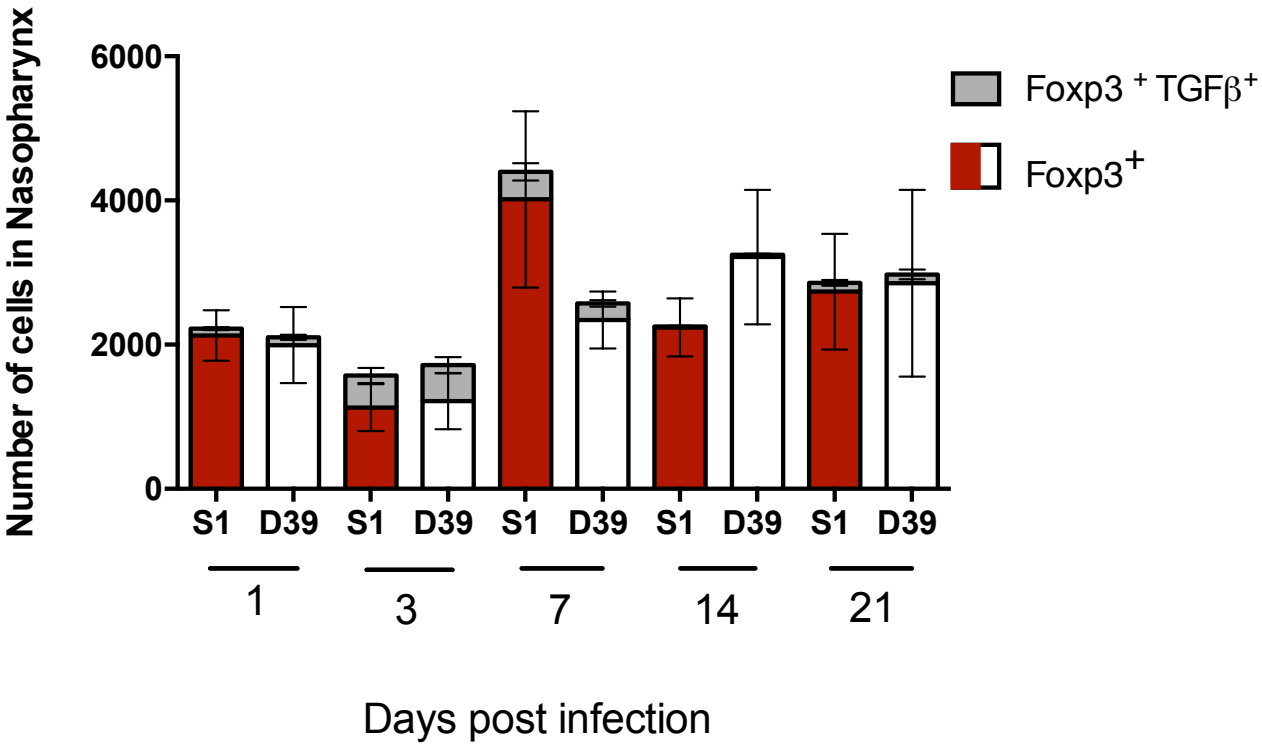


Figure 45. T regulatory cell (Treg) numbers in the nasopharynx over 21 days of colonisation with either serotype 1 (ST217) or serotype 2 (D39). Mice were IN infected with either 10^5 CFU of ST217 sepsis or D39 and culled at 1,2,3,5,7, 14 or 21 days post infection. 5-10 mice/group were culled and tissues were taken for analysis of bacterial load. Nasopharyngeal tissue was prepared as a single cell suspension. Cells were stained for CD45, CD4 and Foxp3 to identify Treg populations present in each time point. Number of Treg cells present in the nasopharynx over 21 days of colonisation (Red/white bars) Each bar represents the mean numbers of Treg cells detected in 5 mice/timepoint. Grey bars represent the proportion of Treg cells expressing TGFβ. Error bars represent the mean ±SEM. Two-way ANOVAs were performed to find any statistical differences between ST217 and D39-infected mice (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001).

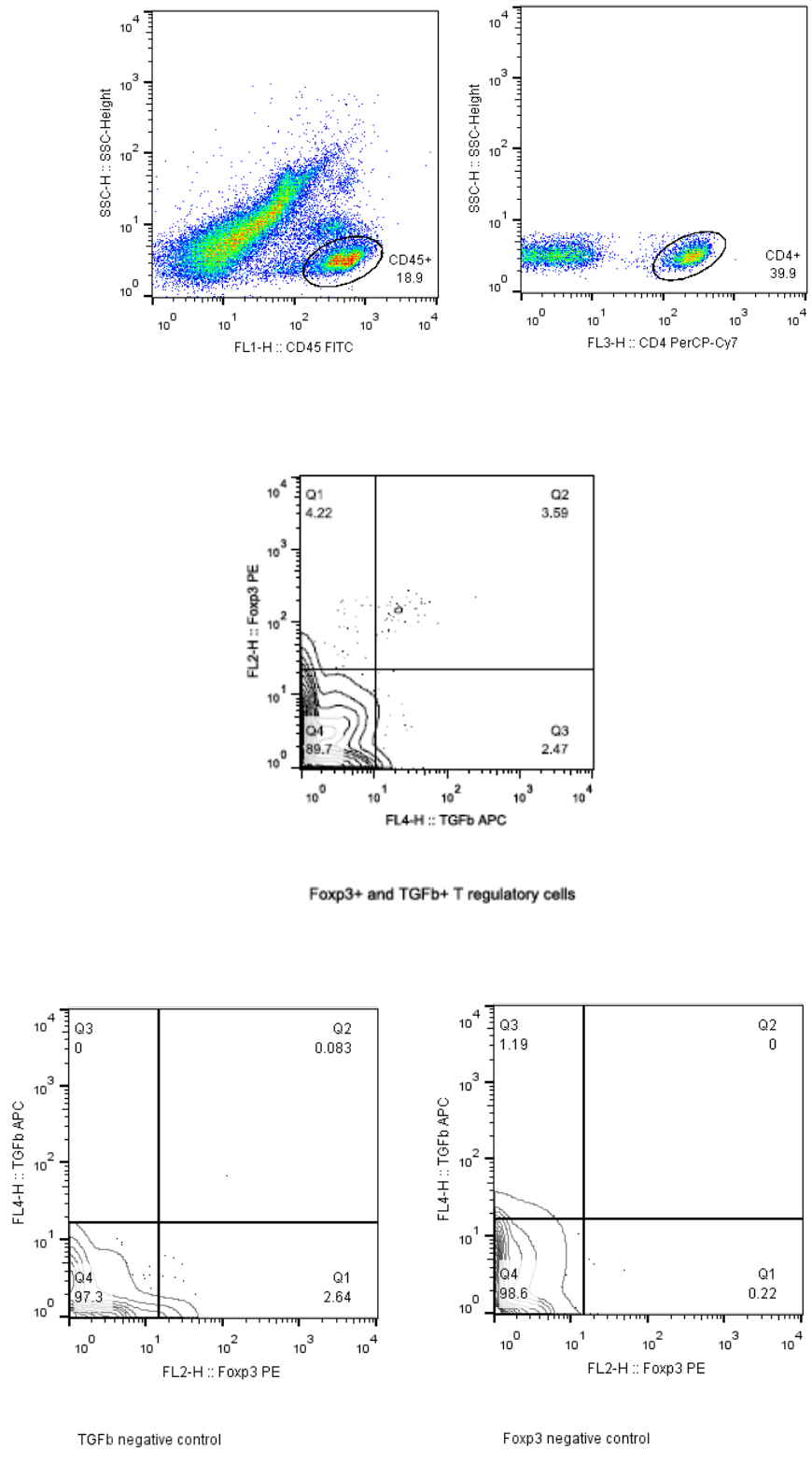


Figure 46. Gating strategy for isolation of CD45$^+$ CD4$^+$ FoxP3$^+$ and TGFβ$^+$ T cells. Cells were first gated using CD45 staining. CD45$^+$ cells that were also positive for CD4 were gated next. Cells that were CD45$^+$ CD4$^+$ and FoxP3$^+$ were classes as Treg cells. The number of Treg cells expressing TGFβ$^+$ were also quantified for comparison between the two serotypes. Negative controls for TGFβ and FoxP3 were performed by fluorescence minus one (FMO) of TGFβ and FoxP3.

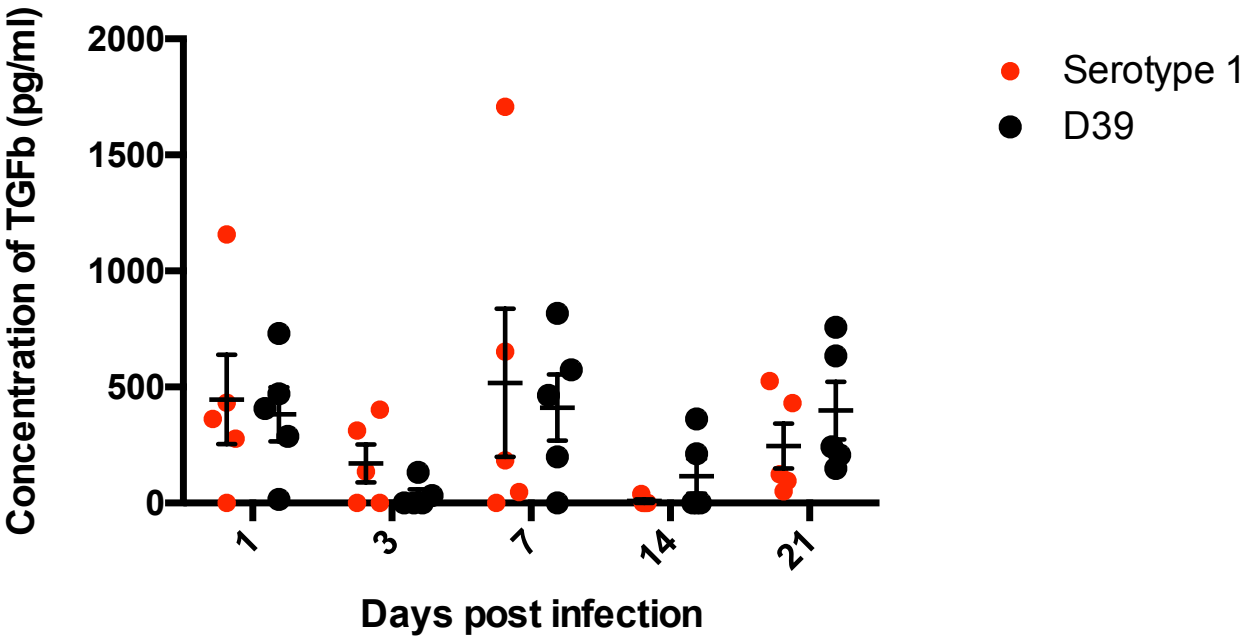


Figure 47. Changes in levels of active TGF-β present in the nasopharynx during serotype 1 (ST217sepsis) and serotype 2 (D39) colonisation. Active TGF-β determined from nasopharyngeal supernatant by luciferase-reporting transformed mink lung epithelial cell assay. Each circle represents a single mouse and error bars shown the mean ± SEM.

Concentrations of active TGF-β present in the nasopharynx were determined over 21 days of colonisation with D39 or serotype 1-infected mice (Figure 47). No significant differences were observed between the two groups of mice at any timepoint throughout the experiment but fluctuations in the concentrations of TGF-β were seen over the timecourse of carriage. Highest mean concentrations were observed at day 7 post infection, whilst the lowest recorded was at day 14, which is also when T regulatory cell numbers were lowest. The patterns of TGF-β concentrations were similar in both ST217 and D39-infected mice, despite higher rates of dissemination of serotype 1.

5.2.3 Effect of high doses of Serotype 1 or Serotype 2 bacteria on nasopharyngeal colonisation.

My studies revealed little differences in the host immune cell profile during serotype 1 nasopharyngeal carriage compared to D39 colonisation. I next repeated these experimental carriage models using higher doses of *S. pneumoniae*, in the same 10μl volume, with the aim of increasing bacterial dissemination and potentially inducing invasive disease. Carriage patterns were compared in mice infected with D39 compared to ST217, along with cellular infiltration of neutrophils, T regulatory cells and Th17 cells.

Results showed that during the early stages of nasopharyngeal carriage (Day 1-3), there were no differences in the CFU counts of D39 infected mice compared to those infected with ST217 (Figure 48A). However, at day 14 post infection, ST217 bacterial load in the nasopharynx was found to be significantly higher than that of D39. Indeed, the 2-log drop in bacterial nasopharyngeal density between days 3 and 14 in D39-infected mice suggested that bacterial clearance was underway. When examining bacterial dissemination into the lungs, only one mouse infected with D39 showed bacterial dissemination into the lungs (Day 3). At day 14 post infection, no colonies of D39 were observed in the lungs but three mice infected with ST217 had colonies present in the lungs. However, there was no significant differences found when comparing lung bacterial densities over 14 days using a Two-way ANOVA. (Figure 48B).

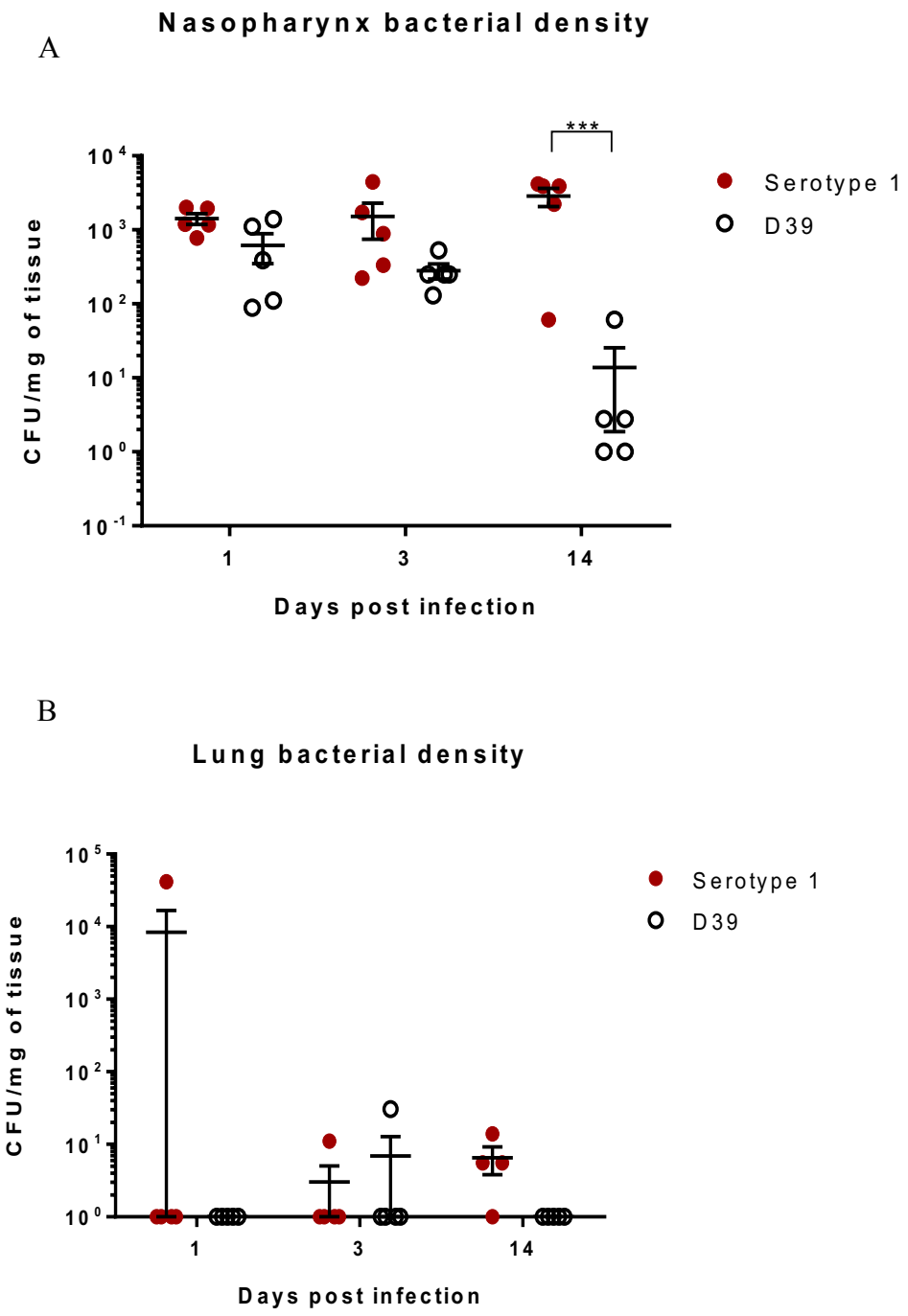


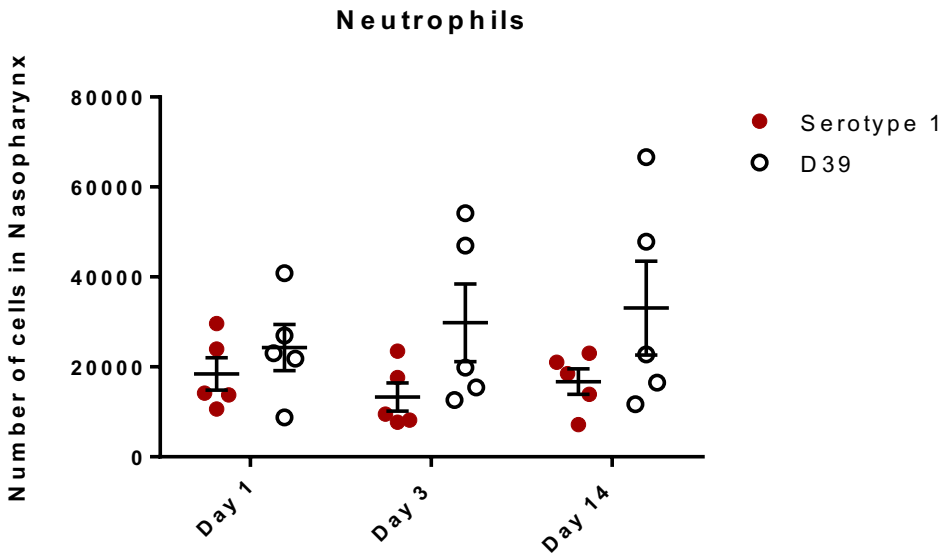
Figure 48 Comparison of Serotype 1 (ST217) and Serotype 2 (D39) in a BALB/c nasopharyngeal carriage model. Mice were intranasally infected with either 10^6 CFU of ST217 sepsis or D39. At 1,3 and 14 days post infection, 5 mice/group were culled and nasopharyngeal and lung tissues were taken for analysis of bacterial load. (A) Bacterial load (cfu/mg) in nasopharynx. (B) Bacterial loads (cfu/mg) in the lungs. Data is presented as follows; each symbol represents an individual mouse and errors bars show the mean ± SEM. Statistical analysis was performed by two-way ANOVA (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001).

5.2.4 Effect of high doses of serotype 1 or serotype 2 bacteria on the host immune system during nasopharyngeal colonisation.

When ST217 and D39 were intranasally administered into BALB/c mice at a concentration of 10^6 in a volume of 10μl, trends of bacterial colonisation for D39 and ST217 varied greatly, with D39 numbers decreasing over 14 days and ST217 colonisation increasing. To determine whether these changes reflected differences in host immune responses generated during 14 days of colonisation, leukocytes were profiled in D39 and ST217 infected mice.

Neutrophil numbers were examined in the nasopharynx to assess whether infection with ST217 induces significant differences in neutrophil numbers, particularly at day 14 when CFU counts are significantly different (Figure 48A). Results show that there was a significant difference (Two-way ANOVA, $P<0.05$) between the numbers of neutrophils induced between D39 and ST217 colonised mice over 14 days (Figure 49A). Colonisation with D39 led to a significantly higher number of neutrophils over the duration of the experiment compared to ST217 colonisation and may reflect the differences in CFU/mg counts at day 14 post infection.

A



B

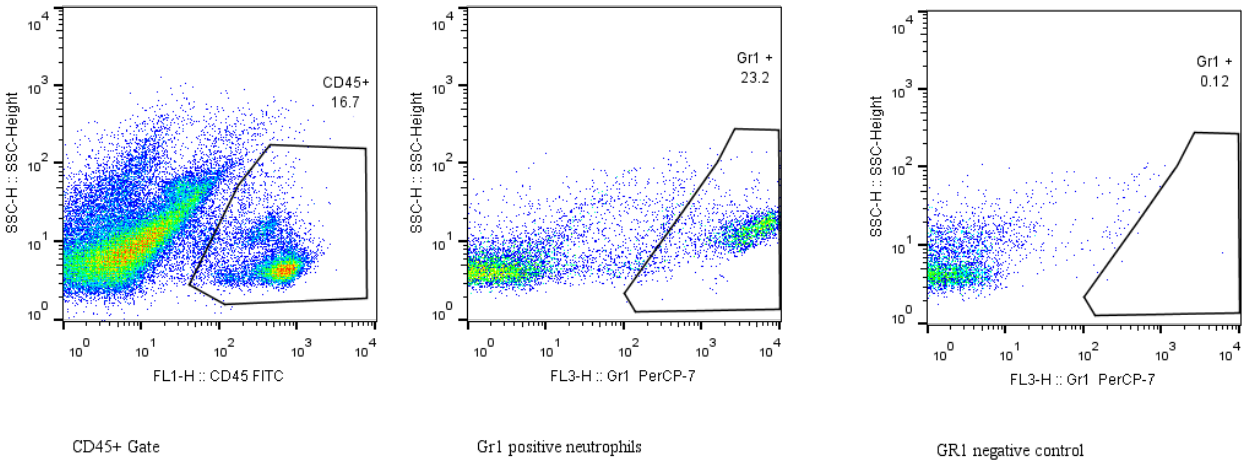


Figure 49 Changes in neutrophil numbers in the nasopharynx during Serotype 1 (ST217) and Serotype 2 (D39) colonisation. Mice were IN infected with either 10^6 CFU of ST217sepsis or D39. At 1,3 and 14 days post infection, 5 mice/group were culled and the nasopharynx was processed to create a single cell suspension. Cells were stained with antibodies against CD45 and GR1, to isolate neutrophils. (A) Number of neutrophils present in the nasopharynx at 1,3 and 14 days of colonisation. Each circle represents a single mouse and error bars shown the mean ± SEM. Two-way ANOVAs were performed to find any statistical differences between ST217 and D39-infected mice. (B) Gating strategy for isolation of $CD45^+$ $GR1^+$ Neutrophils. GR1 negative staining performed using FMO of GR1.

In addition to comparing neutrophil recruitment into the nasopharynx, numbers of Th17 cells were also examined at 1 and 14 days post infection (Figure 50).

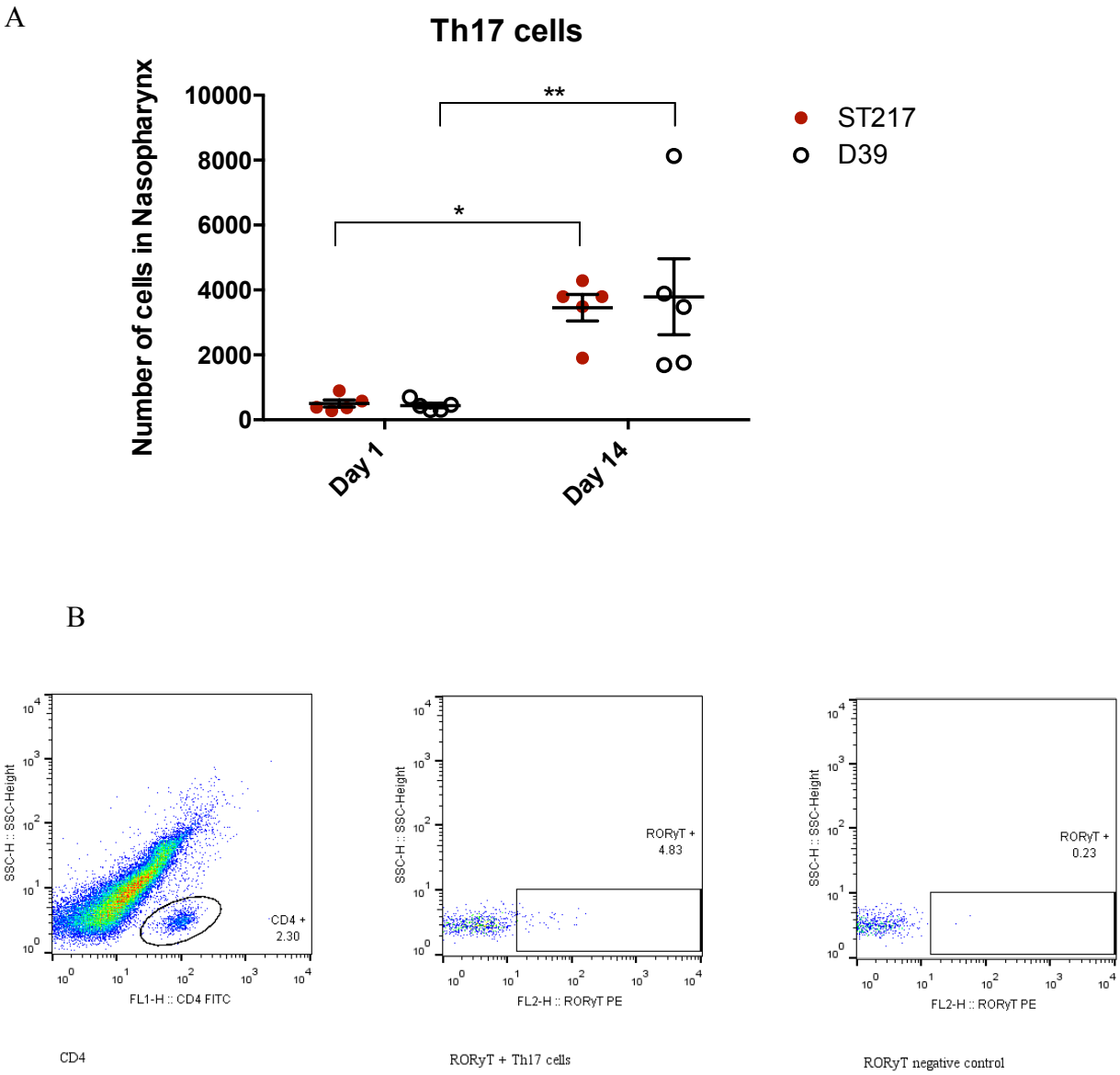


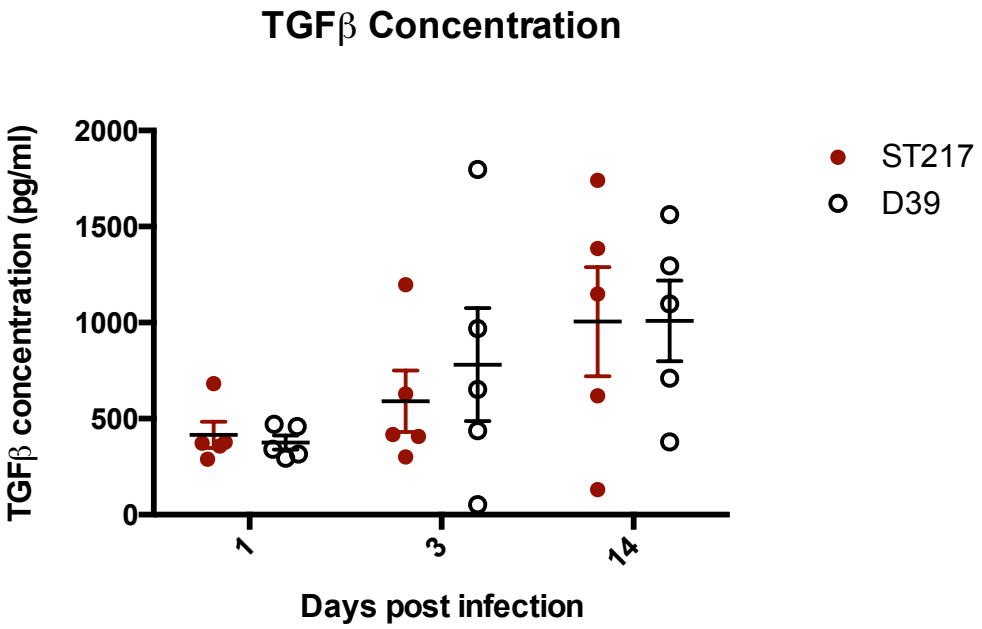
Figure 50 T helper 17 cell (Th17) numbers in the nasopharynx over 14 days of colonisation with either serotype 1 (ST217) or serotype 2 (D39). Mice were IN infected with either 10^6 CFU of ST217sepsis or D39. At 1,3 and 14 days post infection, 5 mice/group were culled and the nasopharynx was processed to create a single cell suspension. Cells were stained for CD4 and RORγt to identify Th17 populations present at each time point. (A) Number of Th17 cells present in the nasopharynx over 14 days of colonisation. Each circle represents a single mouse and error bars shown the mean ± SEM. Two-way ANOVAs were performed to find any statistical differences between ST217 and D39-infected mice (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001). (B) Gating strategy for isolation of $CD4^+$ and $ROR\gamma t^+$ T cells. RORγt negative staining was performed with RORγt FMO.

Th17 cell numbers increased significantly between day 1 and 14 post infection in both ST217 and D39-infected mice.

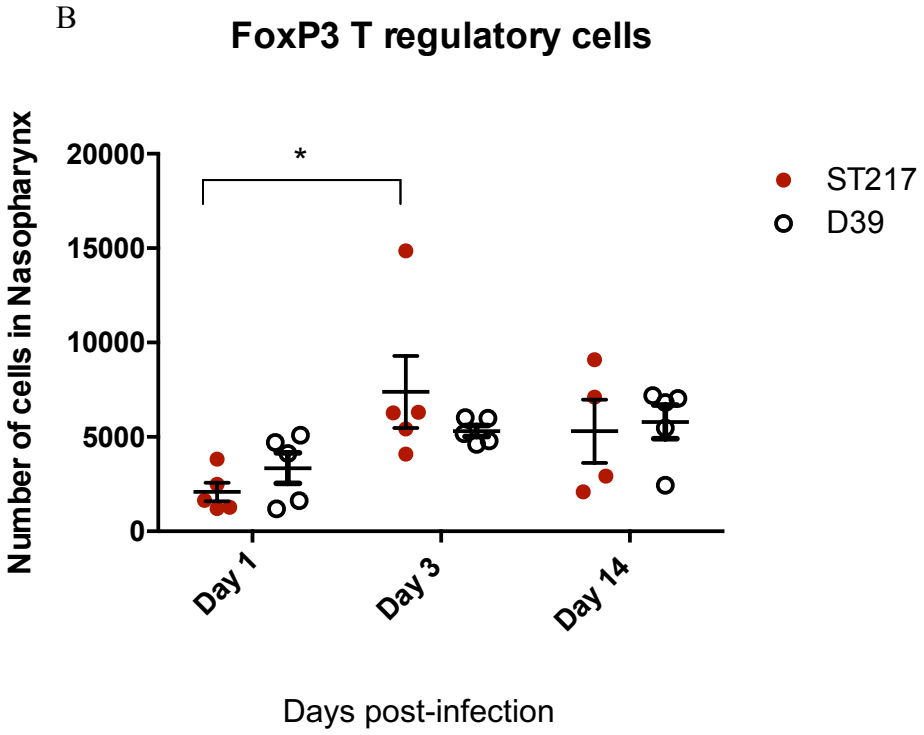
As ST217 bacterial counts remained significantly higher than D39 at 14 days post infection (Figure 48A), it was hypothesised that differences in T regulatory cell numbers or levels of TGFβ could have caused the significant differences in ST217 CFU counts on day 14. Results from flow cytometric assessment of T regulatory cell numbers and quantification of active TGFβ levels in nasopharynx revealed no significant differences between ST217 colonisation compared to that of D39 (Figure 51A&B). Colonisation of ST217 caused significant increases in the number of Foxp3 T regulatory cells between day 1 and 3 post infection, but no significant changes in the number of T regulatory cells in the nasopharynx of D39-infected mice was observed.

Detectable TGFβ levels in nasopharyngeal homogenates increase significantly ($P<0.05$) over time in both ST217 and D39 infected mice but again, no significant differences between carriage of the two serotypes was detected (Figure 51A).

A


TGFβ Concentration
TGFβ concentration (pg/ml)
2000
1500
1000
500
0
1
3
14
Days post infection
ST217
D39

B


FoxP3 T regulatory cells
Number of cells in Nasopharynx
20000
15000
10000
5000
0
*
Day 1
Day 3
Day 14
Days post-infection
ST217
D39

C

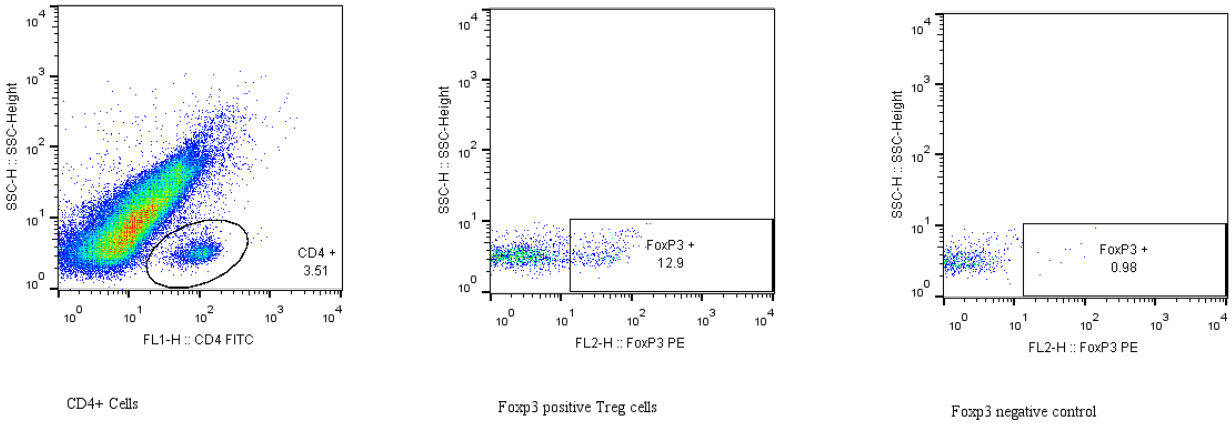


Figure 51 T regulatory cell numbers in the nasopharynx over 14 days of colonisation with either serotype 1 (ST217) or serotype 2 (D39). Mice were IN infected with either 10^6 CFU of ST217 or D39. At 1,3 and 14 days post infection, 5 mice/group were culled and the nasopharynx was processed to create a single cell suspension.. (A) Active TGF-β determined from nasopharyngeal homogenates by luciferase-reporting transformed mink lung epithelial cell assay. Each circle represents a single mouse and error bars shown the mean ± SEM. (B) Nasopharyngeal tissue was prepared as a single cell suspension. Cells were stained for CD4, and Foxp3 to identify Treg populations present at each time point. Each circle represents a single mouse and error bars shown the mean ± SEM. Two-way ANOVA was performed to find any statistical differences between ST217 and D39-infected mice (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001).. (C) Gating strategy for T regulatory cells. $CD4^+$ cells were selected for and T regulatory cells were identified using intracellular staining for Foxp3.

5.2.5 *In vitro* T cell differentiation/expansion in the presence of ST217 induces a pro-inflammatory T cell repertoire.

The aim of this experiment was to expose BALB/c bone-marrow derived macrophages to either D39 or ST217 and then to culture pneumococcal-exposed macrophages with $CD4^+$ T cells harvested from spleens of naïve BALB/c mice. After 3 days of macrophage and T cell co-culture, flow cytometry was performed to examine T cell differentiation or expansion in response to ST217 and D39 (Figure 52).

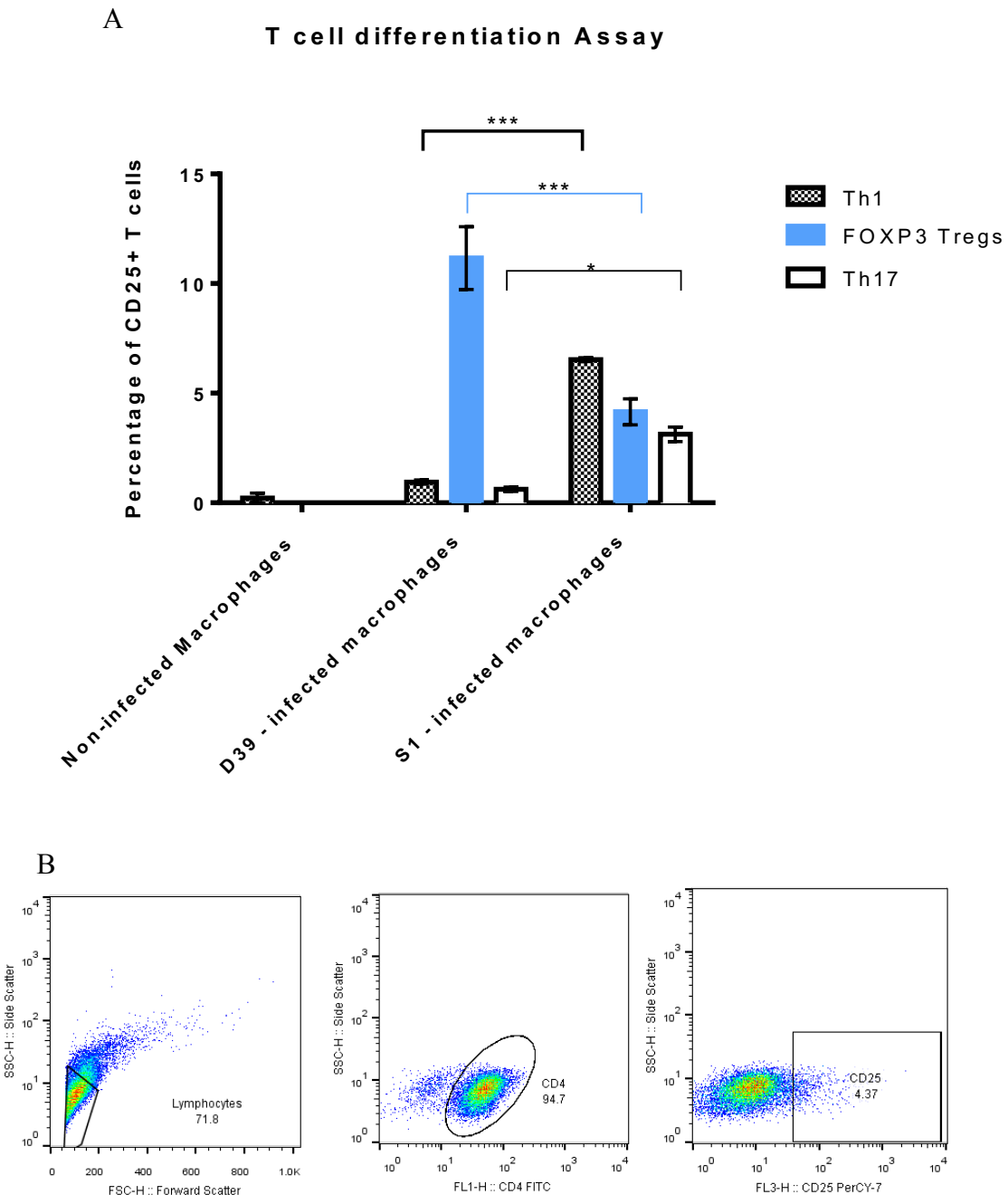


Figure 52 T cell stimulation assays used to assess different T cell subsets induced in the presence of ST217 and D39 antigens. 7-10 week female naïve BALB/c mice were sacrificed and bone marrow from tibia and femurs removed. Bone-marrow (BM) derived cells were cultures for 6 days in the presence of M-CSF (2μg/ml) to stimulate macrophage differentiation. Live ST217 or D39 bacteria were cultured with BM-derived macrophages for 24 hours before removal using antibiotics. Meanwhile, spleens were removed from 7-10 week female naïve BALB/c mice and $CD4^+$ T cells were isolated using magnetic beads. T cells were then co-cultured with activated macrophages for 3 days before flow cytometry staining was performed to analyse T cell subset differentiation. (A) Comparison of T cell differentiation in the presence of macrophages infected with either ST217sepsis (serotype 1) or serotype 2 (D39) or uninfected. Each infection condition was performed in triplicate wells. Error bars represent mean ± SEM. Two-way ANOVAs were performed to test statistical significance of the data (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001).. (B) Gating strategy for activated T cells ($CD4^+$ $CD25^+$ cells)

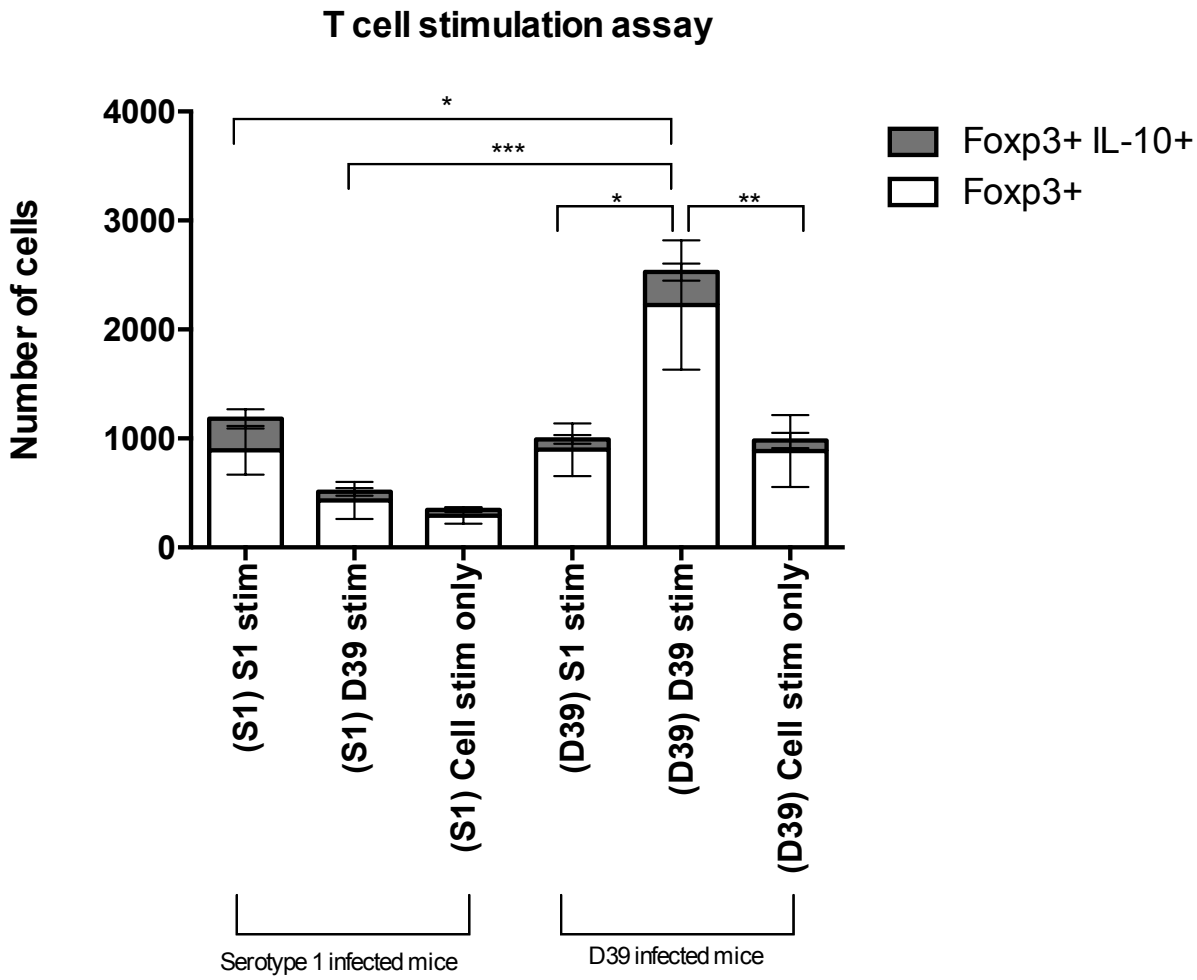
T cell differentiation results suggest that ST217 antigens induce the differentiation or activation of a range of different T cell subsets via their effects on macrophages, with Th1 being the predominant subset. In contrast to this, macrophages infected with D39 predominantly induced the differentiation or activation of $Foxp3^+$ T regulatory cells (Figure 52A). Macrophages co-cultured with D39 induced significantly higher percentages of $Foxp3^+$ T regulatory cells compared to ST217 co-cultured macrophages. Macrophages exposed to ST217 induced significantly higher proportions of Th1 and Th17 cells compared to macrophages exposed to D39. Thus, exposure to ST217 induces a broad T cell subset repertoire of Th1, Th17 and T regulatory cells, whereas D39 predominantly causes high percentage differentiation of $Foxp3^+$ T regulatory cells and very low levels of Th1 and Th17 cells.

5.2.6 *In vitro* T cell re-stimulation assays

T cell re-stimulation assays were used to assess the T cell repertoire induced after 21 days of nasopharyngeal carriage with ST217 compared to D39 (Figure 53). Mice were colonised with serotype 1 (ST217) or serotype 2 (D39) for 21 days before the experiment was terminated and the cervical lymph nodes draining the nasopharynx were removed. Lymph node cells were stimulated with heat-killed D39, ST217 or a cell stimulation cocktail only and numbers of $Foxp3^+$ T regulatory cells and $Foxp3^+$ T regulatory cells expressing IL-10 were quantified using flow cytometry. Results demonstrate that when cells from the lymph nodes of D39-colonised mice were stimulated with heat-killed D39, a significantly higher number of $Foxp3^+$ T regulatory cells were observed compared to the same cells stimulated with serotype 1 (ST217) (Figure 53). In

addition, cells from mice infected with D39 had significantly higher numbers of Foxp3^{+} T regulatory cells after re-stimulation with D39 compared to ST217-colonised mice after re-stimulation with D39. No significant differences were observed when cells from ST217 colonising mice were stimulated with ST217 and cell stimulation cocktail only. When cells from D39-infected mice were stimulated with D39, significant differences were observed in the number of T regulatory cells compared to stimulation with cell stimulation cocktail only. Thus suggesting that low T regulatory cell responses were observed during serotype 1 carriage compared to D39.

A



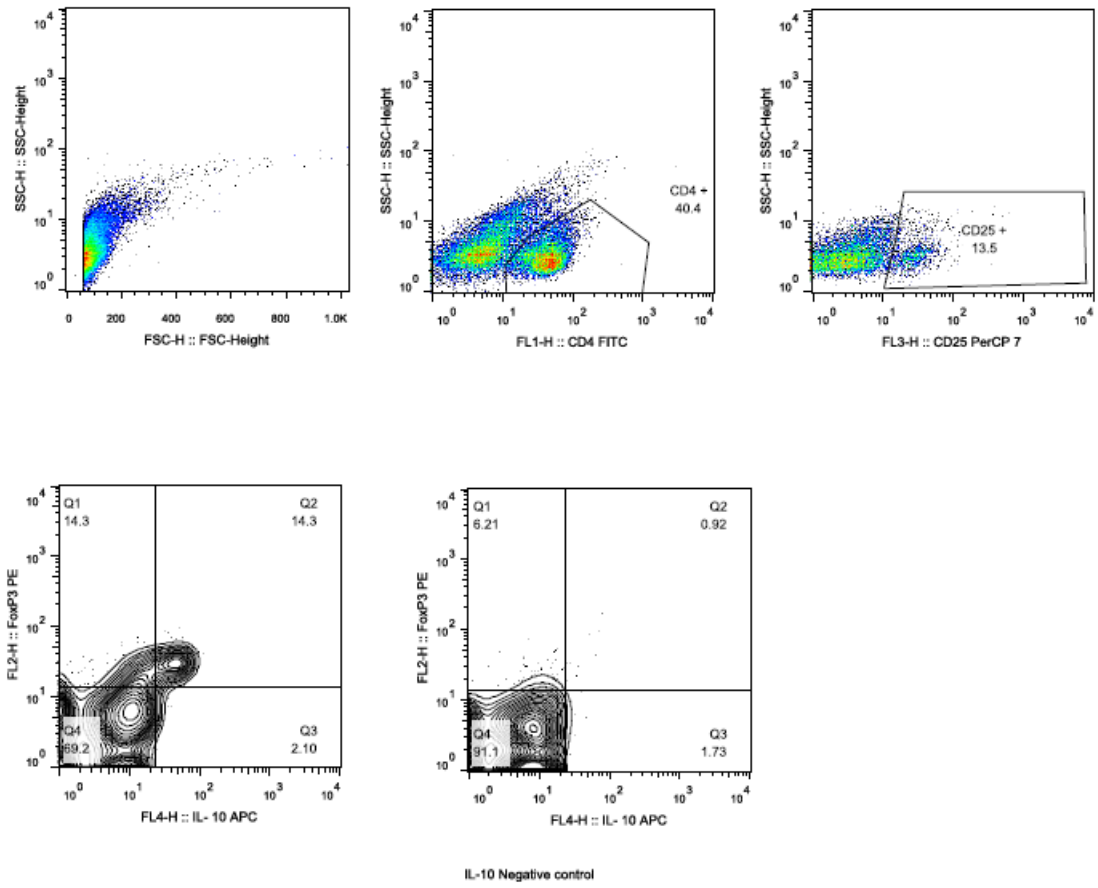


Figure 53 T cell re-stimulation assays with cervical lymph nodes following 21 days of carriage. 6 mice per group were I.N infected with 10^5 CFU of *S. pneumoniae* (ST217 or D39) in 10µl of PBS. At day 21 post infection, mice were culled and cervical lymph nodes (CLN) removed. A single cell suspension was created and cells from each mouse were stimulated with heat killed ST217, D39 or a cell stimulation cocktail only. After 18 hours of incubation, cells were stained with CD4, CD25, Foxp3 and IL-10 to identify populations of T regulatory cells by flow cytometry. (A) White bars represent the number of Foxp3 T regulatory cells ($CD4^+$ $CD25^+$ $Foxp3^+$) induced from both ST217 and D39-infected mice after stimulation with different antigens. Grey bars represent the portion of T regulatory cells producing IL-10 cytokine. Each re-stimulation was performed in duplicate and error bars represent the mean ± SEM. Statistical analysis was performed by Two-way ANOVA (*P < 0.05, **P < 0.01,***P < 0.005, or ****P < 0.001). (B) Gating strategy used to identify $Foxp3^+$ T regulatory cells ($CD4^+$ $CD25^+$ $Foxp3^+$ $IL\text{-}10^+$) IL-10 negative control performed using FMO IL-10 APC antibody.

5.3 Discussion

Streptococcus pneumoniae is a common commensal of the upper respiratory tract and colonisation of the nasopharynx is thought to be a pre-requisite of invasive pneumococcal disease [169] [168]. Ordinarily, in areas where rates of carriage are high, the risk of invasive disease is also high [292] [3]. However, this has not been reported for IPD caused by serotype 1. Serotype 1 is the leading cause of IPD in sub-Saharan Africa but is described as rarely found during nasopharyngeal carriage, even in areas of high disease burden [44]. The aim of the work in this chapter was to understand the interactions of serotype 1 with host immunity and its impact on serotype 1 carriage. Using murine models of experimental nasopharyngeal carriage, mice were inoculated with serotype 1 (ST217) or serotype 2 (D39) and the density and duration of carriage were compared along with rates of bacterial dissemination to other tissues. Comparisons of the host immune responses generated during colonisation with these two serotypes was also performed.

Results showed that serotype 1 is able to colonise the nasopharynx for at least 21 days and maintain stable CFU counts over this time. Carriage patterns between the two serotypes differed, with serotype 1 being carried at significantly higher densities at two time points (day 7 and 14 post infection). Interestingly, significantly higher rates of bacterial dissemination occurred in mice colonised with serotype 1. In particular, serotype 1 pneumococci were found to be present in the brain in the first seven days of nasopharyngeal colonisation. No detectable colonies of serotype 2 were found in the brain over the duration of the experiment. In addition, serotype 1 mice had no bacterial colonies detected in the blood suggesting an alternative route of transmission

from the nasopharynx to the brain that bypassed the blood. The nasopharynx has an anatomical connection to the central nervous system (CNS) through the olfactory nerves, as the posterior nasal cavity is covered by the olfactory epithelium, which contains nerves of the olfactory system. The olfactory nerves travel from the cribriform plate, which is a bone behind the nose, up to the olfactory bulb, located at the inferior side of the brain [278]. Previous reports have shown that bacterial translocation of *Neisseria meningitidis* and *Streptococcus pneumoniae* from the nasal mucosa to the brain can occur without bacteria entering the bloodstream, thus meningitis can occur directly from nasopharyngeal colonisation [102,278]. Data presented here suggest that high rates of serotype 1 meningitis may occur as serotype 1 appears to be able to enter the brain directly from the nasal cavity during carriage without needing to invade the blood. However, the possibility that there was a transient serotype 1 bacteraemia that was missed due to the timing of sampling cannot be excluded, although it is unlikely that high enough CFUs could have seeded into the blood stream from the nasopharynx to eventually cause CNS infection. Indeed, there is no evidence to suggest that there is direct seeding from the nasopharynx to the blood.

In Niger there has been a long history of meningitis epidemics with *Neisseria meningitidis* and *Streptococcus pneumoniae* [293] [246]. A recent surveillance study was conducted to assess whether high concentrations of airborne dust in this area was a risk factor for IPD. Experiments in murine models of nasopharyngeal carriage showed that following dust exposure there were significant increases in serotype 1 titres in the brain and that this was not

associated with bacteraemia, once again suggesting a direct nasopharynx to brain translocation route [246].

Results from *in vivo* models show that serotype 1 dissemination into OE, OB and brain was significantly higher than serotype 2, suggesting natural rates of tissue invasion of the olfactory nerves are high during serotype 1 nasopharyngeal carriage. High rates of serotype 1 meningitis seen in Niger and the other 26 countries making up the African meningitis belt could be due to inhalation of dust particles accelerating the natural tendency of serotype 1 to colonise the olfactory nerves as a route into the brain through damage to mucosal barriers facilitating bacterial transmission.

In chapter 4 it was shown that rates of autolysis are significantly higher in serotype 1 compared to serotype 2. High temperatures, such as those found in sub-Saharan Africa, including Niger, have been found to significantly increase rates of autolysis of serotype 1 [246]. Increased rates of autolysis during carriage will lead to higher concentrations of pneumolysin release which has been previously shown to aid tissue invasion [93]. Perhaps, serotype 1 is found to be the predominant cause of meningitis and invasive disease due to climate factors aiding natural virulence mechanisms such as high rates of autolysis to allow increased bacterial dissemination into other tissues from the nasal mucosa.

Carriage rates of serotype 1 have been described as low despite serotype 1 being the main causative serotype of IPD in sub-Sarahan Africa [238]. Observations of bacterial dissemination to the olfactory nerves during serotype 1 carriage suggest that current swabbing techniques may not be detecting serotype 1 due to observed colonisation of deeper tissues of the nasopharynx.

Knowing that climate is an important factor in the onset of IPD, it would be interesting to assess changes in upper airway bacterial density in deeper tissues such as the OB, and OE when serotype 1-infected mice are exposed to high temperatures and dust/sand inhalation. Colonisation of serotype 1 was found to last at least 21 days in the murine models tested here, but different hosts and climatic conditions could affect carriage duration and levels of tissue invasion and may explain why serotype 1 has previously been described as a poor coloniser of the nasopharynx.

Density and duration of nasopharyngeal carriage has been reported to be controlled by host immune responses. $Th17^+$ $CD4^+$ T cells have been described as important in mucosal clearance of pneumococci through recruitment of macrophages and neutrophils [183] [172]. Further studies have shown that Th1 and Th17 T cell anti-pneumococcal responses are key in bacterial clearance but these can be supressed by T regulatory cells [294]. The presence of commensal bacteria in the nasopharynx has been shown to induce T regulatory cells in mice [295]. In the case of the pneumococcus, colonised children have higher titres of T regulatory cells in adenoidal tissue compared to carriage negative children [296]. Nasopharyngeal carriage can be prolonged with elevation of TGF-β and T regulatory cell numbers whilst high Th17 and Th1 T cells drive clearance of pneumococci from the nasopharynx [297] [172] [173]. Hence, dysregulation of this fine balance between immune tolerance and inflammation may lead to invasive disease.

As serotype 1 has been classically described as a poor coloniser and high levels of dissemination to the brain were observed in this study, it was predicted that colonisation with serotype 1 might fail to induce good T

regulatory responses. When numbers of T regulatory cells present in the nasopharynx were compared over 21 days between serotype 1 and serotype 2 colonised mice, no significant differences were observed. Despite this, carriage of ST217 was significantly higher than D39 at day 7 post infection and tabular comparisons showed significant differences ($p=0.0004$) in the overall carriage density of serotype 1 compared to serotype 2 over the course of infection.

T regulatory cells and TGF-β play important roles in prolonging carriage by reducing inflammation, keeping tissue damage to a minimum to prevent bacterial spread to other tissues [174] [100]. It was hypothesised that due to high titres of bacteria found in the OB, OE and brain, T regulatory cell numbers could be lower during serotype 1 colonisation. However, data from murine models suggests that dissemination of serotype 1 to the OB, OE and brain was not a result of lower T regulatory cell numbers during serotype 1 carriage. As the brain is anatomically linked to the nasopharynx via olfactory nerves, tissue damage may not be required to aid bacterial dissemination.

Neutrophil and macrophage numbers were monitored in the nasopharynx of serotype 1 and serotype 2-colonised mice over 21 days. No differences were seen in numbers of either neutrophils or macrophages between the serotypes except on day 14 post infection when numbers were higher in serotype 2 colonised mice. Depletion of neutrophils in experimental carriage models has been shown to have no effect on bacterial loads in the nasopharynx [176]. The exact role neutrophils or macrophages play in serotype 1 carriage is not clear from this data. Flow cytometric staining was performed to identify macrophage populations in this work. It would be interesting to assess the macrophage

phenotypes present in the nasopharynx in serotype 1 verses serotype 2 infections. Alternatively activated macrophages have previously been described as being present during carriage with D39 pneumococci [100] but little is known about macrophage activation during serotype 1 colonisation. If serotype 1 infection, with high rates of autolysis and pneumolysin release, induced classical activation of M1 macrophages with high IL-12 production, this could drive inflammation and aid tissue damage and therefore dissemination of bacteria [298].

Th17 and Th1 cell numbers in the nasopharynx were monitored over 21 days. No difference in cell numbers between carriage of the two serotypes was detected. Numbers remained consistent over the course of 21 days. Th17 cells have been described as important in reducing carriage duration and so the lack of Th17 induction in these experiments may explain why clearance of carriage was not initiated.

Carriage experiments were repeated with higher doses of bacteria in an attempt to induce progression to invasive disease and potentially see differences in nasopharyngeal colonisation between ST217 and D39. Bacterial densities were similar up to day 3 post infection, however at day 14 carriage density of serotype 1 was significantly higher compared to D39. Serotype 1 carriage remained consistent whereas D39 numbers were steadily declining, suggesting bacterial clearance would have occurred soon after 14 days. The immune responses in the nasopharynx were compared but no differences were found in numbers of T regulatory cells, TGF-β, Th17 or neutrophils when comparing serotype 1 to serotype 2 colonisation despite significant differences in CFU numbers at day 14. There was a significant induction of Th17 cells in

both infection groups which was perhaps responsible for declining numbers of D39. Serotype 1 numbers did not decline over the course of 14 days perhaps due to the ability to invade tissues such as the OB and OE which may be 'hidden' from the host immune response and therefore allow serotype 1 to keep recolonising the nasopharynx. When examining T regulatory cell responses, only Foxp3 was used as a marker. Additional staining for intracellular TGF-β or IL-10 would have more accurately identified functionally active T regulatory cells. For example, GARP has been identified as a maker for activated T regulatory cells in humans and mice [299]. Staining for additional markers such as GARP would have been useful to identify whether there was a difference in the number of active T regulatory cells during carriage.

Length of nasopharyngeal carriage in BALB/c mice is longer than in MF1 mice (Figure 16&38) so future experiments should be extended to 28 days to assess the immune responses involved in bacterial clearance and comparisons of carriage duration between the two serotypes.

In addition to monitoring the host immune responses in murine models of experimental carriage, *in vitro* assays were set up to assess differences in T cell differentiation in the presence of both serotypes. Bone-marrow derived macrophages were infected with live serotype 1 (ST217) or serotype 2 (D39) for 24 hours. When $CD4^+$ T cells were cultured with infected macrophages, those infected with serotype 1 induced significantly higher numbers of Th1 and Th17 cells compared to D39. Macrophages infected with D39 caused differentiation of a significantly higher number of Foxp3 T regulatory cells compared to serotype 1. However, no intracellular cytokine staining was

performed for T regulatory cells so it is not known whether these cells were producing anti-inflammatory cytokines.

T cell re-stimulation assays were used to assess the T cell profile present when cells from lymph nodes of mice colonised with either ST217 or D39 were re-stimulated with heat-killed bacteria of both serotypes. Results showed that mice infected with D39 had significantly higher T regulatory cell responses upon stimulation with D39 compared with stimulation of the same cells with serotype 1. Only weak T regulatory responses were seen after re-stimulation of cells from ST217-infected mice, suggesting that colonisation of serotype 1 induces lower T regulatory responses compared to D39 colonisation. When cells from D39-infected mice were re-stimulated with ST217 compared with D39, it was seen that re-stimulation with the same serotype 1 was significantly greater than cross-serotype, this suggests that capsule potentially plays a more important role than other factors in driving T cell stimulatory responses.

Both T cell differentiation and T cell re-stimulation assays suggest that carriage of serotype 2 leads to higher T regulatory responses compared to carriage with serotype 1. It was hypothesised that carriage with serotype 1 would induce poor immune-modulatory responses such as T regulatory cells and TGF-β and therefore carriage duration would be short due to high levels of inflammation allowing for bacterial dissemination or clearance. Predicted differences in the balance of pro and anti-inflammatory responses between carriage of serotype 1 and serotype 2 were not observed when experimental murine carriage models were used. Serotype 1 did colonise the nasopharynx for at least 21 days and dissemination was significantly higher in serotype 1 infected mice but no consistent differences were observed in the host immune

responses between the two serotypes. This suggests that differences in bacterial dissemination is likely to be driven more strongly by the pathogen rather than excessive inflammation and tissue damage caused by the host immune responses.

These results have demonstrated that serotype 1 is able to successfully colonise the murine nasopharynx for at least 21 days. This is in contrast to what has been published in humans which states that colonisation of serotype 1 is shorter than other serotypes [44]. This is supported by evidence that antibiotic resistance in serotype 1 is lower compared to other serotypes that are associated with long-term colonisation [300]. Murine models of experimental carriage can be limited in their representation of the human nasopharynx where carriage of multiple serotypes is common and therefore competition for nutrients and space will likely affect carriage duration of some serotypes. In addition, environmental factors such as climate, and host factors such as immune status and age are all important factors in determining length of carriage. Results from the previous chapter places emphasis on the importance of pneumolysin in promoting invasion of serotype 1 from the lungs into the bloodstream in the context of pneumococcal pneumonia. It likely that these same virulence factors are used to aid serotype 1 translocation from the nasopharynx to the brain.

Conclusion

These results suggest that serotype 1 can establish long term (more than 21 days) colonisation of the murine nasopharynx. During carriage, high rates of bacterial dissemination were observed which included colonies detected in the brain, lungs and olfactory nerves. Serotype 1 appears to be able to invade local tissues of the upper airway and access the brain without needing to enter the blood and cross the blood-brain barrier, and this is likely to be due to high rates of autolysis and release of highly haemolytic pneumolysin, which together with the influence of climate, may help explain why serotype 1 is the leading serotype to cause pneumococcal meningitis in sub-Saharan Africa. Despite differences in the rates of bacterial dissemination, colonisation of serotype 1 compared to serotype 2 did not induce gross differences in the host immune response beyond a differential ability to induce activation of different T cell subsets. This suggests that the ability of serotype 1 to invade local tissues may be directly related to the pathogen rather than excessive inflammation caused by the host which would damage local tissues and facilitate movement of bacteria to the other tissues.

Discussion

6.0 Discussion

The aim of this research was to further understand serotype 1 *Streptococcus pneumoniae* pathogenesis and explore the role of certain virulence factors and their influence in both carriage and invasive disease.

Streptococcus pneumoniae is a natural commensal of the nasopharynx and many sources have described carriage as an important pre-requisite for IPD [301] [302] [3]. Interestingly, serotype 1 has been described as a poor coloniser of the nasopharynx due to rare detection during routine nasopharyngeal swabbing, even in areas of high disease burden [4] [239,303] [277]. Using murine models of experimental carriage, I have demonstrated that serotype 1 pneumococci can successfully colonise a murine nasopharynx for over 14 days. In addition, serotype 1 could colonise deeper tissues anatomically connected to the upper airway, such as the olfactory bulb and olfactory epithelium, at significantly higher densities compared to serotype 2, strain D39. Upon examination of the brain, serotype 1 colonies were detected in the first 7 days of carriage. As no D39 colonies were found to be present in the brain over the course of infection, it appears that serotype 1 has greater invasive potential compared to D39 and utilises a potential route of dissemination to the brain through the central nervous system, bypassing the blood. Previously published data have shown that *S. pneumoniae* can migrate through the olfactory tissues to reach the brain from the nasopharynx [278], however, this has not, up to now, been explored in the context of serotype 1 infection. These data may partially explain why serotype 1 is the predominant serotype to cause non-bacteraemic meningitis in Africa. Previously published data have shown that environmental factors such as airborne dust and high temperatures which

are common in sub-Saharan Africa increase rates of IPD in murine models of nasopharyngeal carriage [246]. Therefore, serotype 1 is likely to cause high incidents of meningitis because it is able to colonise deeper tissues of the upper airway such as the OB and OE at significantly higher densities compared to serotype 2, D39. The ability of serotype 1 to invade these tissues and reach the brain to cause meningitis is likely to be enhanced by environmental factors such as high temperatures or high concentrations of airborne particulates which have been shown to increase rates of autolysis and impair phagocytosis by macrophages and neutrophils [246]. The combination of these factors is likely to influence disease progression from carriage to meningitis during serotype 1 colonisation of the nasopharynx. [304 60 285]. Further work should be done to examine whether invasion of the olfactory tissues and brain is serotype dependent.

Colonisation of the olfactory tissues is likely to also occur in humans and may provide an explanation for low carriage detection of serotype 1. Bacteria in olfactory tissues would not be detected by current swabbing techniques. Several questions have arisen from these findings, including, does re-seeding from the olfactory tissues to the nasopharynx? In addition, little is currently known about the host immune responses in the olfactory tissues and brain in the presence of pneumococci. Further work is needed to examine differences in the immune cell profiles present in the OB and OE during serotype 1 colonisation compared to D39. This is important to assess whether the immune response initiated during colonisation has a significant effect on progression of serotype 1 to the brain. Further work should also be aimed at

identifying which virulence factors contribute to local tissue invasion and the influence these may have on the host immune response, a factor we know to be important in controlling nasopharyngeal carriage [100 305].

The role of the capsule in colonisation was examined. *In vitro* comparisons of three ST217 isolates (from blood, CSF and nasopharynx) suggested that in the nasopharynx a thinner capsule is needed, in contrast to a thicker capsule found on ST217 isolates from the blood. Presence of a thicker capsule provided better protection from complement deposition and phagocytosis from neutrophils, as shown using *in vitro* assays. Current literature regarding the role of the capsule in colonisation is conflicting, with some reports stating that a thin capsule is required to promote bacterial adherence to nasopharyngeal cells [77]. However, other reports state a thicker capsule is required to protect from the host immune response and therefore heavier encapsulation is associated with prolonged carriage duration [79]. When the three ST217 isolates were compared in a murine model of carriage, there were no differences in their ability to establish nasopharyngeal carriage, despite significant differences in capsule thickness and resistance to complement deposition *in vitro*. Further examination of capsule over 14 days in the nasopharynx demonstrated that the capsule thickness changed significantly over the course of infection, with results suggesting that nasopharyngeal colonisation induces an increase in capsule thickness which allowed the bacteria to colonise for over 14 days. However, murine models are not sufficient to fully mimic changes in capsule thickness that may be observed in humans. Multiple species of bacteria usually colonise the human nasopharynx and at times these may include multiple serotypes of *S. pneumoniae* [306 307,308]. The full

effect of bacterial competition on capsule expression could not be addressed in this work. However, it is worth noting that significant changes in the capsule thickness were seen throughout the duration of carriage, suggesting that capsule thickness should not be defined by niche or even serotype as the host immune responses, competition with other bacteria and nutrient/oxygen availability may significantly impact capsule thickness of *S. pneumoniae.*

Further work should be done to compare serotype 1 colonisation characteristics (capsule thickness, resistance to C3b deposition, colonisation of OE and OB) compared to a serotype such as 19A which has been described as a 'good coloniser' with high detection rates during routine nasopharyngeal swabbing, combined with low rates of IPD [309]. This will be useful to measure whether changes in capsule thickness and dissemination into the olfactory tissues seen during serotype 1 colonisation is common amongst all *S. pneumoniae* serotypes.

The host immune responses were compared between colonisation of serotype 1 and serotype 2 in the nasopharynx. The aim of this work was to ascertain whether there were significant differences in the host immune responses between the two serotypes. Previous publications show the importance of immune-modulating cells in controlling nasopharyngeal carriage with emphasis on the role of T regulatory cells and TGF-β [100]. Colonisation of serotype 1 and serotype 2 induced significant increases in numbers of Th17, Th1 and T regulatory cells in the nasopharynx, however, no significant differences were observed when comparing responses between serotypes, despite serotype 1 colonising the nasopharynx at a significantly higher density and seeding into the olfactory tissues and brain. Thus, any differences in the

host immune responses initiated between the two serotypes are likely to be subtle, or not confined to nasopharynx, and these methods were not sensitive enough to detect any differences in the cellular response. Further work should include more detailed phenotyping of the T regulatory cells present during carriage. Markers are now available to identify activated T regulatory cells, for example GARP, and CD39 which can be used to detect T regulatory cells with highly immunosuppressive abilities [310-312]. Use of the transcription factor Foxp3 alone is not sufficient to assess the phenotype of T regulatory cells present during serotype 1 compared to serotype 2 carriage.

This body of research was performed using two different mouse strains, MF1 and BALB/c. These murine strains differ in their susceptibilities to pneumococcal infection. BALB/c mice are highly resistant to pneumococcal pneumonia due to rapid recruitment of T regulatory cells into the lung in response to pneumococci and high TGFβ levels [173,208]. This recruitment is critical in reducing inflammation and ensuring minimal tissue damage occurs in the lungs. When comparing carriage of serotype 1 in MF1 mice to BALB/c, at 21 days post infection in MF1 mice, all pneumococci had been cleared. However, in BALB/c mice, serotype 1 was still carrying at a high density at day 21 with no sign of a decline in numbers. The high levels of immune modulation present in the BALB/c mice may allow serotype 1 carriage for over 21 days. Further work should be done to examine whether BALB/c are able to successfully clear serotype 1 from the nasopharynx over time. Interestingly, even with significant increases in the T regulatory cell numbers during colonisation, serotype 1 is still able to invade the OB, OE and brain, suggesting that the host immune response is not able to fully prevent bacterial

dissemination and that tissue invasion may be predominantly driven by the bacteria.

When serotype 1 and 2 were compared in a BALB/c murine model of pneumonia, striking differences in disease severity were observed. Firstly, mice infected with serotype 1 displayed 100% mortality, compared to those infected with serotype 2 (100% survival). When other clinically-relevant serotypes were examined in this model, only African serotype 1 isolates caused mortality. Mortality was attributed to high bacterial numbers in the bloodstream. Data showed that African serotype 1 could pass rapidly from the lungs into the bloodstream and cause severe bacteraemia. This was found to be due to high rates of autolysis in African serotype 1 isolates which led to the release of highly haemolytic pneumolysin. Serotype 1 pneumolysin leads to cytotoxic damage of lung epithelial cells which is the likely cause of bacterial dissemination into the bloodstream. Neutralisation of pneumolysin by liposomes (lipid vesicles acting as decoy pneumolysin binding sites) significantly reduced the damage to lung epithelial cells and was seen to reduce mortality rates of infected mice.

Whilst it is known that pneumolysin aids bacterial invasion of host tissues [93], I have now demonstrated that this is the main driving force behind serotype 1 pathogenesis in the lung. By calculating the amount of pneumolysin produced, the haemolytic activity of that pneumolysin and the rate of bacterial lysis, I can now determine the amount of pneumolysin and therefore haemolytic units released per minute which can predict the invasive potential of different serotypes and lineages of *S. pneumoniae*. African serotype 1 bacteria release significantly higher concentrations of haemolytic pneumolysin due to high

rates of autolysis compared to other clinically relevant serotypes. This is predicted to drive serotype 1 pathogenesis in the lungs of BALB/c mice and facilitates bacterial dissemination from the lungs into the bloodstream to cause high mortality rates in a murine strain that has been widely published as being highly resistant to pneumococcal pneumonia. Serotype 1 invasion into the olfactory tissues and brain during nasopharyngeal colonisation is also likely to be driven by high rates of autolysis and pneumolysin. I hypothesize that low carriage rates and high incidents of IPD reported with African serotype 1 infection is due to release of pneumolysin in the nasopharynx that facilitates bacterial invasion of local tissues including the olfactory tissues and brain. Higher bacterial loads of serotype 1 compared to serotype 2 in the nasopharynx of BALB/ mice is likely to be attributable to high concentrations of serotype 1 pneumolysin driving bacterial proliferation and therefore increasing rates of tissue invasion. To fully assess the role of pneumolysin, autolysin and bacterial capsule in serotype 1 pathogenesis, isogenic mutants of serotype 1 need to be produced. If a pneumolysin or autolysin-negative mutant was developed and disease pathogenesis during pneumococcal pneumonia in BALB/c mice was compared to wild-type serotype 1, the importance of these virulence factors could be fully ascertained. Unfortunately, there have been many challenges associated with mutagenesis of serotype 1 *S. pneumoniae*. Whilst many pneumococcal research groups can successfully construct pneumolysin/autolysin knockout bacteria in a variety of serotypes, no-one has been able to successfully genetically manipulate serotype 1.

Pneumolysin is known to drive many of the host immune responses to pneumococcal infection including TGFβ release from epithelial cells and IL-1β production via inflammasome activation[100,116,313]. *In vitro* analysis of T cell differentiation in the presence of serotype 1 compared to serotype 2 showed significantly lower T regulatory cell differentiation in the presence of serotype 1. These data suggest that serotype 1 infection leads to differentiation of pro-inflammatory T cell subsets such as Th17 and Th1. Perhaps the difference in the repertoire of T cell subsets is influenced by not only the amount of pneumolysin present but also the haemolytic activity of the toxin. To assess this, direct comparisons between ST217 and ST306 could be performed as they both release similar quantities of pneumolysin upon lysis but ST306 has a non-haemolytic pneumolysin. Several differences in the pathogenesis of these two sequence types in both carriage and invasive disease suggest that the haemolytic activity of pneumolysin plays an important role[259].

When studying epidemiological data for serotype 1, mortality rates from Europe and Africa are often combined, which can skew the data as ST306, which is the dominant sequence type in Europe, causes low mortality compared to African sequence types such as ST3081 and ST217[108,242,245,255,314]. When comparing ST217 and ST306 in murine models of infection, I observed significant differences in disease severity and mortality rates. Therefore, studies concerning serotype 1 should be clearly separated into European and African isolates as not all sequence types of serotype 1 have the same disease pathogenesis.

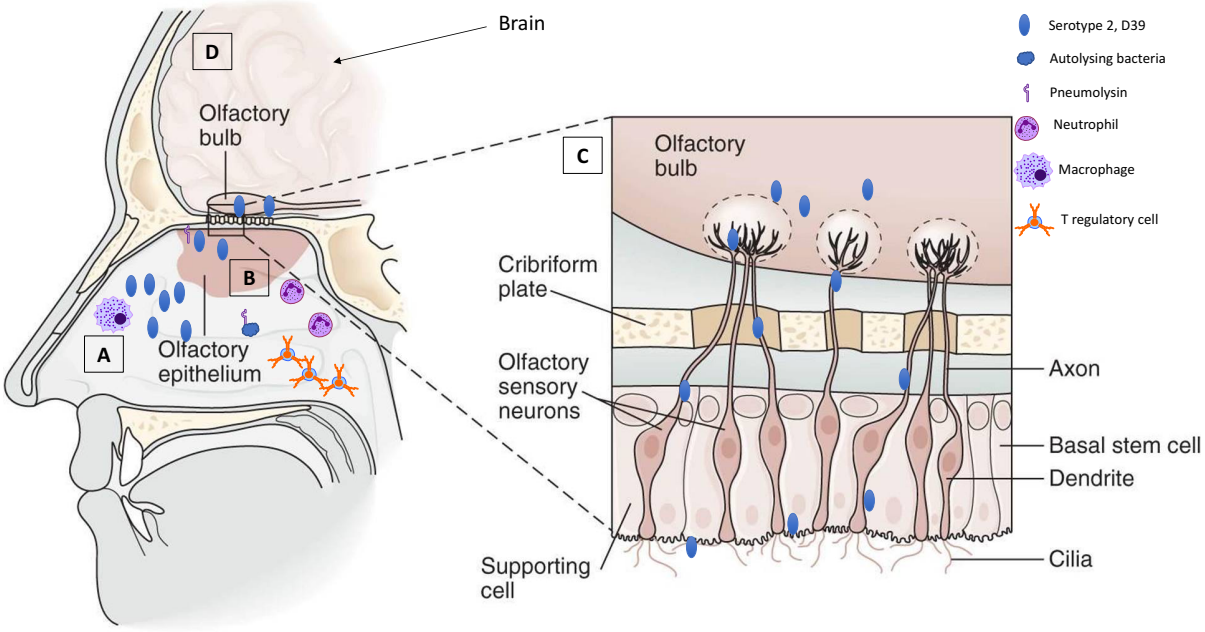
Before this research was performed, very little was known about the influence of serotype 1 capsule and pneumolysin on mucosal invasion and the

associated immune response. As a result, scientists are keenly awaiting epidemiological data, particularly from Africa, on the impact of PCV13 on rates of serotype 1 carriage and invasive disease. As African serotype 1 causes disease in healthy young adults, it is not clear whether a vaccine that removes a serotype 1 reservoir in young children will result in a decrease in disease rates across the adult population. High mortality rates observed in a mouse strain highly resistant to pneumococcal pneumonia, during African serotype 1 infection suggests that this serotype is highly invasive compared to other serotypes and therefore may explain why high disease rates are observed in young, otherwise healthy adults. I have been able to identify two key virulence factors in African serotype 1 disease pathogenesis; pneumolysin and autolysin. Pneumolysin is known to enhance tissue invasion because of damage caused to epithelial cells [284]. In areas such as sub-Saharan Africa, environmental factors such as high temperatures and presence of Harmattan winds are likely to exacerbate this process as high temperatures increase rates of autolysis and dust exposure decreases the ability of macrophages and neutrophils to phagocytose *S. pneumoniae* [246]. Thus, a combination of a serotype producing highly haemolytic pneumolysin and environmental factors which increase the rates of pneumolysin release may explain why serotype 1 is the leading cause of IPD in Africa. As there has been much emphasis placed on the importance of pneumolysin in serotype 1 disease pathogenesis, future therapeutics could be aimed at targeting pneumolysin, whether this may be through future vaccine design or administration of liposomes or anti-pneumolysin antibodies to bind and neutralise pneumolysin, to reduce excessive tissue damage and bacterial dissemination into the blood or brain.

Serotype 1 nasopharyngeal colonisation

Figure 54 summarises differences in serotype 1 nasopharyngeal colonisation compared to serotype 2. Carriage density of serotype 1 is greater than that of serotype 2 in murine models of infection. Studies performed in chapter 5 demonstrate that serotype 1 can successfully colonise the olfactory tissues at significantly higher densities than serotype 2. In addition, serotype 1 is also able to invade the brain during nasopharyngeal carriage. Few differences are seen in the host immune responses between the two serotypes, which suggests that differences in carriage density and invasive potential are primarily driven by the bacteria. Previous data have shown that serotype 1 has significantly faster rates of autolysis which allows the release of pneumolysin. Studies in chapter 4 have shown that pneumolysin produced by serotype 1 has a higher haemolytic activity compared to other serotypes and causes high levels of cytotoxicity in host cells along with disruption of tight junctions between lung epithelial cells. Although cytotoxicity levels have only been performed using lung epithelial cells, we would predict that pneumolysin has similar effects on nasopharyngeal cells. Therefore, it is assumed that high rates of autolysis in serotype 1 bacteria lead to high concentrations of haemolytic pneumolysin released in the nasopharynx. This may cause damage to local epithelial cells, allowing pneumococci to invade the olfactory epithelium and bulb. Excess pneumolysin produced by serotype 1 is then likely to facilitate further translocation into the brain. This may explanation why high rates of pneumococcal meningitis caused by serotype 1 are seen in Africa.

D39 nasopharyngeal colonisation



Serotype 1 nasopharyngeal colonisation

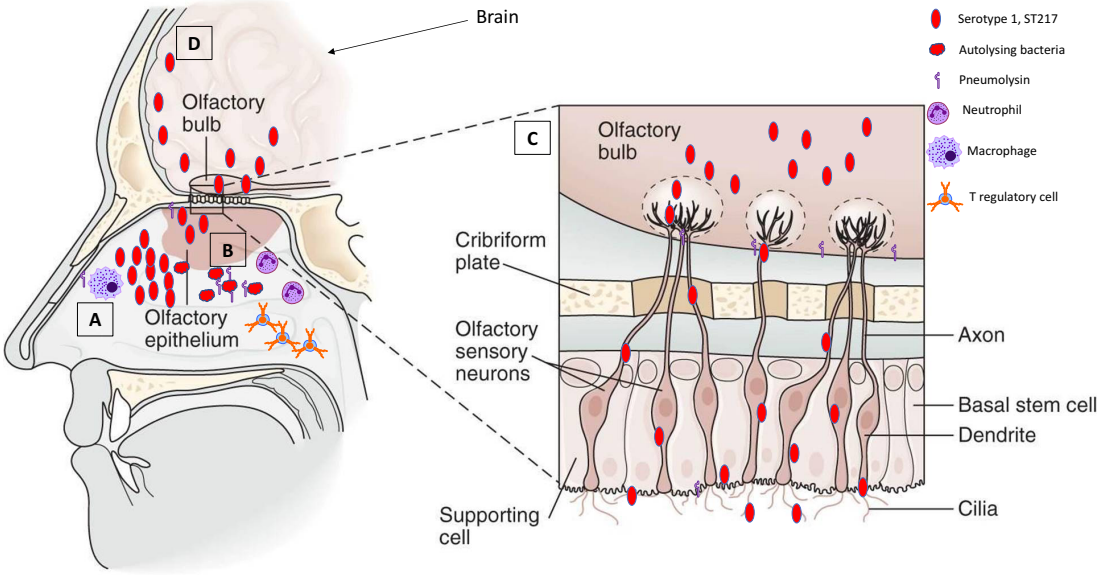


Figure 54. Invasion of the brain by serotype 1 ST217 during nasopharyngeal carriage. The top figure represents nasopharyngeal colonisation with D39 and the lower figure colonisation with serotype 1 (ST217). (A) Shows colonisation of the nasopharynx (NP). Serotype 1 (red) has been shown to colonise the NP at a significantly higher density that D39 (blue). (B) Pneumococcal colonisation can also occur in the olfactory epithelium which contain olfactory neurons. (C)There is evidence that pneumococci can adhere to these neurons and migrate up these neurons to the Olfactory bulb (OB). The OB is in close proximity to the brain (D). D39 can migrate into the olfactory bulb via olfactory neurons but it is only serotype 1 that is able to move from the OB into the brain. Adapted from Faud AS *et al* Harrisons Principles of Internal Medicine, 17th edition.

Comparisons of pneumococcal pneumonia caused by serotype 1 compared to serotype 2

Figure 55 summarises the role of pneumolysin and autolysin in serotype 1 pneumococcal pneumonia. Results from this study demonstrated that serotype 1 has significantly higher rates of autolysis compared to serotype 2. Autolysis enables the release of pneumolysin from the cell. It was found that the haemolytic activity of serotype 1 pneumolysin was significantly higher than other clinical serotypes which resulted in higher levels of cytotoxicity of lung epithelial cells and significant disruption of tight junctions between epithelial cells that form the barrier between the lungs and bloodstream. *In vivo*, this equated to serotype 1 causing bacteraemia and resulting in 100% mortality in murine models of pneumococcal pneumonia compared to serotype 2 which resulted in no detectable bacteria in the blood and 100% survival rates. Figure 55 shows a model whereby serotype 1 in the lung undergo high levels of autolysis, leading to the release of larger quantities of pneumolysin. This pneumolysin causes damage to lung epithelial cells which allows bacteria to translocate into the bloodstream. Once in the blood, serotype 1 causes widespread bacteraemia, leading to high mortality rates. The host immune response during pneumonia was not studied in this work but it is likely that excess pneumolysin in the lungs causes high levels of inflammation which causes further damage to host cells. In the absence of high levels of pneumolysin, as with a serotype 2 infection, high levels of TGF-β are produced which reduces inflammation and prevents excess tissue damage. This allows the bacteria to be contained within the lung and no dissemination into the bloodstream is observed.

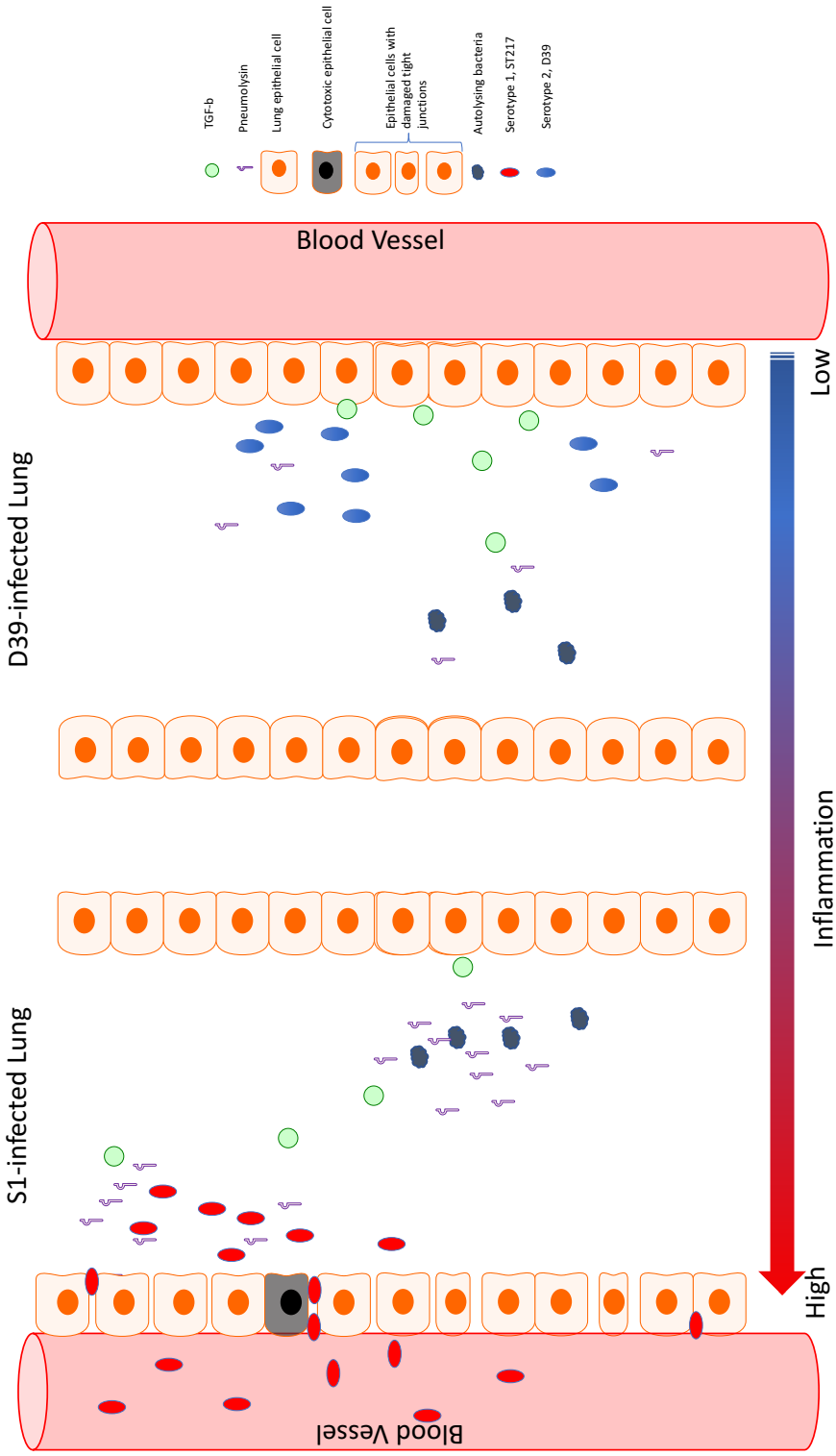


Figure 55. Comparison of pneumococcal pneumonia in serotype 1 compared to serotype 2 infection. In a serotype 1 (ST217/ST3081) infected lung, levels of bacteria lysis are high, resulting in release of high concentrations of pneumolysin. Pneumolysin causing damage to tight junctions between lung epithelial cells and levels of cell cytotoxicity are high. High levels of pneumolysin and local tissue damage equate to high inflammation. Damage to lung epithelial cell barriers allow serotype 1 bacteria is disseminate out of the lung into the bloodstream and cause bacteraemia. In a D39 infected lung, rates of bacterial lysis are significantly lower and therefore less pneumolysin is released. This results in less tissue damage and lower levels of inflammation. As no cytotoxic damage occurs, barriers between lungs and blood remain stable and D39 are contained in the lung and are subsequently cleared by the host immune response.

The aim of this work was to understand the mechanisms of disease pathogenesis of African serotype 1. Using both *in vitro* and *in vivo* models of infection, results have shown that serotype 1 pathogenesis is driven by production of highly haemolytic pneumolysin which is released in vast quantities due to rapid rates of autolysis. Detailed analysis has shown that the number of haemolytic units of pneumolysin released per minute is higher in African serotype 1 isolates compared to other clinically important strains. Using murine models of nasopharyngeal carriage, serotype 1 was shown to successfully colonise the nasopharynx for over 14 days. In comparison to D39, serotype 1 invades olfactory tissues and is able to enter the brain. In murine models of invasive pneumococcal disease, high rates of autolysis and therefore release of haemolytic pneumolysin led to rapid dissemination of serotype 1 from the lungs into the bloodstream, causing 100% mortality compared to 100% survival observed with infection with D39 and clinically relevant serotypes.

These findings have implications in the design of future vaccines and therapeutic targets. Pneumolysin has been shown to be the main virulence factor involved in disease pathogenesis. Targeting pneumolysin may help reduce tissue invasion and bacterial dissemination during nasopharyngeal carriage and in the context of serotype 1 IPD.

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8.0 Appendix

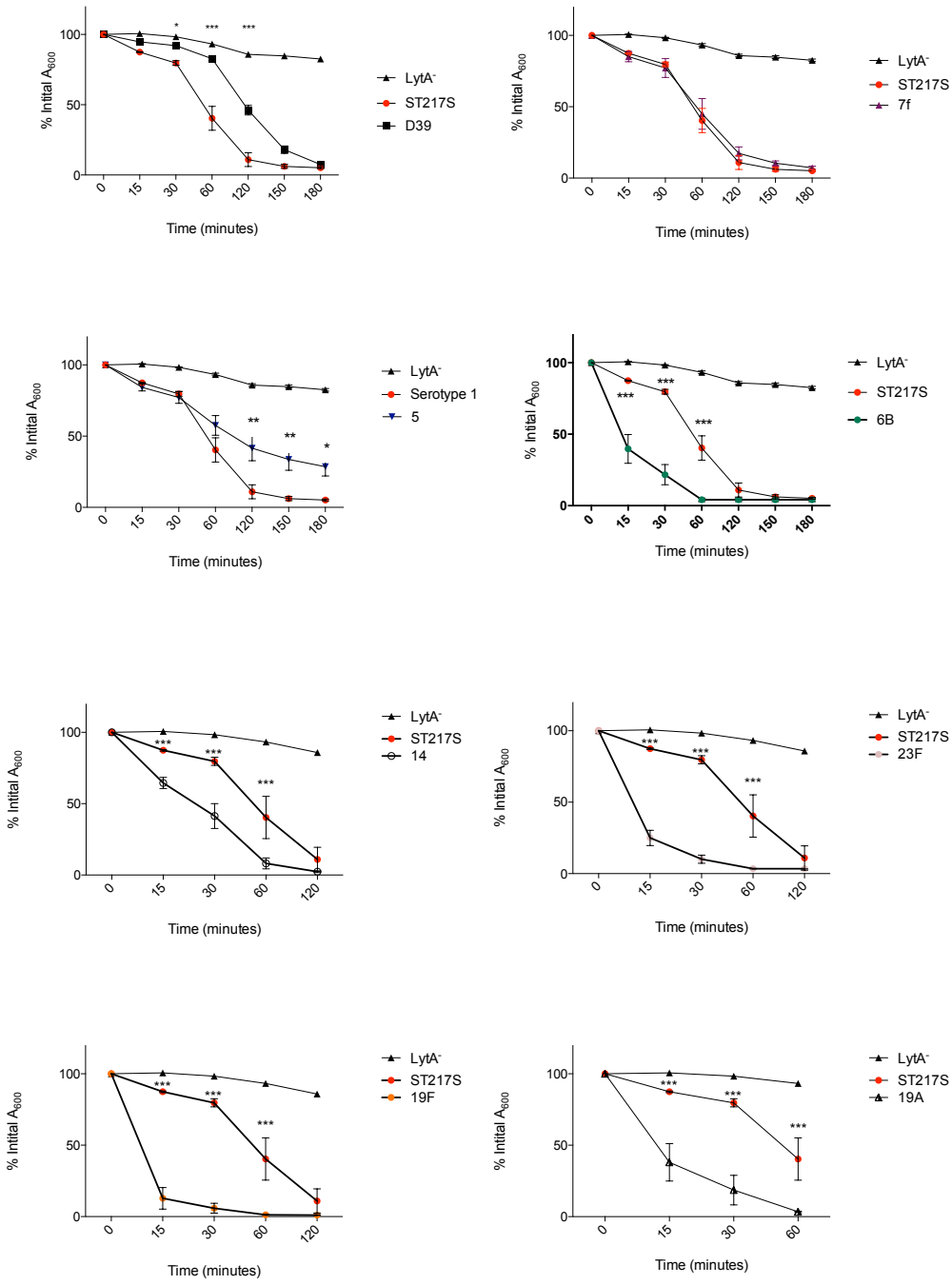

Figure 56.Triton X-100-induced autolysis assays comparing rates of autolysis in serotype 1 compared to other clinical serotypes . Bacteria (OD_{600} 1.0) were incubated at 37^0C and 175rpm with 0.01% Triton X. At 15 - 30 minute intervals, OD_{600} was measured and converted to a percentage of the original OD_{600} reading. LytA negative D39 was used as a control. Statistical analysis was performed by Two-way ANOVA. Rates of autolysis were measured in triplicates; data is presented as mean ± SEM. (ns=non-significant. *P<0.05, **P<0.01, ***P<0.001, P<0.0001).