The human lung immune responses to nasopharyngeal pneumococcal colonisation in healthy adults

Thesis submitted in accordance with the requirements of the Liverpool School of Tropical Medicine for the degree of Doctor in Philosophy

by

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Declaration

I, Elena Mitsi, declare that this thesis is the result of my own work and effort. In some instances, work was done in collaboration with colleagues and external collaborators. Table A details in full the attribution of work and responsibilities related to the project. The research of this thesis was carried out at the Liverpool School of Tropical Medicine. The contents of this thesis have not been presented, nor are currently being presented, wholly or in part, for any other degree or qualification.

Table A: List of collaborators and contributors to the work presented in this thesis

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Statistical support	Simon Jochems

Abstract

The human lung immune responses to nasopharyngeal pneumococcal colonisation in healthy adults.

Pneumococcal pneumonia burden remains high globally, affecting the youngest, the oldest and immunocompromised individuals worldwide. Colonisation of the human nasopharynx with *S. pneumoniae* is a pre-requisite for the development of pneumococcal disease and the primary reservoir for transmission. Paradoxically, it is also the main source of naturally acquired immunity. Currently, there is limited knowledge on how nasopharyngeal pneumococcal colonisation impacts on the lung immune responses in humans. Experimental human pneumococcal colonisation offers a safe way to study the dynamics between a known start-point colonisation episode and the lung immune responses.

The data presented in this thesis show that colonisation of the nasopharynx with *S. pneumoniae* is an immunising event for the human lung, affecting both the innate and adaptive arm of immunity. Alveolar macrophage activation and function were altered post colonisation, leading to increased opsonophagocytic capacity that persisted for up to three months post the challenge. The overall skewed $CD4^+$ Th-1 responses observed in the lung indicate that $CD4^+$ T cells may prime alveolar macrophage through IFN-γ secretion. *In vitro* stimulation of lung lymphocytes with pneumococcus suggest that TCR-γδ T cells can stand as an additional source of IFN-γ *in vivo*. As described before, pneumococcal colonisation seeded the human lung with cognate, IL-17 secreting, $CD4^+$ T cells- the only observed memory population amongst lung T cells. Antibody levels against the capsule of the challenge strain were also elevated post colonisation. Pneumococcal cells found in the lung days to weeks after clearance of nasal colonisation suggest that they may be the stimulus for the observed enhanced lung immunity. Collectively, the data presented here support the use of respiratory tract as route for effective pneumococcal vaccination. On the other hand, they emphasize that heavily colonised individuals with defective alveolar macrophage function or elderly due to lack of colonisation are at increased risk to develop pneumonia.

Publications, Presentations and Prizes

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Zaidi, S. R., A. M. Collins, **E. Mitsi**, J. Reine, K. Davies, A. D. Wright, J. Owugha, R. Fitzgerald, A. Ganguli, S. B. Gordon, D. M. Ferreira & J. Rylance (2017) Single use and conventional bronchoscopes for Broncho alveolar lavage (BAL) in research: a comparative study (NCT 02515591). *BMC Pulm Med,* 17**,** 83.

Oral presentations

2018 – Plenary session, Annual Scientific meeting of Liverpool Glasgow Malawi Wellcome Trust (Chester, UK)
Title: Cross-talk of alveolar macrophage and T cells boost the lung immunity post nasopharyngeal pneumococcal colonisation

2018- Plenary Session "The lung" at the 11th International Symposium on Pneumococci and Pneumococcal Diseases, ISPPD-11 (Melbourne, Australia)
Title: Cross-talk of alveolar macrophage and T cells boost the lung immunity post nasopharyngeal pneumococcal colonisation

2016 – Talk at the 10th International Symposium on Pneumococci and Pneumococcal Diseases, ISPPD-10 (Glasgow, UK).
Title: Human Pneumococcal Carriage leads to enhanced alveolar macrophage phagocytosis capacity.

Poster presentations

2017 –Oral poster at the 8th European Meeting on the Molecular Biology of the Pneumococcus (Europneumo) (Stockholm, Sweden)
Title: Human nasal pneumococcal carriage leads to increased innate and humoral immunity in the lungs

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Abbreviations

AM	Alveolar macrophage
APC	Antigen-presenting cell
B cell	B lymphocyte
BAL	Bronchoalveolar lavage
BCR	B cell receptor
Bmem	Memory B cell
CAP	Community acquired pneumonia
CFU	Colony forming unit
ChoP	Phosphorylcholine
COPD	Chronic obstructive pulmonary disorder
CPS	Capsular polysaccharide
CyTOF	cytometry by time-of-flight
EHPC	Experimental human pneumococcal challenge
FBS	Fetal bovine serum
GM-CSF	Granulocyte macrophage- colony stimulation factor
HI	Heat-inactivated
HIV	Human immunodeficiency virus
iBALT	inducible bronchoalveolar lymphoid tissue
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPD	Invasive pneumococcal disease
IQR	Interquartile range
IVIG	Intravenous immunoglobulin
LPS	Lipopolysaccharide
MAIT cell	Mucosal associated invariant T cell
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
NET	Neutrophil extracellular trap
NK cell	Natural killer cell
OPA	Opsonophagocytic assay
PAFR	Platelet-activating factor receptor

PAMP	Pathogen-associated molecular patterns
PcpA	Pneumococcal choline-binding protein A
PCV	Pneumococcal conjugate vaccine
Ply	Pneumolysin
PPV	Polysaccharide pneumococcal vaccines
PRR	Pattern recognition receptor
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
Spn	Pneumococcus
TCR	T cell receptor
TGF	Transforming growth factor
Th cell	T helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
TRM cell	Tissue-resident memory cell
URT	Upper respiratory tract
WHO	World Health Organisation

Chapter 1

General introduction

1.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae (also known as pneumococcus) is a lancet-shaped, gram-positive, opportunistic bacterial pathogen, commonly found in the human nasopharynx (Figure 1.1A). It has a thick, multi-layered cell wall made mainly of peptidoglycan and teichoic acids and is surrounded by a polysaccharide capsule (CPS), which is composed of repeating units of simple sugars (monosaccharides) (Figure 1.1B) (Brown, Hammerschmidt and Orihuela 2015). CPS is the major virulence factor and immunodominant surface structure of *S. pneumoniae*. Pneumococcal strains sharing a unique capsular structure are grouped into serotypes and currently there are more than 95 recognised pneumococcal serotypes, with the majority of them described as immunologically and structurally distinct from each other (Weiser, Ferreira and Paton 2018). Some serotypes are antigenically similar, forming serogroups, which are denoted by a number, and each serotype within the group denoted by a letter (Geno et al. 2015, Rodrigo and Lim 2014).

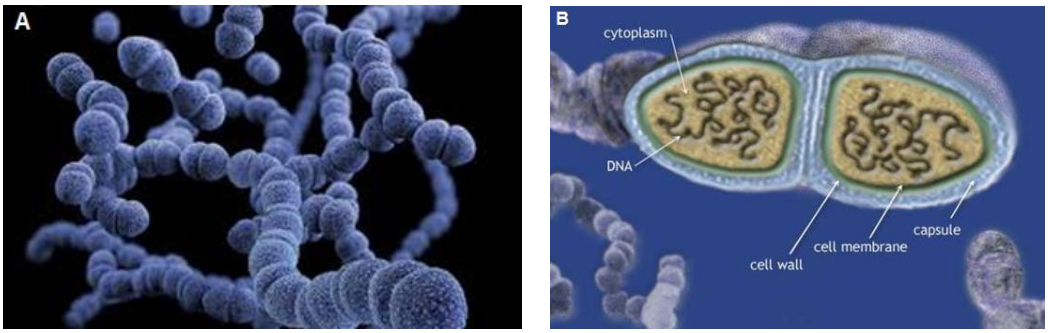


Figure 1.1. Structure of *Streptococcus pneumoniae*. A) Image illustrates *S. pneumoniae* chain formation (source: Centres of Disease Control and Prevention Public Health Image Library). B) Simple presentation of the main compartments of *S. pneumoniae* diplococcus (Image source: online microbiology notes website).

Pneumococcus is a highly adapted commensal with a relatively simple life cycle. Colonisation, transmission and occasionally invasion to sterile human sites are the three distinct stages observed during his life cycle within its human host (Figure 1.2).

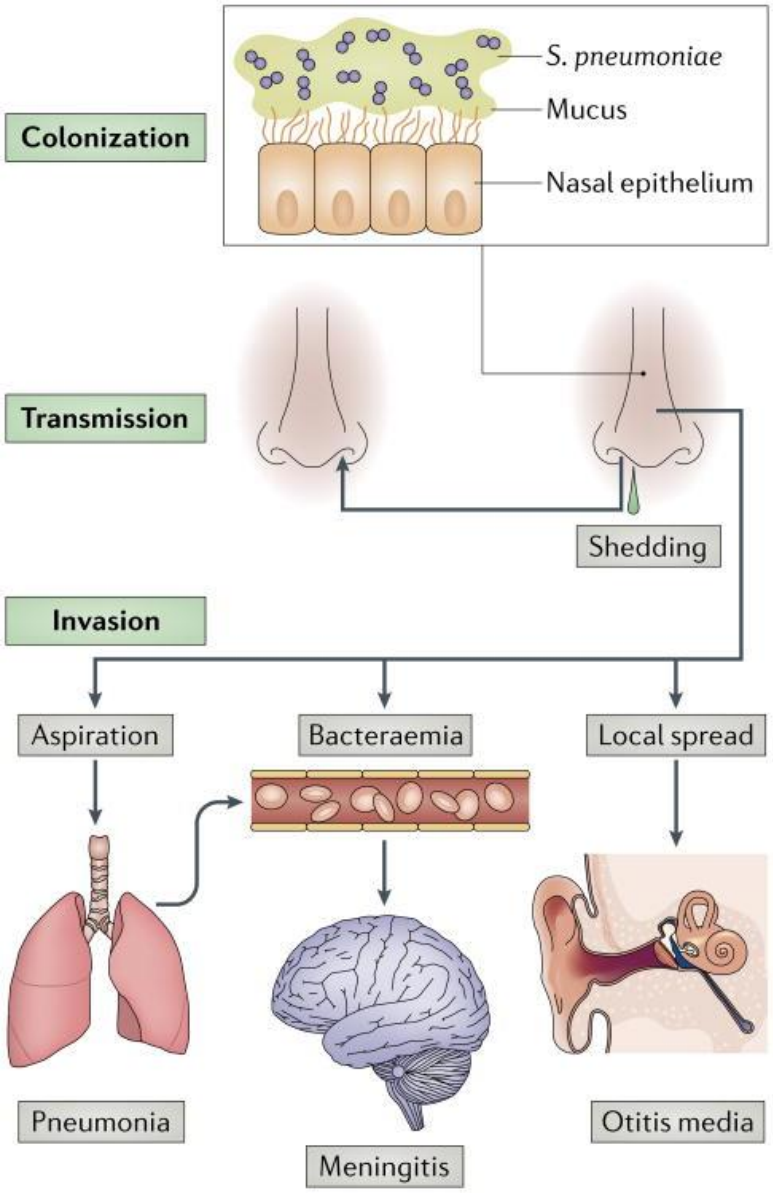


Figure 1.2. The life cycle of *S. pneumoniae* and the pathogenesis of pneumococcal disease. *S. pneumoniae* colonises the mucosal surfaces of the upper respiratory tract (URT), a state known as pneumococcal carriage. Pneumococcal carriers can shed bacteria in nasal secretions and thereby transmit the bacterium. Dissemination beyond its niche along the naso/oro-pharyngeal epithelium, either by aspiration, translocation through the epithelium or local spread, can lead to invasive diseases, such as pneumonia, meningitis and otitis media. Image source: Nature microbiology review (Weiser et al. 2018).

1.2 Nasopharyngeal pneumococcal colonisation

Pneumococcus has evolved to colonise the mucosal surfaces of the human upper respiratory tract (Kadioglu et al. 2008). This state is known as pneumococcal colonisation or carriage. As the majority of colonised adults will not develop clinical symptoms, pneumococcal colonisation is considered an asymptomatic state of pneumococcal infection (Bogaert, De Groot and Hermans 2004). In children, although, high densities of colonisation, especially in the presence of viral infection, have been associated with rhinitis symptoms (Rodrigues et al. 2013). Prevalence of colonisation varies considerably between countries, while within a country it appears to persist at approximately a constant level (Numminen et al. 2015). Nasopharyngeal pneumococcal colonisation is also the main source of pneumococcal transmission between individuals. Commonly colonisation precedes invasion and, therefore, it is also considered a pre-requisite for the development of pneumococcal disease (Simell et al. 2012).

Data from longitudinal studies have demonstrated that carriage is a dynamic process (Murad et al. 2019). The timing of acquisition and clearance is normally influenced by factors including serotype, age, and previous pneumococcal exposure (Turner et al. 2012) (Hill et al. 2008).

1.2.1. Epidemiology of pneumococcal colonisation

Rates of nasopharyngeal pneumococcal colonisation are age-dependent. In young children, colonisation rates are particularly high, ranging from 20-50% in developed countries, (McCullers 2006), whereas its prevalence is significantly higher in low-middle countries. In Gambia, over 90% of healthy children under 5 years of age carry pneumococcus (Adegbola et al. 2014). Colonisation rates decline with increasing age (Waroux et al., 2014) and stabilise at 1-10% in adults. However, adults with small children at home may have a higher colonisation rate (Hussain et al. 2005). Unlike in children, carriage in the elderly is considered a rare event. Controversially, a study conducted in a Dutch elderly cohort with influenza-like-illness detected an ample reservoir of pneumococci, including serotypes 6B and 3, in the saliva of the seniors (Krone et al. 2015), implying change in the niche of colonisation with advanced age. However, this finding has not yet been replicated elsewhere. Appropriate qPCR assays are crucial when saliva sample is used in pneumococcal detection, as the human oral cavity contains

pneumococcus-like viridans which otherwise can be misidentified as *S. pneumoniae* (Carvalho Mda et al. 2007).

In infancy, the peak of pneumococcal colonisation, over 80% of children acquire pneumococcus within the first month of their life (Hill et al. 2008). Also, young children experience multiple carriage episodes in the length of a year. A study in Thailand reported a median of seven pneumococcal acquisitions during the first 24 months of life (Turner et al. 2012). In addition, simultaneous colonisation with up to four pneumococcal serotypes has been observed in young children, which is described as a common phenomenon in low- and middle-income countries (Satzke et al. 2015).

Not only carriage rates decrease with age, but so also does the density and duration of the colonisation episode. This is most likely due to the progressive acquisition of immunity as a result of prior pneumococcal colonisation episodes. However, large variations in the duration of carriage have been observed in different cohorts. Longitudinal studies conducted in rural Gambia villages have demonstrated that increased age was associated with shorter colonisation episodes (Hill et al. 2010), as well as decrease in density (Roca et al. 2012). In a study done in Sweden, the mean carriage duration of penicillin-resistant pneumococci in children under five was 43 days as compared to 25 days in those over the age of five (Hogberg et al. 2007). The increased frequency of influenza A and B infection in younger than older children and adults is an additional driver of the inverse correlation between age and pneumococcal density (Peltola, Ziegler and Ruuskanen 2003).

Pneumococcal serogroup is also an important parameter of the carriage duration and density. Serotypes with thicker capsules tend to have longer durations of nasopharyngeal carriage in young children compared to those with thinner capsules (Cobey and Lipsitch 2012). In terms of density, a study conducted in healthy pre-school children in day-care in Portugal used molecular methods of serotyping and found differences in pneumococcal density amongst different serotypes, such as 3 and 35, describing an hierarchy of distinct pneumococcal serotype carriage densities (Rodrigues et al. 2016).

Overall, the fall of carriage rates with age in a serotype-independent manner, as well as the declined pneumococcal density in the colonised adults, suggests that

frequent exposure to circulating strains during childhood builds up naturally-acquired immunity to pneumococcus. Epidemiological and modelling data have demonstrated that the immunising effect of carriage is likely mediated by a combination of serotype-dependent and serotype-independent mechanisms (Weinberger et al. 2008, Granat et al. 2009, Cobey and Lipsitch 2012). On the other hand, population-based studies have shown that natural anti-pneumococcal capsule IgG and IgM levels fall in the elderly populations and antibody function may also decline (Simell et al. 2008, Goldblatt et al. 2009). These observations do not explain the low colonisation-high disease rates paradox commonly seen in this age group (Figure 1.3) but add value to the role of these antibody types to protection against pneumococcal disease.

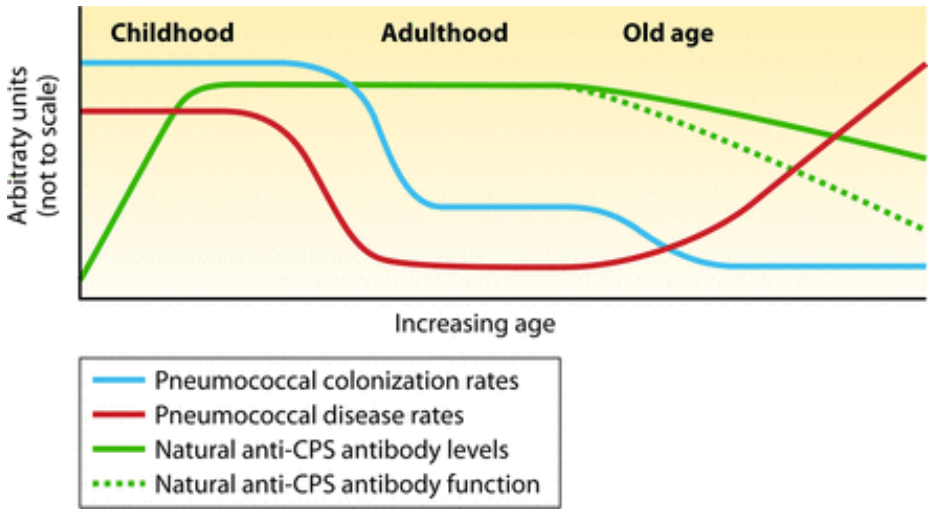


Figure 1.3. Schematic of pneumococcal colonisation, disease rates and antibody levels in different age groups. Pneumococcal colonisation and disease rates are high in young children, fall dramatically during adulthood, whereas in elderly colonisation is infrequent, and rates of pneumococcal disease increase. Naturally acquired pneumococcal anti-CPS antibody levels rise with recurrent exposure. Young adults have high levels of naturally acquired antibodies, occasional episodes of colonisation, and low rates of disease. In the elderly, antibody levels and functional activity decline (Adler et al. 2017).

1.2.2 Mechanisms of pneumococcal colonisation

Pneumococcal colonisation is a constant interplay between various pathogen- and host-specific factors. Many of these factors have dual functions in disease pathogenesis, as well as in pneumococcal carriage and transmission into new hosts.

1.2.2.1 The role of capsule on colonisation

The polysaccharide capsule attached to the bacterial surface is the main pneumococcal virulence factor, but its presence is also important during the colonisation phase of the bacterium. In murine models, unencapsulated mutants are capable to colonise the nasopharynx but at reduced density and duration compared to the encapsulated parent strains (Cohen et al. 2012, Hyams et al. 2010, Nelson et al. 2007). In humans, pneumococcal isolates lacking capsule (unencapsulated pneumococcal strains) are occasionally found as nasopharyngeal coloniser, but they do not cause invasive pneumococcal disease as septicaemia. However, one study demonstrated that strains of a distinct cluster of unencapsulated *S. pneumoniae* are capable to cause conjunctivitis in humans (Valentino et al. 2014).

The majority of CPSs serotypes (all but four) are negatively charged. In early colonisation, the pneumococcal capsule is suggested to have a physiochemical effector mechanism by limiting mucus-mediated clearance of pneumococci (Nelson et al. 2007). Later on, to neutralise the inhibitory effect of negatively charged capsule on adhesive interaction with epithelial host cells, the bacteria are able to bind positively charged host molecules (Fab antibody fragments) on their surface and thereby enhance pneumococcal adherence (Weiser et al. 2003).

While pneumococcus requires the capsule in order to evade clearance by the host immune mechanisms, excessive capsule expression has been shown to inhibit adherence to the epithelium (Talbot, Paton and Paton 1996). To achieve balance, the pneumococcus spontaneously switches capsule expression between two phenotypes. This high-frequency phenotypic variation (also known as phase variation) results in different display of various bacterial features and be recognised by colony morphology between two phenotypes: opaque and transparent (Simell et al. 2012, Weiser et al. 1994). Opaque colonies have a greater amount of capsule than the transparent form. Using animal models, it has been shown that transparent pneumococcal colonies promote an efficient and more stable nasopharyngeal colonisation (Weiser et al. 1994),

whereas the opaque variants with thicker capsule and resistance to opsonophagocytic killing survive better in the bloodstream (Kim and Weiser 1998).

During disease pathogenesis, the capsule confers resistance to complement mediated opsonophagocytosis (ingestion of bacteria by the host phagocytic cells) (Bruyn, Zegers and van Furth 1992, Watson, Musher and Verhoef 1995). It forms a physical barrier that limits access of antibodies and complement to the pneumococcal surface (Brown et al. 1982). By reducing binding of C-reactive protein, the capsule further inhibits complement activation of the classical complement pathway.

1.2.2.2 Adherence of *S. pneumoniae* to the upper respiratory tract epithelium

The glycocalyx overlying the upper respiratory tract epithelium is composed of gel-like mucin glycoproteins and contains antimicrobial peptides and immunoglobulins (Rose and Voynow 2006). Although, this mucoid layer forms the first host defence against acquisition of colonisation, it also provides an environment rich of nutrients for *S. pneumoniae*.

After *S. pneumoniae* has achieved transit from the lumenal mucus to the epithelial surface (Nelson et al. 2007), it seeks access and adherence to the epithelium (Figure 1.4). Until very recently, our understanding of *S. pneumoniae*–host cell interactions came primarily from models that use cultured human epithelial cells. The latest findings on pneumococcus-epithelium interaction come from an *in vivo* model of experimental human pneumococcal colonisation (EHPC) (Gritzfeld et al. 2013), whereby human mucosal samples were obtained by minimally-invasive curette microbiopsy (Jochems et al. 2017). This human study revealed that *S. pneumoniae* colonisation is associated with epithelial surface adherence, micro-colony formation and migration across the epithelial barrier (micro-invasion), without overt disease. The micro-invasion phase triggered inflammatory and innate immune responses, which were associated with clearance (Figure 1.5) (Weight et al. 2019).

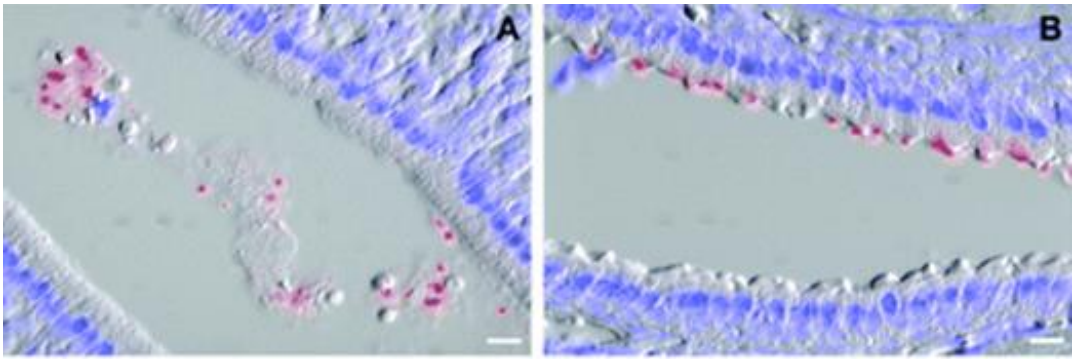


Figure 1.4: Phases of early colonisation. Pneumococcal cells A) entrapped in mucus and B) adhered to the nasal epithelium. Pneumococci stained with red dye and epithelial cells with blue. Image source: (Nelson et al. 2007).

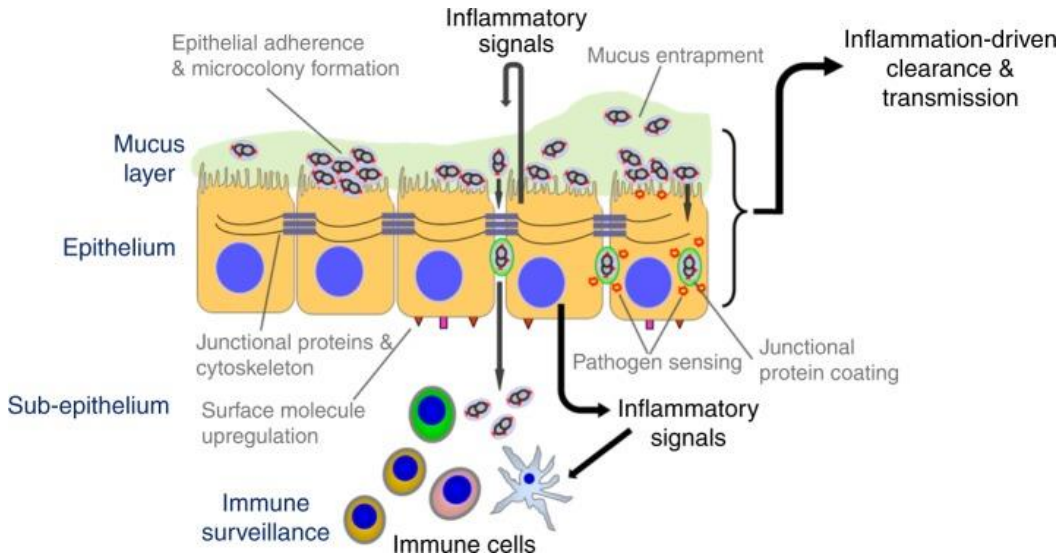


Figure 1.5: Model of pneumococcal colonisation at the human mucosal epithelium. Pneumococcal adhesion and micro-colony formation on the epithelial surface may lead to micro-invasion; internalisation of the bacteria and/or transmigration across the epithelial barrier (micro-invasion). Image source: (Weight et al. 2019).

1.2.2.3 Pneumococcal surface proteins and enzymes with involvement to cell adherence

Once pneumococcus has accessed the epithelial surface it secretes enzymes and utilises surface proteins to bind to host cell-surface carbohydrates and initiate colonisation.

Phosphorylcholine (ChoP) moieties, component of the cell wall teichoic acid, bind to the platelet-activating factor receptor (PAFR) on epithelial cells and serve as an anchor for a number of choline-binding proteins (Cundell et al. 1995). One of these adhesins, the choline-binding protein A (CbpA; also known as PspC) binds the secretory component of the polymeric immunoglobulin receptor (pIgR), mediating adherence to the mucosal epithelium (Zhang et al. 2000). CbpA also binds the host proteins factor H, regulator of the alternative complement pathway, enabling the pneumococci to evade the host immune response and aiding adherence to epithelial cells (Hammerschmidt et al. 2007). Another choline-binding protein, the pneumococcal choline-binding protein A (PcpA), is also able to facilitate pneumococcal adherence to nasopharyngeal and lung epithelial cells (Khan et al. 2012). Also, if it is present, pilus- a multimeric filamentous surface structures attached to the cell wall- promotes pneumococcal adherence to the epithelium (Bagnoli et al. 2008).

Zinc metalloprotease ZmpA (also known as IgA1 protease) expressed by *S. pneumoniae* is a protease that cleaves the hinge region of human IgA1. The remaining bound Fab fragments on the surface of pneumococcus have been associated with increased adherence-promoting properties, as they may neutralise the inhibitory effect of negatively charged capsules on adhesive interaction with host cells. This coating of pneumococcus with anticapsular polysaccharide antibody has been shown to unmask the bacterial ChoP ligand, allowing for increased adherence mediated by binding to the PAFr on epithelial cells (Weiser et al. 2003).

S. pneumoniae also encodes extracellular glycosidases, some of which have been reported to enhance adherence by modifying host glyco-conjugates to reveal glycan receptors (King 2010). One of them, the pneumococcal neuraminidase (NanA) cleaves N-acetylneuraminic acid from mucin, decreasing the viscosity of the mucus and exposing host epithelial cell surface receptors for pneumococcus. This action facilitates pneumococcal adherence to the epithelium and increases colonisation (Tong et al. 2000,

Manco et al. 2006). Neuraminidase enzymatic action has been related with the synergy between pneumococcus and influenza viruses in causing illness. In animal models, neuraminidase was found to contribute both to epithelial adherence and biofilm viability and its effect on the latter was independent of its sialidase activity (Wren et al. 2017).

The role of these pneumococcal factors in enabling and promoting colonisation is summarised in table 1.1.

Table 1.1. Pneumococcal factors with described role on colonisation.

Pneumococcal factor	Function
Polysaccharide capsule	Negatively charge capsule limit clearance of pneumococci mediated by mucus during early colonisation (Nelson et al. 2007) Protection against opsonophagocytic killing (Melin et al. 2010)
Phosphorylcholine (ChoP)	Binds to PAFr on epithelial cells, increasing adhesion (Cundell et al. 1995)
Pneumococcal choline-binding protein A (PcpA),	Contributes to pneumococcal adherence to nasopharyngeal and lung epithelial cells (Khan et al. 2012).
Pneumococcal surface protein C (PspC)	Binds pIgR and mediates adherence to the mucosal epithelium (Zhang et al. 2000)
IgA1 protease	Cleaves human IgA1; encapsulated pneumococci coated with the Fab fragments have been associated with increased ability to adhere to the epithelium (Weiser et al. 2003).
Neuraminidase A	Cleaves terminal sialic acids from host glycopeptides and reveals adhesion receptors on the epithelium (Tong et al. 2000, Manco et al. 2006)
Pilus	Promotes epithelial adherence (Bagnoli et al. 2008)

1.2.2.4 *S. pneumoniae* interactions with the nasopharyngeal flora

The nasopharyngeal microbiome is a dynamic ecosystem, complex and extensive. It is established in the first year after birth and varies throughout a person's lifetime (Shak, Vidal and Klugman 2013). Recent studies have shown that early in life certain URT microbiome profiles (Biesbroek et al. 2014) are associated with an increased risk and frequency of subsequent respiratory infections and disease severity (de Steenhuijsen Piters et al. 2016). Early interactions between infectious agents such as viruses and the respiratory microbiome have shown to modulate host immune responses potentially affecting the course of the disease and future respiratory health (Unger and Bogaert 2017). Recent findings on nasal microbiome derived from the EHPC model suggest that not only establishment of colonisation, as initially was thought, but even exposure to *S. pneumoniae* can confer large changes to the microbiota profile directly following pneumococcal exposure (de Steenhuijsen Piters et al. 2019).

During colonisation, *S. pneumoniae* interact with the nasopharyngeal microbial community, developing both synergistic and antagonistic relationships. Some of these interactions have already been described and mainly focus on the relationship between pneumococcus and other respiratory pathogens. An antagonistic relationship has been identified between *S. pneumoniae* and *S. aureus* that may, in part, be explained by the production of pneumococcal hydrogen peroxidase (H_2O_2), which results in lethal bacteriophage induction in *S. aureus* (Regev-Yochay et al. 2006, Selva et al. 2009). On the other hand, pneumococcal colonisation in the nasopharynx has also been associated with carriage of two other major nasopharynx-based pathogens, *H. influenzae* and *Moraxella catarrhalis*. Although there are exceptions, most studies find a positive association between the presence of *S. pneumoniae* and *H. influenzae* and between *S. pneumoniae* and *M. catarrhalis* in the human nasopharynx (Mackenzie et al. 2010, Pettigrew et al. 2008). The epidemiologic association between *S. pneumoniae* and *H. influenzae* was recently extended by the observation that their carriage densities are also positively correlated (Chien et al. 2013). Interestingly, in many murine and *in vitro studies*, *S. pneumoniae* and *H. influenzae* seem to inhibit each other, dependent on the order in which they are being given (Lijek and Weiser 2012).

Many respiratory viruses predispose individuals to bacterial infection through the disruption of the airway-epithelial barrier, which facilitates the adhesion of bacterial

pathogens (Avadhanula et al. 2006). Primary infection with influenza virus enhances pneumococcal colonisation by liberating host-derived nutrients (Siegel, Roche and Weiser 2014) and by decreasing mucociliary clearance (Pittet et al. 2010, Short et al. 2012). Recent data from an EHPC clinical trial showed that transient infection with live attenuated influenza vaccine (LAIV) strains impaired neutrophils activity and monocytes infiltration in the human nasopharynx (Jochems et al. 2018), leading to a transient increase in pneumococcal density (Rylance et al. 2019). In line with this observation, clinical trials in children have shown that LAIV increases the number of colonising *S. pneumoniae*, which would facilitate transmission (Thors et al. 2016).

1.3 Pneumococcal transmission

A human nasopharynx colonised by pneumococci acts as the reservoir and source of pneumococcal transmission from individual to individual. Pneumococcal transmission is very common between and from young children, as their nasopharynx is readily colonised with high densities and their social behaviour involves intimate contact with others. Also, rhinorrhoea, frequently caused by respiratory viruses, is common in preschool children. Clinical surveys in day-care attending children demonstrated a relationship between secretion volume and pneumococcal colonisation density (Rodrigues et al. 2013). Climatic conditions seem to affect pneumococcal transmission, as higher transmissibility was found during the cooler and drier months, when airway secretions are more copious and is more likely to occur in conjunction with viral infections of the upper respiratory tract (Numminen et al. 2015, Musher 2003).

Apart from day-care centres, pneumococcal colonisation and contagion have been reported to occur under the same conditions in military camps, shelters for the homeless, prisons, and nursing homes (Musher 2003). Airborne transmission was described for the first time in 2010 in a ferret model of co-infection with influenza A virus. The infected ferrets were able to transmit the bacteria to other ferrets in the same cage or up to 1m away (McCullers et al. 2010). Prior to this observation, a study of prevalence in adults had showed that carriage could be detected in adults twice as often when they had an upper respiratory tract infection, implying that acquisition of pneumococci was favoured during viral co-infections (Regev-Yochay et al. 2004). Under the same lines, a EHPC study of a young adult cohort demonstrated that asymptomatic

viral URT viral infection is associated with higher colonisation rate post the bacterial challenge (Glennie et al. 2015), which may have implication in increased change of bacterial shedding and transmission.

Preliminary data from the experimental human pneumococcal challenge model shows that healthy adults, who were not in contact with children, shed bacteria through the nasal or oral route and, therefore may acts as pneumococcal reservoir and source for transmission to others (Mitsi et al. 2019). Recently, using a pneumococcal model of exposure, research within the EHPC group demonstrated that hands contaminated with pneumococci can serve as vehicles for transmission, leading to acquisition of nasopharyngeal colonisation (Connor et al. 2018).

Aside from social behaviour, climatic conditions and co-infection with respiratory virus, there are also bacterial factors that can promote shedding. The role of capsule type and amount of shedding and transmission was tested in a murine model using isogenic serotype- switch and cps- promoter switch mutants, showing reduction in shedding and transmission compared to wild type, when the capsule was switched. Mutants with lower expression of CPS and thinner capsules were shed and transmitted poorly (Zafar et al. 2017a). Encapsulation may facilitate shedding by allowing escape from the mucus that lines the airway surface, with a thicker capsule or capsule of certain serotypes being more effective (Weiser et al. 2018).

Pneumolysin (ply) is a major pneumococcal virulence factor. It is the single pneumococcal cytotoxin, which triggers strong pro-inflammatory host responses. Its inflammatory property was tested in a mice model in association with bacterial shedding and transmission. Ply-knockout mutant caused moderated URT inflammation and had reduced ability to transit to littermates (Zafar et al. 2017b). These findings with Ply provide a link between pneumococcal virulence and transmission, suggesting that factors such as Ply that contribute to the disease state by enhancing inflammation can also promote the transmission of *S. pneumoniae* (Weiser et al. 2018).

1.4 Pneumococcal disease and its spectrum

The "asymptomatic" phase of pneumococcal colonisation is widely considered as the essential step that enables transmission to a new host and precedes development of pneumococcal disease. As noted above, induction of pro- inflammatory chemokines and cytokines, upregulation of target receptors and damage to the respiratory epithelium caused by viral infection of the URT increase bacterial loads in the nasopharynx. This facilitates bacterial transmission but also increases the likelihood of penetration of host tissues and progression to localised (non-invasive) or invasive disease (Figure 1.6).

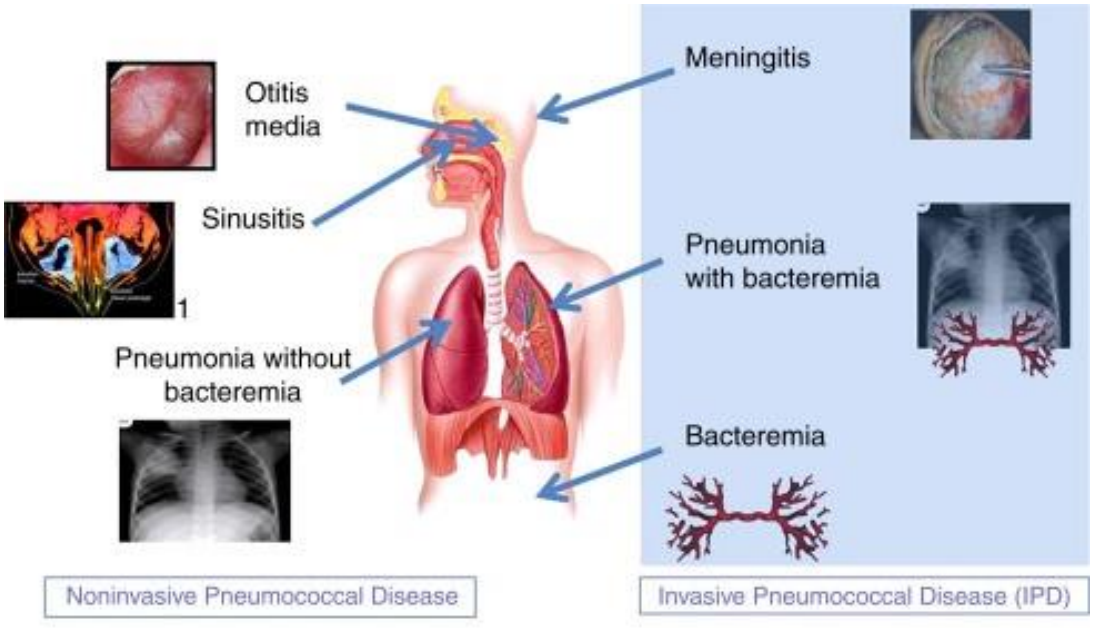


Figure 1.6. Spectrum and pathway of disease caused by *S. pneumoniae.* Translocation of *S. pneumoniae* to local tissues (middle ear, nasal sinuses, lungs) causes non-invasive pneumococcal diseases. Tissue penetration and access to the bloodstream or meninges leads to invasive disease (Image source: WedMD 2014).

Pneumococcal spread to local tissues causes simple mucosal infections, such as otitis media of the middle ear or sinusitis. *S. pneumoniae* can also be aspirated into the distal pulmonary alveoli. Without effective clearance by the lung mucosal immune mechanisms, this results in non-bacteraemic pneumonia, whereby infection is confined to the lung mucosa. However, if bacteria spread to normally sterile sites around the alveoli, including the bloodstream, the infection progresses to invasive pneumococcal

disease, such as bacteraemic pneumonia and empyema (Jover et al. 2008, Bogaert et al. 2004).

Invasive pneumococcal disease (IPD), including meningitis, can occur independent of lung mucosal involvement. It requires breaching of epithelial and/or endothelial barriers and penetration of tissues, ultimately providing access to the bloodstream. In the case of meningitis, this involves breaching the blood–brain barrier. Invasion mainly involves interaction between ChoP moieties and PAFR on the surface of cytokine-activated respiratory epithelial and vascular endothelial cells, followed by hijacking of the PAFR recycling pathway to gain entry (Cundell et al. 1995). There are also marked differences in the capacity of specific *S. pneumoniae* strains to cause invasive disease, which highlights the vast genetic and phenotypic heterogeneity of this bacterium. Also, the range of pneumococcal disease differs in severity and frequency (Figure 1.7). The severity of disease caused by *S. pneumoniae*, is inversely related to incidence (Jambo et al. 2010) and pneumonia dominates the burden of disease caused by this bacterial pathogen (Wahl et al. 2018).

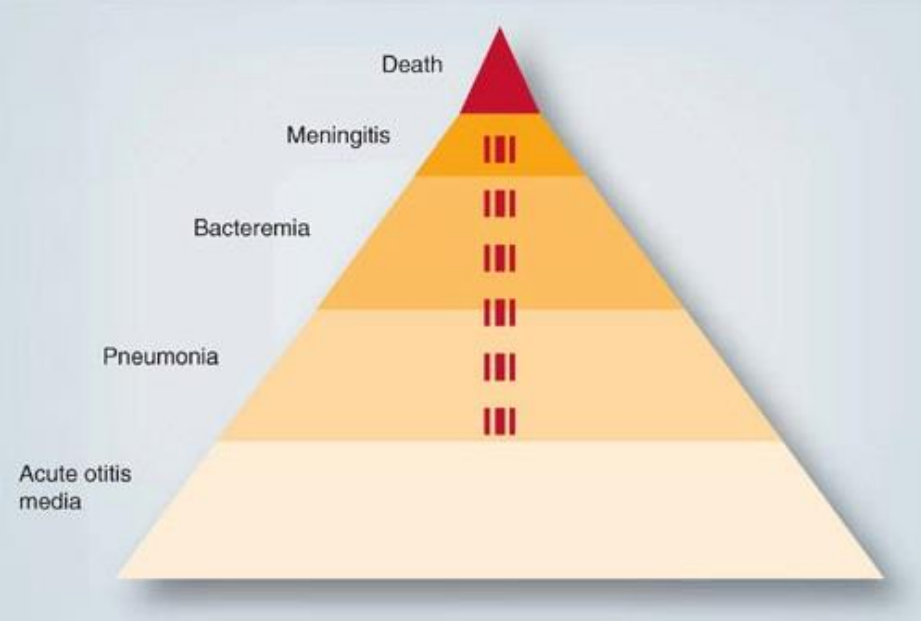


Figure 1.7. Hierarchic chart of pneumococcal diseases. Illustration of pneumococcal disease relative association with frequency and severity. (Image source: Expert Reviews Vaccine, 2019).

Progression to invasive disease is more likely in young children, elderly people and patients with specific lifestyle traits and comorbidities (Figure 1.7). In 2015, pneumococcal deaths was estimated to be over 500,000 deaths in children under 5 years of age worldwide (Wahl et al. 2018). Pneumococcal pneumonia incidence rates vary with age, affecting disproportionally young children and the elderly. In resource-rich settings, there is a trend of increasing incidence of pneumococcal meningitis in the elderly population, which frequently results in death, with higher mortality rates in this than in any other age group (Adler et al. 2017).

1.4.1 The burden of pneumococcal pneumonia

In 2015, 10 million episodes of childhood pneumococcal pneumonia were reported worldwide, with 76% mortality rate in Africa and 36% globally (Wahl et al. 2018). In numbers, this is translated to approximately 400,000 pneumococcal pneumonia deaths in children under 5 years of age in one year (Wahl et al. 2018). In the developed world the highest burden of pneumococcal disease arises in the elderly population, in which community-acquired pneumonia (CAP) incidence sharply increases following the age of 65. In 2014, over 70% of cases of CAP occurred in people aged over 65 (Daniel et al. 2016). In the United States, an estimated 600,000 episodes of pneumococcal CAP occur annually, with a total cost to the society of 4.85 billion dollars (Huang et al. 2011). Within Europe, CAP is the leading cause of death due to infection, with approximately 90% of deaths due to pneumonia occurring in people aged >65 years. Pneumonia places a considerable burden on healthcare resources and society, with associated annual costs in Europe estimated at approximately €10 billion, mainly due to hospitalisation and lost working days (Torres et al. 2013). Over the next two decades, the incidence of CAP is expected to double as the population ages and develops more co-morbidities (Wroe et al. 2012).

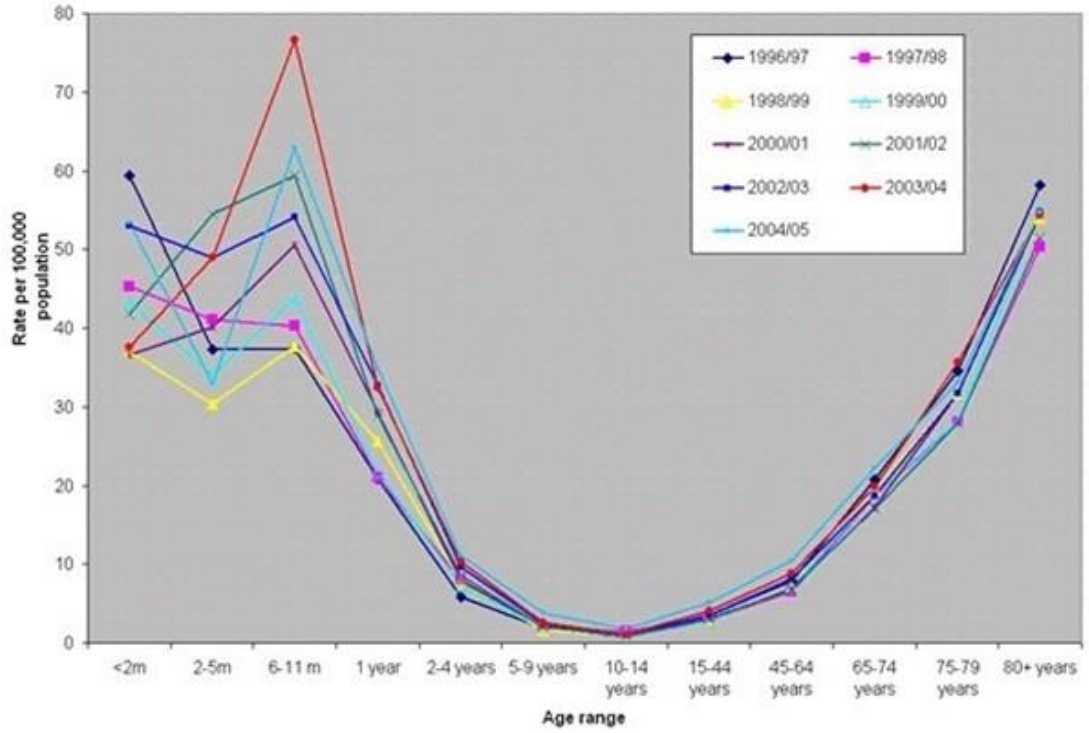


Figure 1.8. **Invasive pneumococcal disease rates per age group**. IPD incidence rate per 100,000 in England and Wales. Reproduced from Public Health England, July 2012.

Other risk factors than age are recognised for CAP, including smoking, alcoholism, conditions such as chronic obstructive pulmonary disorder (COPD), immunosuppressive conditions and co-infection with HIV. Patients infected with HIV are 25 times more likely to develop pneumonia, with CAP being a frequent respiratory complication in HIV patients even in the highly active antiretroviral therapy (HAART) era (Cilloniz et al. 2014, Klugman, Madhi and Feldman 2007). In HIV infected persons who have advanced disease, are not under HAART and with low $CD4^+$ T cell counts, there is a progressive loss of control of colonisation and invasion of *S. pneumoniae*, coupled with the inability to resolve pneumonia. Data on the effects of HIV on the physiological functions of alveolar macrophages in the lung are conflicting (Jambo et al. 2014). Alveolar macrophages are the gatekeeper of mucosal lung tissue integrity, and therefore compromised physiological functions can be detrimental for the lung health.

Pneumococcal pneumonia can be effectively treated with antibiotics. However, patients with bacteraemic pneumonia require longer courses of intravenous (IV) antibiotics than patients with non-bacteraemic pneumonia (Jover et al. 2008), and growing trends in antibiotic resistance are being witness globally. The local host immune

response plays an important role in regulating the containment of *S. pneumoniae* within the nasopharyngeal cavity or restrain its replication in the lung.

1.5 Innate immunity to S. pneumoniae

A brisk local host immune response to *S. pneumoniae* involving phagocytes (neutrophils and macrophages), B cells (antibodies against pneumococcal polysaccharides and proteins) and T cells rapidly eliminates colonisation, whereas a poor mucosal immune response results in protracted colonisation. Both innate and adaptive immunity play a role in these host defence responses against S. pneumoniae (Bogaert et al. 2004).

Innate immunity provides the early line of defence against microbes. It consists of cellular and biochemical defence mechanisms that are in place even before infection, responding rapidly to pathogens that enter to the human body. During pneumococcal infection the epithelial cells, through the specialised sets of receptors (pattern recognition receptor, PRR), can trigger such innate immunity to prevent pneumococcal invasion.

1.5.1 Mucociliary escalator

The mucociliary escalator is the layer of fluid, antimicrobial compounds and mucins (glycoproteins coated with negatively charged sialic acid) that lines the respiratory epithelium from the nasopharynx to the proximal bronchial tree (Knight and Holgate 2003, Tilley et al. 2015). The effectiveness of the mucociliary escalator depends on hydration, mucins produced by secretory cells and on the coordinated function of the ciliated cells that provides the force and direction of the escalator (Tilley et al. 2015). *S. pneumoniae* expresses enzymes that cleave sialic acid, e.g. NanA, in attempt to access the epithelium. Inhibition of mucociliary function by cigarette smoke (Nuorti et al. 2000) or viral co-infection (Pittet et al. 2010) increases the ability of pneumococcus to establish colonisation, be aspirated into the lungs and develop infection within them. Also, ciliary beat frequency and mucociliary clearance slow with aging (Ho et al. 2001).

1.5.2 Antimicrobial compounds

Respiratory secretions contain products with antimicrobial functions, inhibiting bacterial growth. Antibacterial compounds include (I) lactoferrin, b) lysozyme and c) a wide range of antimicrobial peptides (e.g. defensins and cathelicidin).

Lactoferrin is an iron binding protein found in nasal secretions and neutrophil granules. It sequesters iron -essential for bacterial respiration- and has direct microbicidal effects. However, *S. pneumoniae* is relatively resistant to lactoferrin as the latter is bound to the pneumococcal surface by PspA, which blocks its activity (Shaper et al. 2004). Pneumococcus has developed similar restriction mechanisms against the action of lysozyme.

Lysozyme breaks down peptidoglycans (PG) found in the bacterial cell wall. In respiratory fluids the major source is probably airway epithelium and associated glands, but it is also produced by neutrophils. While lysozyme can restrict the growth of *S. pneumoniae in vitro,* modifications to its cell wall structure render it relatively resistant to degradation. These peptidoglycan modifications reduce overall fitness of the bacterium, but this is outweighed by the benefits of resistance to the degrading activity of lysozyme (Davis et al. 2008).

Defensins and cathelicidins are two other antimicrobial peptide families that contribute to the host defence at mucosal sites. The α defensins, also known as human neutrophil peptides (HNP), are small peptides found in neutrophil azurophilic granules, whereas β defensins (hBD) 1-4 are all found in respiratory fluids, with hBD2 release stimulated by direct activation of epithelial cells, as well as by cytokines such as interleukin (IL) 1β and tumour necrosis factor (TNF) (Kota et al. 2008) (Kim, Min et al.2010). Respiratory epithelial cells constitutively express hBD1, whereas hBD2 & 3 are inducible by *S. pneumoniae*, and both restrict pneumococcal growth and promote its lysis *in vitro* (Lee et al. 2004, Scharf et al. 2012). The human cathelicidin LL-37, the only known human member of cathelicidins, is found in neutrophil-specific granules and exhibits broad spectrum microbicidal activities (Ganz 2004). Exemplifying the overlap of phagocytic and epithelial host defences, the mRNA for the human cathelicidin has also been found in human airway epithelia (Bals et al. 1998). The pneumococcal toxin pneumolysin has been shown to induce the release of LL-37 from human lung mast cells, reducing pneumococcal viability (Cruse et al. 2010).

In the lung, surfactant protein A and D (SP-A and D respectively) are constitutively synthesised and secreted into alveolar lining fluid by type II pneumocytes and non-ciliated bronchial epithelial cells. SP-A and D bind to exposed mannose and glucose residues on the surface of bacteria, leading to agglutination of pathogens, inhibition of microbial growth, and increase recruitment of phagocytes. SP-A and D also function as opsonins that increase macrophage phagocytosis of *S. pneumoniae* (Lawson and Reid 2000). In particular, SP-A has been shown to promote phagocytosis of *S. pneumoniae* mouse alveolar macrophages *in vitro* (Kuronuma et al. 2004). Although, it binds to the pneumococcus, SP-A does not have to be bound to the organism to enhance phagocytosis: "priming" of macrophages with the protein seems to be sufficient (Kuronuma et al. 2004).

C-reactive protein (CRP), an acute-phase protein, has also been detected in the upper respiratory tract of healthy individuals and is also elevated during inflammation. The concentration of CRP is lower in the respiratory tract than in serum but is sufficient to contribute to innate immunity, and is locally produced by epithelial cells (Gould and Weiser 2001, Gould and Weiser 2002). CRP has several functions in relation to cell-surface phosphorylcholine-expressing bacteria (such as *S. pneumoniae* and *Haemophilus influenzae*), which includes activation of complement by the classical pathway, enhancing opsonisation and inhibition of the attachment of bacteria to epithelial cells (Gould and Weiser 2002). These functions assist in clearance of colonising pneumococci from the upper respiratory tract.

1.5.3 Complement system

The complement system consists of a series of host proteins found in serum, epithelial lining fluid and cell surfaces that form protease cascades. It assists host immunity by identifying and opsonising invading pathogens and links the innate and adaptive immunity by promoting antigen recognition and improving antibody efficacy. The complement system is organised into three pathways known as classical, mannose-binding lectin (MBL) and alternative pathways. All converge on the activation of protein C3 to C3b which together with its degradation product iC3b, are deposited on the pneumococcal surface as opsonins. This deposition strongly stimulates phagocytosis by neutrophils and macrophages via complement receptors 1,3,4 and the complement receptor of the IgG superfamily (CRIg);

termed the classical pathway. This is the major mechanism by which complement activity facilitates the killing of S. pneumoniae (Brown et al. 2002). The classical pathway is also important in early lung defence against *S. pneumoniae,* where pulmonary cells synthesize and secrete complement components (Kerr et al. 2005).

The importance of complement to host immunity against pneumococcus is demonstrated by the increased incidence of pneumococcal infections in patients with inherited complement deficiencies, such as those with deficiency of the classical component C2 who have reduced ability of complement-dependent phagocytosis of *S. pneumoniae* (Yuste et al. 2008). However, the pneumococcus has evolved multiple mechanisms of inhibiting complement activity. These mechanisms involve pneumococcal factors, such as the capsule and pneumococcal surface proteins (Table 1.2).

Table 1.2. Pneumococcal factors with inhibitory function on complement activity.

Pneumococcal factor	Function
Polysaccharide capsule	Inhibition of C3 protein surface binding (Melin et al. 2010) Prevention of C3b conversion to iC3b, and subsequent opsonisation via the alternative complement pathway (Hyams et al. 2010)
Pneumococcal surface protein A, PspA	Inhibits the deposition of C3 onto the surface of pneumococci and interferes with both the classical and alternate complement pathways (Ren et al. 2004) Competes with the binding of CRP to cell-surface phosphocholine and, thus, inhibits complement deposition on the pneumococcal surface (Mukerji et al. 2012)
Pneumococcal surface protein C, PspC	Binds complement factor H which inhibits the alternative pathway C3 convertase and protects cells from complement attack (Dave et al. 2001).
Pneumolysin	Stimulates the classical complement pathway in the absence of pneumolysin-specific antibody, diverting the inflammatory response from intact pneumococci (Paton, Rowan-Kelly and Ferrante 1984)

1.5.4 Pattern Recognition Receptors (PRRs)

Upon access to the epithelium, the innate immune system initially recognises *S. pneumoniae* by several pattern-recognition receptors (PRRs). These receptors can recognise highly conserved microbial structures known as pathogen-associated molecular patterns (PAMPs) and initiate an inflammatory response. PRRs include transmembrane Toll-like receptors (TRLs), cytosolic NOD-like receptors (NLRs), DNA sensors, scavenger receptors and C-type lectins.

1.5.4.1 Toll-like receptors

From the 10-known human TLRs, only three have been described in host defence against the pneumococcus. These are TLR2, TLR4 and TLR9.

- TLR2: Amongst the diverse ligands recognised by TLR2 are pneumococcal lipoteichoic acid (LTA) (Schroder et al. 2003) and mainly lipoproteins (LP) (Tomlinson et al. 2014). Responses to surface lipoprotein-deficient Δlgt pneumococcal strain, such as macrophages NF-kB activation and TNF-α release, are reduced compared to wild-type bacteria (Tomlinson et al. 2014). Structural differences in LTA between pneumococcal strains alter their ability to stimulate TLR2 *in vitro* which may contribute to differences in virulence (Draing et al. 2006). Overall, our knowledge on the role of TLR2 in protection against pneumococcus derives from mice models, where it is involved in both protection against pneumococcal colonisation and disease (Paterson and Orihuela 2010).

- TLR4: It is best known as a specific receptor for lipopolysaccharides (LPS), but also recognises other ligands including pneumolysin (Malley et al. 2003). The role of pneumolysin-TLR4 recognition during nasopharyngeal carriage is ambiguous. Malley et al. 2003 showed that TLR4 −/− mice have increased colonisation levels and heightened risk of invasive disease (Malley et al. 2003). In contrast other work described that TLR2, but not TLR4, was necessary for efficient clearance of colonisation (van Rossum, Lysenko and Weiser 2005). Furthermore, TLR4 plays only a limited role in pneumonia, systemic infection or meningitis. As indicated, this is probably due to in part redundancy between different TLRs (Paterson and Orihuela 2010).

- TRL9: It recognises unmethylated cytosine-phosphate-guanosine (CpG) motifs present in the bacterial DNA (Ishii and Akira 2006). TLR 9 −/− mice display enhanced susceptibility to pneumococcal pneumonia, a phenotype more severe than that seen with mice lacking TLR2 or TLR4 (Albiger et al. 2007). Unlike TLRs 2 and 4, which are on the cell surface, TLR9 is expressed within endosomal compartments, thus it may serve to amplify/modulate responses following phagocytosis.

1.5.4.2 NOD-like receptors

NOD1 and NOD2 are intracellular PRRs, acting in the cytosol to recognize and respond to bacterial cell wall components. They function via activation of the inflammasome, a cytosolic multiprotein complex which activates caspase-1. The latter in turn promotes the cleavage, and thus the activation of IL-1β and IL-18, with the active cytokines to lead to a downstream inflammatory response. It has been shown that NOD2 sensing of lysozyme-digested peptidoglycan promotes macrophage recruitment, through CCL2 signalling, and hence facilitate clearance of *S. pneumoniae* colonisation in mice (Davis, Nakamura and Weiser 2011).

1.5.4.3 Scavenger receptors

Scavenger receptors (SR-A, MARCO, CD36, mannose-receptor) represent a large, diverse family of surface glycoproteins expressed predominantly on macrophages, dendritic cells and endothelial cells (Hussell and Bell 2014). These PRRs act as phagocytic receptors mediating direct, non-opsonic phagocytosis of pathogenic microbes (Areschoug and Gordon 2009). Both SR-A and MARCO bind pneumococci, although the bacterial features that are recognized are not yet known. Mice lacking either receptor have increased susceptibility to pneumococcal pneumonia (Arredouani et al. 2004, Arredouani et al. 2006). Also, data from a mouse colonisation model indicate that MARCO is an important component of anti-*S. pneumoniae* responses in the murine nasopharynx during colonisation, which amplifies TLR2- and NOD2-dependent NF-κB activation and signalling (Dorrington et al. 2013). In addition, MARCO expression on alveolar macrophages (AMs) is downregulated after influenza infection, contributing to severity of secondary pneumococcal infections (Sun and Metzger 2008).

The mannose receptor (CD206) interacts with glycoproteins and glycolipids that are found on the surface of pathogens. Recognition of non-opsonised bacteria results in the suppression of alveolar macrophages in humans, rats, mice and rabbits — an effect that is dependent on the expression of mannose receptors by these cells (Zhang et al. 2005, Hussell and Bell 2014). This suggests that, unless other receptors are co-ligated or the bacteria are 'flagged' for immune recognition in some way, signalling through the mannose receptor prevents alveolar macrophages from initiating pro-inflammatory responses against microorganisms. This may be of benefit in preventing inflammatory responses against commensal bacteria in the airways.

1.5.5 Phagocytes

Professional phagocytes, such neutrophils, monocytes, macrophages and dendritic cells, detect foreign particles, such as bacteria, through their surface receptors. Binding of the pathogen to the receptors initiate its internalisation in a phagosome, which normally acidifies and fuses with lysosomes in order to destroy the contents. Phagocytes are central players in host defence against infection, such as pneumococcal infection.

1.5.5.1 Neutrophils

Neutrophils are among the first cells recruited during many stages of *S. pneumoniae* pathogenesis. In mice, neutrophils are recruited during asymptomatic nasopharyngeal colonisation (Lu et al. 2008, van Rossum et al. 2005), whereas in healthy adults they are abundant in the nose, even in absence of pneumococcal colonisation (Jochems et al. 2017). In the lungs, neutrophils are mainly recruited to the alveoli during invasive infection. They comprise a crucial component of innate immunity, controlling bacterial and fungal infection through a combination of both oxidative and non-oxidative mechanisms. The oxidative burst, which generates reactive oxidative species (ROS) through an NADPH oxidase system, was long thought of as the neutrophil's primary mode of killing pathogens. Other than ROS, neutrophils also produce a multitude of antimicrobial molecules in their granules. The two major granule subsets are the azurophilic and specific granules. The azurophilic granules, are the first to fuse with the phagolysosome and contain defensins, bactericidal permeability-increasing protein (BPI), as well as serine proteases such as elastase, cathepsin G and proteinase 3. Weiser and colleagues showed that human neutrophils kill *S. pneumoniae* via serine proteases

cathepsin G and neutrophil elastase, offering an explanation to Chedaki-Higashi syndrome (Standish and Weiser 2009). Neutrophils from Chedaki-Higashi syndrome patients have a diminished capacity to kill pneumococcus (Root, Rosenthal and Balestra 1972), pointing out that non-oxidative mechanisms play an important role in neutrophil-mediated killing. Additionally, in the absence of NADPH oxidase-generated ROS, mice tolerate greater numbers of neutrophils in the lung without a concomitant increase in lung injury (Marriott et al. 2008). Neutrophils can also produce extracellular traps (NETs), which trap pathogens within chromatin structures, and subsequently kill them with various antimicrobial proteins (Brinkmann et al. 2004). Pneumococcus, though, shows resistance to killing by NETs via the expression of a surface endonuclease encoded by endA, which allows pneumococci to degrade the DNA scaffold of NETs and escape (Beiter et al. 2006).

1.5.5.2 Monocytes

Monocytes consist 1-6% of circulating blood cells. They are attracted to inflamed tissues through chemokines (MCP-1, RANTES, MIP-1β)(Deshmane et al. 2009), where they differentiate into macrophages. In mice it has been shown that pneumococcal colonisation leads to TLR2-dependent recruitment of monocytes into the nasal mucosa, facilitating clearance of pneumococci (Zhang, Clarke and Weiser 2009). Weiser and colleagues have further elucidated the mechanism of gradual pneumococcal clearance from the nasopharynx by recruited monocyte/macrophage either colonisation alone (Davis et al. 2011) or in the context of influenza co-infection (Nakamura, Davis and Weiser 2011). They have shown that digestion of pneumococci by phagocytes results in CCL2 release, and CCR2-dependent recruitment of the additional monocytes/macrophages to the airway lumen (Davis et al. 2011). During influenza-pneumococcal infection, this CCL2 auto-feedback loop is inhibited by the synergistic type I IFN response and leads to increased pneumococcal density in the mice nasopharynx (Nakamura et al. 2011). In agreement with the findings in murine models, a recent EHPC study showed that in humans acquisition of pneumococcus in the nasopharynx induces recruitment of monocytes (with a peak at Day 9 post the intranasal challenge) and that monocyte function was associated with the clearance of colonisation (Jochems et al. 2018). Data from a co-culture model of human monocytes and $CD4^+$ T cells have

suggested that live pneumococci trigger Th1-biased response via monocyte production of IL-12p40, whereas heat-killed pneumococci trigger a Th17 response through TLR2 signalling (Olliver et al. 2011). During pneumococcal pneumonia, monocytes are recruited to the lungs- although at a later point compared to neutrophils- and contribute to bacterial resolution from the pneumonic regions of the lung (Goto et al. 2004). But prior pneumonia development, the highly differentiated macrophages of the lung- the alveolar macrophages- fight to restrain pneumococcal replication.

1.5.5.3 Alveolar macrophages

Alveolar macrophages are the most numerous cell type in the alveolar spaces and can readily be recovered in high purity (>95%) by bronchoalveolar lavage. Studies conducted in murine models have shown that alveolar macrophages derive from fetal-liver monocytes. The latter migrate to the lung and differentiate into AMs during the first weeks of life following exposure to granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by alveolar type II epithelial cells (Mass et al. 2016). These fetal monocyte-derived alveolar macrophages are long-lived cells and are maintained via self-renewal (Morales-Nebreda et al. 2015).

Alveolar macrophages play a critical role in homeostasis, host defence, and tissue remodelling (Lambrecht 2006, Svedberg et al. 2019). Strategically located, AMs function as a first line defence of the lung against inhaled microorganisms and damage to these cells is an important factor in increased host susceptibility to airborne bacterial infection (Gwyer Findlay and Hussell 2012). This is due to expression of numerous cell surface receptors and their strong phagocytic and microbicidal capacity (Guilliams et al. 2013). Interaction with mannose receptor, SIPRa, CD200R or activation of TGFβR signalling result in suppression of AM in humans (Hussell and Bell 2014). On the other hand, stimulation of alveolar macrophages through TLR2, TLR4 or TLR9 inhibits IL-10R signal transduction and releases these cells from the suppression that is usually mediated by epithelial cell-produced IL-10 (Fernandez et al. 2004) (Figure 1.9). Signalling via one TLR in AMs markedly changes the pattern of expression of other TLRs on the same cells (Maris et al. 2006).

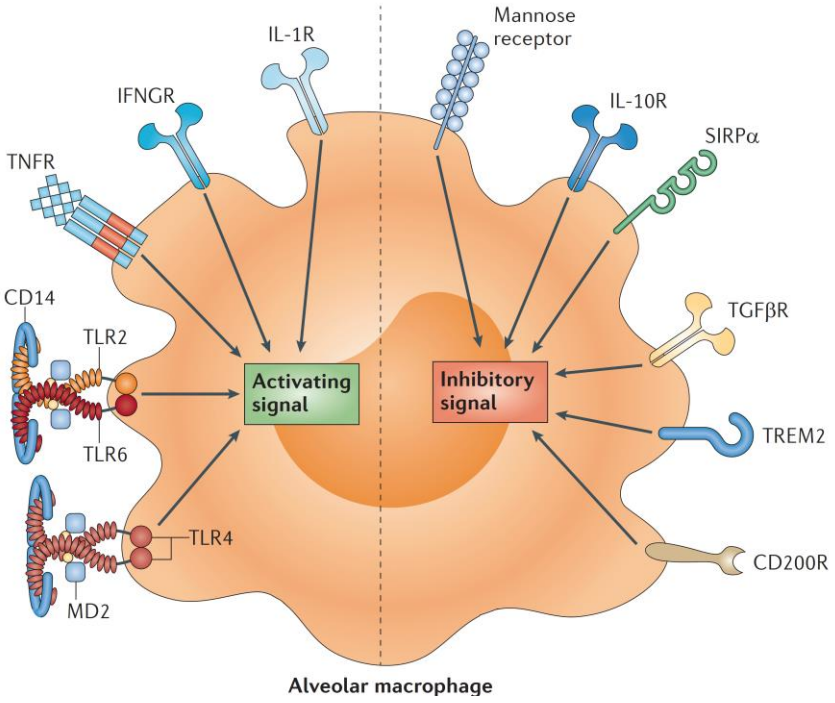


Figure 1.9. Alveolar macrophage surface receptor that promote either cell activation or suppress inflammatory responses. Image source: (Hussell and Bell 2014).

Once activated, AMs generally have a greater phagocytic capacity (Hussell and Bell 2014), release large quantities of ROS and NO and have higher pro-inflammatory cytokine production, such as TNFα, which aid in pathogen destruction (Steinmuller et al. 2000). Once their phagocytic capabilities are overwhelmed, AMs institute an inflammatory response that results in leukocyte chemotaxis (Dockrell et al. 2003). They secrete chemokines like CXCL8 (IL-8), CCL2 (MCP-1), and CX3CL1, promote neutrophil and monocyte recruitment to the lung; and growth factors such as macrophage-colony stimulating factor (M-CSF), which augment monocyte survival.

AMs also express major histocompatibility complex (MHC) class II antigens ((HLA-DR,DQ,DP) and exhibit antigen presenting activity, although this is reduced relative to pulmonary dendritic cells and interstitial macrophages (Misharin et al. 2013) Flow cytometric analysis has shown that human alveolar macrophages are unique, compared to macrophages in other tissues, in that they express high levels of CD11c (integrin alpha X), CD169 (siglec-1),scavenger receptors (e.g., MARCO, CD68, CD204, SR-A) and pattern

recognition receptors (TLR4, TLR2), as well as receptors for Fcγ (CD64) and SIRP-α (CD47), along with receptors for various cytokines, chemokines, and signalling molecules (Morrell et al. 2018, Bharat et al. 2016, Yu et al. 2016). Conversely, they are low in expression for CD11b (Mac-1), which may reflect their special function of tolerance versus immunity. A study of murine pneumococcal infection showed that the initial inflammation and its subsequent resolution induced the influx of a population of AMs with dramatically increased expression of CD11b (monocyte-derived AMs), which continued after the bacterial resolution (Kirby, Raynes and Kaye 2006).

The polarisation of macrophages into "M1" and "M2" gene expression phenotypes has formed a base for partially understanding the complexity of tissue macrophage function over the time course of tissue injury and repair (Murray et al. 2014). In controlled cell culture conditions, classically activated or M1 macrophages are induced by exposure to interferon gamma (IFN-γ) and LPS and appear to be primed towards microbicidal functions, i.e. to produce more ROS and more proinflammatory cytokines, as well as IL12 and IL23. M2 or alternatively activated macrophages are induced by IL-4 released from Th2 cells. Their primary role appears to mediate tissue repair, through the production of anti-inflammatory cytokines, including transforming growth factor–β (TGF-β). In humans, there is no general consensus about whether alveolar macrophages in the healthy state are M1- or M2-like *in vivo* (Hussell and Bell 2014). More recent findings from several independent groups suggest that this concept incompletely describes and underestimates the complexity of macrophage gene expression *in vivo*, which might include simultaneous expression of M1 and M2 genes during injury and repair (Xue et al. 2014). Furthermore, the applicability of this paradigm to human lung macrophages is not known.

1.6 Adaptive immunity to the pneumococcus

Although it needs induction before being able to mount quick responses, the adaptive immune system is highly specific, has memory and is comprised of a humoral response and a cell-mediated response.

1.6.1 Humoral immunity to *S. pneumoniae*

Capsule-specific antibodies produced by activated B cells play an important role in protection against disease and potentially against colonisation. Antibodies to the pneumococcal capsular polysaccharide are produced following vaccination against pneumococcus or through natural exposure after a colonisation episode. In adults, pneumococcal colonisation confers complete protection against acquisition of the same strain up to one year later, as shown in the EHPC model (Ferreira et al. 2013). Similarly, serotype-specific protection against colonisation is present in toddlers for at least several serotypes, demonstrating that this also plays a role in children (Weinberger et al. 2008). However, studies of anti-capsular immunity have shown that the serotype-specific immunity elicited by natural exposure to pneumococci is imperfect, frequently allowing repeated colonisations with the same serotype. Three separate studies have found evidence of strong immunity for one serotype (individuals who have carried type 14 are approximately 90% less likely to carry it again); however, they suggest that such immunity for all other serotypes, if it exists, is much less protective (Goldblatt et al. 2005, Hill et al. 2008, Weinberger et al. 2008). Additionally, an epidemiological study in Bangladeshi infants and young children attributed the observed decrease in the rates of subsequent pneumococcal acquisition to their association with the previous heterologous carriage, pointing out the importance of serotype-independent immunity (Granat et al. 2009).

Pneumococcal proteins, with highly conserved patterns amongst the pneumococcal serotypes, have long been on the target of new vaccines strategies, as they could provide serotype independent protection. PspA is considered one of the most important antigens capable of inducing a protective immune response. In adults, anti-protein antibody responses are weak and although experimental pneumococcal colonisation elicited serum IgG against 18 proteins, including PspA and PspC, (Wright et al. 2012, Ferreira et al. 2013), baseline IgG levels to them did not correlate with protection against acquisition of colonisation (Ferreira et al. 2013). Similar to the pneumococcal protein, baseline CPS-specific IgG do not correlate either with pneumococcal colonisation protection in adults (Pennington et al. 2016). To study the role of naturally acquired immunity against invasive pneumococcal disease, a recent study used human intravenous immunoglobulin preparation (IVIG), representing natural

IgG responses to *S. pneumoniae*, and suggested that this type of protection depends on antibody to protein antigens rather than the capsule (Wilson et al. 2017).

Among the different antibody classes, IgG seems to have a major role in the immunity against pneumococcus post vaccination. Circulating anti-pneumococcal IgG in mice previously colonised was the predominant antibody response and associated with reduced bacteraemia, whereas antibody knock-out mice sustained lethal bacteraemia (Cohen et al. 2011). In bronchoalveolar lavage fluid (BALF), levels of IgG and IgA were elevated in the colonised mice and were associated with reduced bacteraemia in the lung. In a PCV vaccination trial of young adults, serum levels of IgG to CPS-6B were strongly correlated with those measured in the nasal washes four weeks post vaccination and resulted in high reduction in rates of 6B colonisation (Collins et al. 2015, Mitsi et al. 2017). This reduction in colonisation following PCV vaccination was likely conferred by antibody-mediated bacterial agglutination on the mucosal surface that led to bacterial sequestration, that may aid mucociliary clearance and prevent acquisition of colonisation (Mitsi et al. 2017). In mucosal surfaces, IgA is the dominant antibody, with IgA1 making up approximately 90% of the total IgA in the upper and 70% in the lower respiratory tract (Burnett 1986). *S. pneumoniae* produces protease IgA1, which cleaves human IgA1, and thus circumvent killing by this important mucosal immunoglobulin (Janoff et al. 2014) . Cleaved fragments of IgA1 facilitate bacterial adherence to the respiratory epithelium and can thus even promote colonisation acquisition (Weiser et al. 2003). Therefore, mucosal (nasal and lung) protection requires other classes of immunoglobins such as IgG (Janoff et al. 2014). In the respiratory mucosa, it is unclear yet whether immunoglobulin to *S. pneumoniae* measured in mucosal (lung and nasal) secretions are locally synthesised by plasma cells or diffuse from vascular compartments via the peripheral circulation. Interestingly, sampling of the nasal mucosa pre and after experimental pneumococcal colonisation revealed that B cells were depleted from the nasal mucosa upon pneumococcal colonisation and this observation was associated with an expansion of Spn polysaccharide-specific and total plasmablasts in blood (Jochems et al. 2019).

1.6.2 T cell mediated immunity to *S. pneumoniae*

Cellular adaptive immune responses are driven by T lymphocytes, which recognise internalised antigen presented on MHC II molecules. CD4 expressing helper T cells secrete cytokines to affect other cells in the local milieu, while CD8 expressing cytotoxic T cells induce apoptosis of host cells infected with intracellular pathogens. In adaptive immunity, the presentation of antigens to naive T cells in lymph nodes by dendritic cells drives the activation of T cells and directs their development into specific effector T cells or cells with a memory phenotype.

1.6.2.1 T-helper 1 mediated immunity

T-helper 1 (Th1) are a subset of $CD4^+$ T cells that initiate pro-inflammatory responses, which increase phagocytosis and intracellular killing. There are contradictory reports on the role of the Th1 cytokine IFN-γ in protection against pneumococcal infection (Rubins and Pomeroy 1997, Rijneveld et al. 2002, Nakamatsu et al. 2007, Sun et al. 2007, Sun and Metzger 2008). However, these studies used murine models, and human Th1 responses remain poorly understood. Interestingly, Kemp et al. found that IFN-γ-producing T cells disappear from the circulation in patients with pneumococcal pneumonia or bacteremia, suggesting that these cells are engaged in the *in vivo* immune response (Kemp et al. 2002). Furthermore, it has been demonstrated that peripheral blood mononuclear cells (PBMCs) from healthy adults living in a region with a high incidence of pneumococcal carriage and disease respond to pneumococcal antigens with both IFN-γ and IL-17 production, indicating that exposure to pneumococci results in T cell-mediated immunological memory (Mureithi et al. 2009). As mentioned earlier, live pneumococci trigger a Th1-biased response *in vitro* (Olliver et al. 2011). Furthermore, Th1 cells induce B cell isotype switching to IgG2 antibody, which correlates with increased phagocytic ability in mice (Lefeber et al. 2003). Despite these evidences, it is not known whether Th1 responses improve adaptive immune mediated protection against subsequent infection.

1.6.2.2 T-helper 2 mediated immunity

A child's immune system in the first few years of life is Th2-skewed so as to avoid inflammation-induced immunopathology (Adkins, Leclerc and Marshall-Clarke 2004, Schaub et al. 2008). The Th2 subset produces IL-4, IL-5 and IL-13, which drives B-cell class switching to IgG and IgE antibody production (Leiva et al. 2001, Mosmann and Sad 1996). Dysregulation of Th2 responses are associated with allergy and predispose for respiratory conditions such as asthma. House bust mouse-sensitised children have early defective antibody responses to S. pneumoniae and H. Influenzae that are associated with asthma development (Hales et al. 2012). However, a distinct IL-4 signature in the lungs of a mice with pneumococcal pneumonia highlighted a Th2 environment as a risk factor for infection (Kang et al. 2009).

1.6.2.3 T-helper 17 mediated immunity

Th17 cells have pathogenic properties, but not all actions by Th17 cells are deleterious; they have been shown to confer protection against infections with extracellular bacteria such as *S. pneumoniae*. Th17 cells secrete the pro-inflammatory cytokine IL-17, which is essential for recruitment and activation of neutrophils and macrophages to the nasopharynx (Marques et al. 2012).

Many studies support a key role for Th17 responses in preventing or reducing pneumococcal carriage mainly in mouse models, whereas in human findings are contradictory (Lundgren et al. 2012, Lu et al. 2008, Zhang et al. 2011, Hoe et al. 2015). In mice, Malley and colleagues demonstrated that $CD4^+$ T cells protect against nasal colonisation through antibody-independent acquired immunity (Malley et al. 2005). The importance of IL-17-secreting Th17 cells has been highlighted by the finding that these cells orchestrate recruitment and activation of neutrophils, monocytes and macrophages to sites of inflammation in the upper respiratory tract, leading to the clearance of pneumococci (Zhang et al. 2009). Although systemic and mucosal antibodies also contribute, the protection against lung infection afforded by prior colonisation is lost in the absence of $CD4^+$ T cells or IL-17 (Wilson et al. 2015). Importantly, it has been found that the site of subsequent infection determine the basis of protection; Th17 responses being critical against lung infection whereas antibodies are needed against invasive infection (Cohen et al. 2011). On the other hand, IL-17-based protection against

pneumococcal infections seems to be diminished or lost when preceded by a viral infection such as influenza (Kudva et al. 2011). The reasons for this are not fully understood.

In humans, IL-17A is produced following *in vitro* stimulation of tonsillar and peripheral blood mononuclear cells with pneumococcal antigens such as pneumolysin and whole cell antigen (Lu et al. 2008, Mureithi et al. 2009, Schmid et al. 2011). IL-17A increased pneumococcal killing by human neutrophils both in the absence and in the presence of antibodies and complement, but this protective affect was abrogated in the absence of IL-17A receptors, as shown in mutant mice in the same study (Lu et al. 2008). In children findings on Th17 responses in response to colonisation are conflicting. Studies have shown that high IL-17 concentrations are associated with low pneumococcal nasopharyngeal carriage density in children (Hoe et al. 2015). In Gambia- an area with very high pneumococcal carriage rates- high levels of IL-17 were not associated with the absolute interruption of pneumococcal carriage (Mureithi et al. 2009). Comparison of Th17 responses to S. pneumoniae between adults and children in two different setting (Sweden and Bangladesh) showed differential levels of Th17 responses to pneumococci in children and adults between the two countries. Swedish children produced lower levels of IL-17A in response to pneumococcal antigens compared to adults, whereas no such difference was noted from the samples from Bangladesh, where responses by children and adults were both significantly higher than those in Sweden (Lundgren et al. 2012). In healthy adults, experimental pneumococcal colonisation resulted in an increased number of pneumococcal specific, IL-17A secreting $CD4^+$ T cells in both blood and lung of the colonised individuals, whereas human alveolar macrophage treatment of with exogenous IL-17A augmented pneumococcal killing (Wright et al. 2013).

1.6.2.4 T regulatory T cells

Regulatory T cells (Treg) play a key role in the control of various aspects of the immune response including maintenance of immune tolerance and prevention of autoimmunity (Sakaguchi et al. 2006). The cytokine TGFβ drives the differentiation of naïve T cells to Tregs (Huber et al. 2004) and they, in turn, limit excessive inflammation by secreting IL10 and other mechanisms. Intracellular expression of Foxp3 is still

considered the most specific single marker of Treg, although a combination of phenotypic expression of $CD4^{+}CD25^{high}CD127^{low}$ has also been established as a useful phenotypic marker for natural Treg (Zhang et al. 2011).

Neill and colleagues demonstrated elevated TGF-β1 and IL-10 levels in nasal washes of Spn colonised individuals post experimental human pneumococcal challenge compared to their non-colonised counterparts. Also, mice with low density and prolonged carriage episodes were shown to have a higher level of TGF-β1 compared to high-density colonised mice (Neill et al. 2014). In young children (<6 years of age), the majority of Tregs in the blood are characterised as naïve, whereas over 50% of Treg found in adenoidal tissues express the markers CD45RO and CD69, and among them, a significant proportion expressing $Foxp3^{high}CD25^{high}$ (Zhang et al. 2011). The latter subpopulation is characterised as effector/memory Tregs with potent suppressive properties (Sakaguchi et al. 2010). Zhang et al described that children with confirmed pneumococcal colonisation during the study period had significantly higher levels of effector/memory Tregs in their tonsils, suggesting that local colonisation promotes antigen-specific Treg in vivo in local lymphoid tissues adjacent to the site of colonisation (Zhang et al. 2011). Later in the same year, another group used pneumococcal specific tetramers bound to epitopes of Ply and demonstrated that tonsils from both children and adults with recurrent tonsillitis contain high proportions of pneumococcal-specific Tregs, implying that pneumococcal-specific Treg become a stable part of the repertoire with age (Pido-Lopez et al. 2011). The same study described that $CD25^{hi}$ Treg depletion from cultures of tonsil mononuclear cells resulted in high increase in the proliferative responses of the remaining $CD4^{+}$ T cells after stimulation with pneumococcal antigens. A few years later, a study described the relation between Th17 and Treg in nasal-associated lymphoid tissue with age, showing that Th1 and Treg are inversely correlated, with amounts of Th17 specific for pneumococcus increasing with age and being greater in carriage-negative individuals (Mubarak et al. 2016). In contrast, mice that are resistant to invasive pneumococcal pneumonia have significantly more Tregs in their lungs than susceptible mice, with this being mediated through TGF-β signalling (Neill et al. 2012). Collectively, these data suggest that maintenance of Th17/Treg balance is critically important in control of pneumococcal colonisation (Figure 1.10).

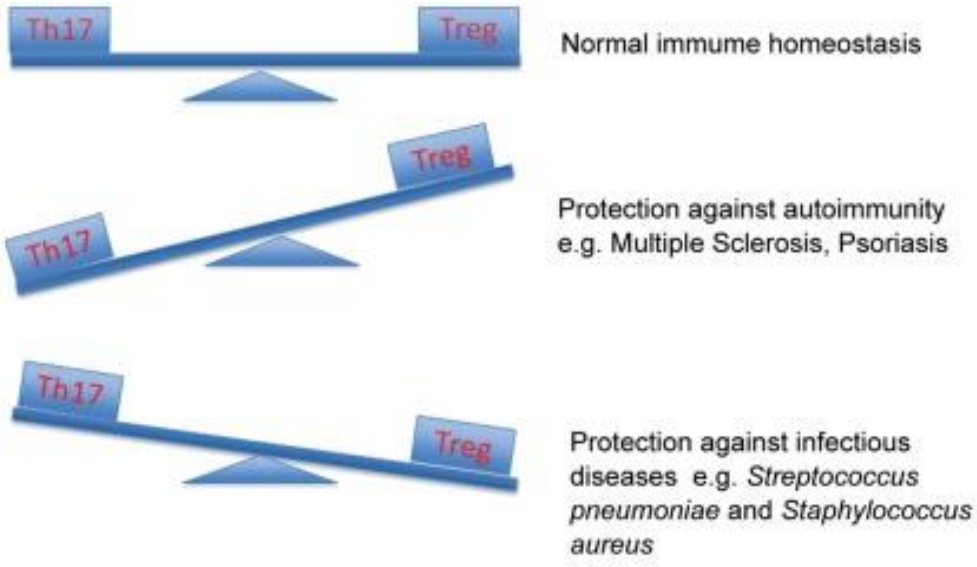


Figure 1.10. Relationship between Th17 and Tregs under normal immune homeostasis and under different diseases contexts. Source of image (Hoe et al. 2015)

1.6.2.5 TCR-γδ T cell mediated immunity

γδ T cells are T cells with a distinctive T-cell receptor (TCR) on their surface. They are considered to be unconventional T cells and a bridge between innate and acquired immunity (Nishimura et al. 2004). The nature of antigens recognised by γδ TCRs is diverse and still incompletely characterized. TCR γδ T cells express a wide array of nonclonal receptors, including PRRs and receptors to recognise natural killer (NK) cell. They can be directly activated through TLRs and C-type lectins (including dectin-1) and indirectly by microbial products presented via antigen-presenting cells (Ivanov, Paget and Trottein 2014). TCR γδ T cells appear to modulate the inflammatory response to other organisms in the respiratory tract despite being present in low numbers. In a murine model of acute *Streptococcus pneumoniae*-mediated lung inflammation, resolution of inflammation following bacterial clearance was associated with a > 30-fold increase in γδ T cell number (Kirby et al. 2007). Another study used cytometry by time-of-flight (CyTOF) mass cytometry to better characterize the phenotypic response of TCR γδ T cells to *S. pneumoniae* lung infection and found that lung infection caused a significant increase in TCR γδ T cells, which expressed high IFN-γ and IL-17A levels (Wanke-Jellinek et al. 2016). The γδ T cells of influenza infected mice were less able to produce IL-17 in response to *S.*

pneumoniae, which contributed *to* influenza induced *S. pneumoniae* infection (Li, Moltedo and Moran 2012). γδ T cells are probably also involved into the resolution of inflammation by inducing cytotoxic cell death of inflammatory monocytes (Kirby et al. 2007). However, the mechanism by which *S. pneumoniae* induces their activation remains unclear.

1.6.2.6 CD8 T cells mediated immunity

CD8 T cells are usually associated with responses to intracellular organisms. Genetic mutations in humans affecting transporter associated with antigen processing (TAP) proteins required for CD8 function are associated with an increased incidence of *S. pneumoniae* infection (Gadola et al. 2000). In a mouse model of lung infection CD8 deficient mice had greater levels of bacteraemia with several serotypes of *S. pneumoniae* and poorer survival with serotype 3 (Weber, Tian and Pirofski 2011). The mechanism appears to be the role of CD8 (CD8+CD28-) in inhibition of $CD4^+$ T cells (particularly Th17) mediated inflammation via the secretion of IL10 and TGFβ (Mertens et al. 2009). In the EHPC model, basal levels of $CD8^+CD161^+$ T cell clusters measured in nasal mucosal tissue were significantly lower in colonised than in non-colonised subjects. Combined with *in vitro* assessment of blood mucosal associated invariant T (MAIT) cells responses post pneumococcal stimulation, these data implied a protective role for this CD8 type against pneumococcal colonisation. (Jochems et al. 2019) However, the role of CD8 T cells for adaptive immunity to *S. pneumoniae* requires further characterisation.

1.7 Vaccination against *S. pneumoniae*

The currently licensed pneumococcal vaccines target the polysaccharide capsule and have shown success in decreasing IPD but confer sub-optimal protection against pneumonia. They are divided in two categories: a) polysaccharide pneumococcal vaccines (PPV) and b) pneumococcal conjugate vaccines (PCVs).

1.7.1 Polysaccharide pneumococcal vaccine

The established 23-valent pneumococcal vaccine (PCV-23) contains purified capsular polysaccharides for the following 23 serotypes: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. PCV23 elicits a T-cell-independent immune response, as purified CPS bind directly to B cell receptors which

leads to B cell differentiation into plasma cells and release of anticapsular IgG and IgM in adults (Durando et al. 2013), but hardly any class switching, affinity maturation, or immunological memory (Rose et al. 2005) (Figure 1.10). PPV are poorly immunogenic in young children (Pollard, Perrett and Beverley 2009), and hence are mainly recommended for use in adults >65 years old (Adler et al. 2017). A Cochrane review in 2013 concluded that PPV23 effectively prevents pneumococcal bacteraemia and meningitis, including in the elderly (Moberley et al. 2013). It has minimal effect at the mucosal level and thus has not been shown to reduce rates of colonisation. The protective efficacy of this vaccine for non-bacteraemic pneumonia remains controversial despite many years of use, and there are theoretical concerns about immunological harm resulting from B cell depletion (Clutterbuck et al. 2012).

Conflicting results have been obtained concerning the immunogenicity of the pneumococcal polysaccharide vaccine in HIV infected patients. A double-blind, randomized, placebo-controlled clinical trial showed that the vaccine was ineffective in HIV-1 infected Ugandan adults, aged 15–55 years (French et al. 2000). Invasive pneumococcal events were more frequent in the PPV23-vaccinated arm, with a big proportion of isolates to be vaccine-specific serogroups. All-cause pneumonia, but not pneumococcal pneumonia, was significantly more frequent in the vaccinated group. Collectively, these data triggers thoughts on whether the vaccine might even have been harmful in this population. In slight contrast to this, a retrospective case-control study performed in the USA demonstrated protection against invasive pneumococcal infections among White but not Black HIV-infected adults (Breiman et al. 2000).

1.7.2 Pneumococcal conjugate vaccines

The pneumococcal conjugate vaccine (PCV) exists in two formulations that target either 10 (PCV10) or 13 serotype s (PCV13) (Pollard et al. 2009). Serotypes included in PCV10 are 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F. The PCV13 formulation includes the additional serotypes 3, 6A, and 19A. PCV13 is currently recommended in immunisation schedule for infants in the UK (Public Health England and Department of Health 2018) and many other countries. In the PCVs, purified CPS are covalently linked to a carrier protein and therefore they induce a T cell-dependent response (Figure 1.11). This type of response stimulates the formation of antigen specific memory B cells (B_{MEM}) and

improves affinity maturation, class switching, and levels of IgG (Papadatou, Tzovara and Licciardi 2019, Clutterbuck et al. 2012). The widespread use of PCVs among children sharply decreased the circulation of the vaccine-type serotypes amongst them (Wahl et al. 2018). As a result, the incidence of IPD and pneumonia in both vaccinated children and unvaccinated adults has reduced dramatically. Most of the overall efficacy of PCVs has been attributed to herd immunity for populations at risk, such as elderly and immunocompromised and a recent study suggested that preschool-aged children, rather than infants, are responsible for maintaining the indirect benefits of PCVs (Weinberger et al. 2019).

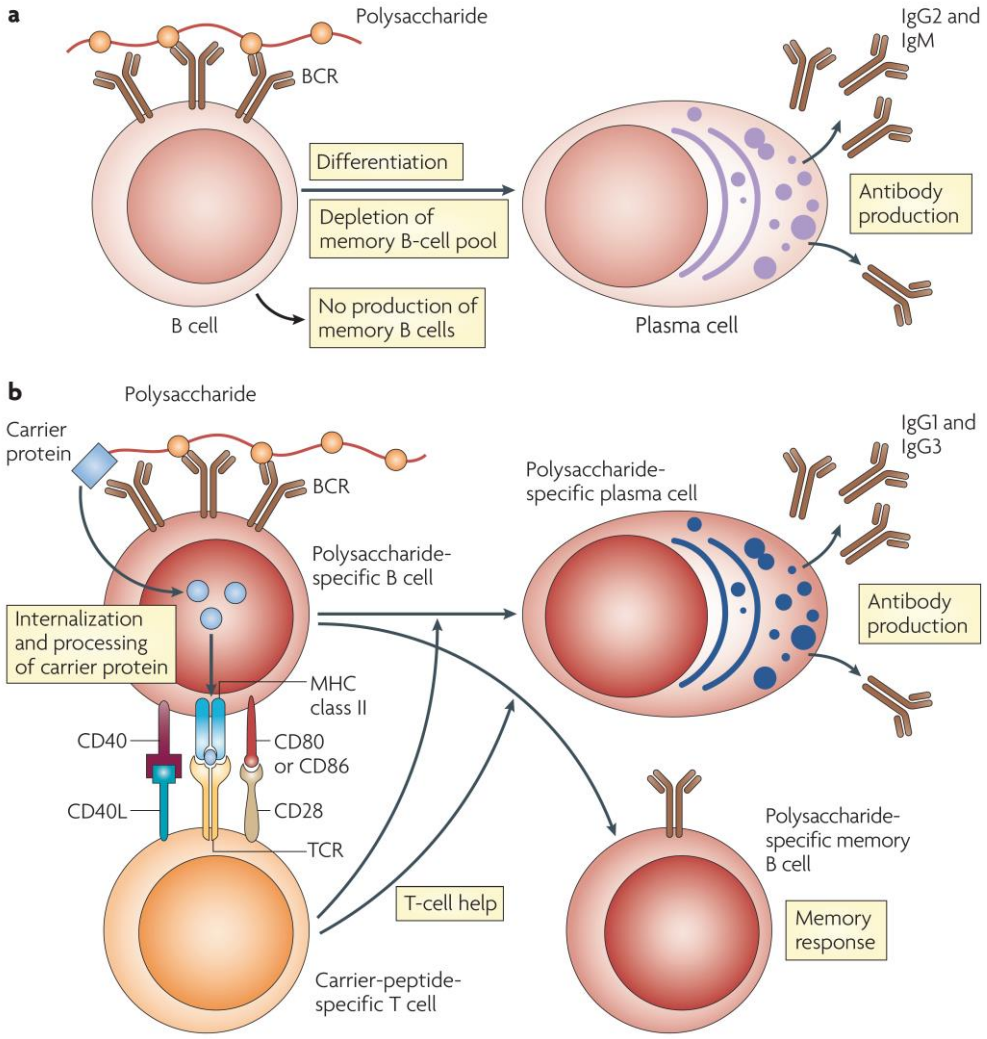


Figure 1.11. The immune responses to polysaccharide and protein-polysaccharide conjugate vaccines. A) Polysaccharides stimulate B cells by cross-linking the B-cell receptor (BCR) and drive the production of immunoglobulins. This process results in a lack of production of new memory B cells. **B)** The carrier protein from protein–polysaccharide conjugate vaccines is processed by the polysaccharide-specific B cell, and peptides are presented to carrier-peptide-specific T cells, resulting in T-cell help for the production of both plasma cells and memory B cells. Image source: (Clutterbuck et al. 2012)

1.7.3 Protection against nasopharyngeal colonisation

Nasopharyngeal colonisation precedes pneumococcal disease; therefore, prevention of carriage has been used as a proxy for vaccine efficacy. In the context of experimental human pneumococcal colonisation, a 78% reduction in colonisation acquisition following intranasal challenge with live 6B was observed in PCV13 vaccinated volunteers compared to the control arm (Collins et al. 2015). Within the same study, assessment of colonisation rates with a multiplex (lytA and CPS-6B specific gene) qPCR assay revealed low density colonisation, identifying additionally colonised participants than previously detected with classic culture. The latter findings suggest that the impact of PCV13 is likely due to a reduction in colonisation density rather than a reduction in the rate of colonisation (German et al. 2019).

Data from healthy, adult participants enrolled in EHPC studies have also been useful to assess naturally circulating serotypes. Samples taken in the first seven years following PCV13 introduction in the UK showed that circulating serotypes responsible for natural nasopharyngeal colonisation, are mostly non-vaccine types. However, there is an ongoing circulation of serotypes 3, 19A and 19F despite their inclusion in the PCV (Adler et al. 2019, Moore et al. 2015). Further data from a UK based study in children under the age of five and their household contacts revealed ongoing circulation of serotypes 3 and 19A, six years after PCV13 introduction (Southern et al. 2018). In particular, Spn3 vaccine escape has been attributed to Spn3 ability to release capsule and, thus, bypass antibody-mediated killing and protection by anti-CPS antibodies (Choi et al. 2016). Collectively, these studies imply that PCV13 fails to protect against nasopharyngeal colonisation by these serotypes and therefore fails to confer herd immunity against them.

1.7.4 Protection against pneumonia

PCVs confer optimal protection against IPD. The incidence of IPD caused by vaccine serotypes declined dramatically following the introduction of PCV7 and this was evident in many countries including the UK (Durando et al. 2013). Fall in PCV7 efficacy over time, due to serotype replacement, resulted to PCV13 introduction and PCV7 succession. A post licensure study reported a 90% protection against IPD caused by PCV7 serotypes and 73% for the additional PCV13 serotypes (Andrews et al. 2014). However, the

protective effect of PCVs against non-bacteraemic pneumonia is less well documented. Following the introduction of PCV7 in the UK, incidence rates of bacterial pneumonia and empyema from 2006-2008 in children less than 15 years old in England, dropped by 19% and 22% respectively. Incidence rates declined across all ages groups, despite vaccination being targeted at children under the age of 2 years old, implying the generation of herd immunity. However, this data was collected using International Classification of Disease version 10 (ICD10) codes and was therefore not specific to bacterial pneumonia caused by *S. pneumoniae.* Data also relied on correct coding by physicians (Koshy et al. 2010).

A five-year prospective cohort study (2008-2013) was conducted in two large UK hospitals to evaluate the impact of childhood PCV13 vaccination, against CAP in hospitalised adults. This study performed urine multiplex immunoassays and could distinguish between pneumococcal vaccine serotypes. It finished three years after the introduction of PCV13, therefore possible variations in serotype distribution, in response to wide vaccine coverage, were possible to determine. They reported an overall 16% decline in pneumococcal CAP per year, with an 88% reduction in the incidence of CAP caused by PCV7 serotypes and 30% reduction in the incidence of CAP caused by additional PCV13 serotypes. However, the incidence of CAP caused by non-vaccine serotypes increased from 2 years after PCV13 introduction. Changes to serotype distribution are shown in Figure 1.12 (Rodrigo et al. 2015). In the Netherlands, a large randomised controlled trial (RCT) (CAPITA study) aimed to determine vaccine efficacy for vaccine type invasive and non-invasive pneumococcal pneumonia, in adults greater than 65 years old, receiving either PCV13 or a control vaccination. Although, the study reported 45% vaccine efficacy against vaccine-type caused non-bacteraemic CAP and 75% against IPD, there was no protective effect against all-cause CAP, overall pneumococcal pneumonia or mortality (Bonten et al. 2015). In the Gambia, a RCT found similar results in infants (6-51 weeks old) receiving a nine-valent PCV. Vaccine efficacy was 37% for radiologically confirmed pneumonia, compared to 77% for IPD caused by vaccine-serotypes (Cutts et al. 2005).

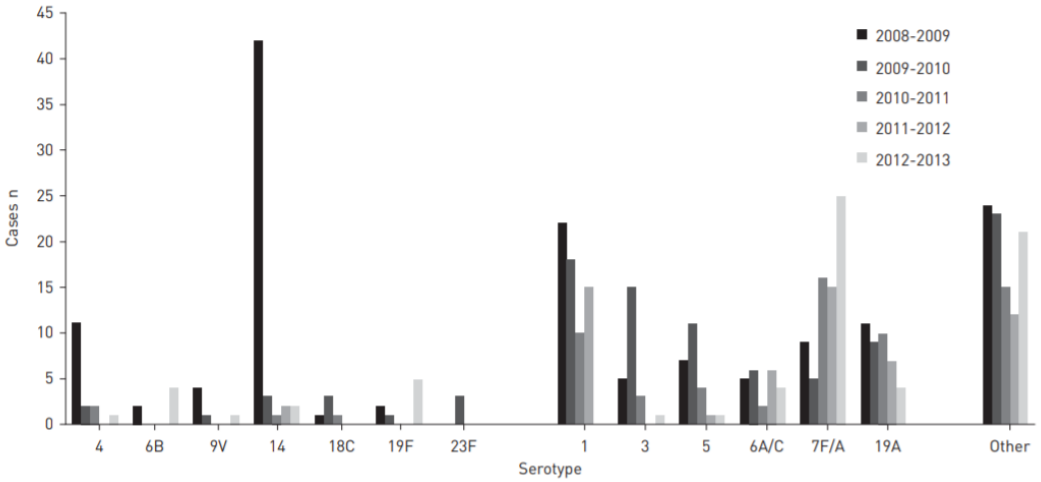


Figure 1.12. Variation in individual serotype numbers by study year among adults with pneumococcal community-acquired pneumonia in two UK hospitals (total n=2229 pneumococcal CAP cases). PCV13 was introduced PCV13 in 2010. Incidence of pneumococcal CAP caused by five PCV7 serotypes (4, 9V, 14, 18C and 23F) decreased over the study period. The incidence of CAP caused by 6B and 19F increased (not statistically significant). Pneumococcal CAP caused by five PCV13 additional serotypes (1, 3, 5, 6A/C and 19A) decreased over the study period. However, the incidence of pneumococcal CAP caused by 7F/A increased. The incidence of CAP caused by non-vaccine serotypes increased from 2012-2013. Image source: (Rodrigo et al. 2015).

Overall the research shows a discrepancy in direct vaccine efficacy against IPD compared to non-invasive CAP. This could reflect diagnostic challenges for uncomplicated CAP, as sputum samples are often inadequate and sputum culture has poor sensitivity, particularly when antibiotics have been administered (Musher, Montoya and Wanahita 2004). Diagnosis usually relies on chest radiography which cannot distinguish causative pathogens. Urinary antigen assays are available but may be unhelpful in children who are concurrently colonised (Dion and Ashurst 2019). Therefore, most often the causative pathogen remains undetected. Other respiratory pathogens including viruses are responsible for a significant burden of CAP, and national surveillance often depends on ICD10 coding which are nonspecific and may be inaccurate (Nouraei et al. 2016).

Suboptimal vaccine efficacy could also reflect poor humoral immune responses to PCV13 at a pulmonary mucosal level. Since there is clinically documented efficacy for PCV7, new generation of PCVs can be licensed on the basis of non-inferiority of induced IgG levels to CPS (WHO,2013). Collectively, data from three controlled efficacy trials

conducted in infants, estimated a capsular IgG concentration of 0.35µg/ml, which could be used as a correlate of protection against IPD in serum, applied to infants one month after vaccination (Siber et al. 2007). However, bronchoscopy is an invasive technique which is unethical to conduct in large scale clinical trials and no such level of protection has been accepted for anti-CPS IgG in BAL. Therefore, the protective effect of antibody responses in the lung is difficult to determine.

1.7.5 Drawbacks of PCVs

Prevention of pneumococcal infection with vaccination is safe and effective and their use prevents from excessive use of antibiotics, which has resulted in antibiotic-resistant strains. Despite the significant impact of PCVs on invasive disease, in part via the conferred herd immunity, which has minimised the spread of pneumococcus within the community, there are a number of drawbacks to PCVs.

1. No broad and suboptimal protection. Protection of the current PCVs is limited to a subset of serotypes, of which the prevalence varies geographically. Furthermore, two serotypes contained in PCV13: SPN3 and SPN19A, have continued to be detected in colonisation studies in fully PCV13 vaccinated children up to 2015/16, with no evidence of a decline in rates of colonisation for these 2 serotypes (Southern et al. 2018), which partially masks PCV13-mediated herd immunity in adults (LeBlanc et al. 2019) .

2. Serotype replacement. In the era post introduction of PCVs, colonisation and disease caused by vaccine-type serotypes have been reduced. However, it's is now well established that non-vaccine type (NTVs) pneumococcal serotypes have filled in the gap in the human niche, contributing to carriage and disease (Lewnard and Hanage 2019). This phenomenon is called serotype-replacement. The effects of replacement on public health have varied geographically. Striking differences have emerged between the UK and USA, countries that otherwise often resemble each other epidemiologically. Similar to other European settings, the UK has had rising non-vaccine serotype invasive pneumococcal

disease, most notably in older adults, mainly driven by PCV7 and less by PCV13 introduction of to the paediatric immunisation schedule. Such impacts of serotype replacement have not been reported in the USA, where incidence of non-vaccine serotype invasive pneumococcal disease in young children and older people has been stable since the introduction of the same vaccine (Lewnard and Hanage 2019). In addition, serotype replacement has resulted in poor matching to serotypes circulating in developing countries, which suffer the largest burden of disease (Feikin et al. 2013).

3. High cost. The conjugation of capsular polysaccharide with the protein carrier, although increases vaccine immunogenicity, has a major impact on the vaccine cost. The high cost of PCVs per dose has hampered its implementation in many developing countries. However, through GAVI Vaccine Alliance nearly 60 GAVI-supported countries have introduced pneumococcal vaccines into their routine programmes. The importance of sustaining PCV vaccination after transitioning from GAVI support was highlighted by a modelling studied in Kenya, which estimated that IPD caused deaths will doubled per year in the country within 10 years of PCV discontinuation. Importantly, the study estimated that PCV continuous use in Kenya post GAVI support period will require an estimated additional US$15·8 million annually compared to vaccine discontinuation (Ojal et al. 2019).

1.7.6 Novel pneumococcal vaccine development

Due to the high manufacturing costs of PCVs, combined with the lack of broad protection against pneumococcal disease, there is a need of novel vaccination strategies to complement current vaccination and treatment options. As a result, different research groups work on the development of novel pneumococcal vaccines (whole cell or protein-based vaccines), with some of them currently in preclinical and clinical trials.

One such vaccine being tested is the Whole Cell pneumococcal Vaccine (WCV), which is based on a killed, non-capsulated pneumococcal strain. The pneumococcal WCV

was used in two clinical trials (USA and Kenya) to study safety, dose tolerance and immunogenicity (Moffitt and Malley 2016). In healthy US adults WCV was shown to be safe and well tolerated; significant IgG responses to pneumococcal antigens were elicited, including PspA and pneumolysin. A third Phase 2 trial of the WCV have been completed in Kenyan adults and toddlers who are also receiving PCV immunisation (ClinicalTrials.gov Identifier: NCT02097472). Results of this trial, which would include evaluations of safety, tolerability and impact on pneumococcal carriage are not available yet.

The development of a protein-based pneumococcal vaccine is a topic that has attracted the interest of many researchers in the past and present, as immunisation with pneumococcal protein antigens could have the potential to induce serotype-independent protection. Several pneumococcal proteins, such as PspA, PspC, PhtD and PsaA, highly conserved among different pneumococcal isolates, have been proposed as new vaccine-candidates against pneumococcal infections (Miyaji et al. 2013). Their immunogenic properties have been tested in mice administration as single proteins or combined. However, their low immunogenicity presents a challenge for formulating vaccines, and thus an adjuvant is generally required for inducing sufficient immune responses. Molecular adjuvants, such as cytokines, toll-like receptors agonists and nucleic acids, can be incorporated into the formulation with the antigen to improve immunogenicity (Paranjpe and Muller-Goymann 2014). Lately, the use of delivery vehicles, such as nanoparticles, emulsions, and hydrogels, as adjuvant has gained attraction (Wang et al. 2015). Such formulations can improve the antigen exposure to the immune cells and also improve uptake by the antigen presenting cells. Although there are currently no approved intranasal or pulmonary protein vaccines, several preclinical formulations using serotype-independent antigens against *S. pneumoniae* have been explored (Goncalves et al. 2019).

1.8 Experimental Human Pneumococcal Challenge Model

The study of controlled colonisation in healthy volunteers has proven to be instrumental to dissect the dynamics of mucosal carriage of bacteria that precedes invasive bacterial infection. The described experimental human pneumococcal challenge model developed by Prof. Jeff Weiser and his colleagues in United States, where both serotypes 23F and 6B were used in dose-escalation studies (McCool et al. 2002). The Experimental Human Pneumococcal Challenged (EHPC) model, developed and based in Liverpool School of Tropical Medicine (Gritzfeld et al. 2013), is currently the only controlled human infection model (CHIM) with *S. pneumoniae* in the world, whereas its establishment in a low income country, Malawi, is ongoing. EHPC is safe and reproducible and enables investigation of pneumococcal biology, as well as the human host immune responses against the pathogen.

Intranasal inoculation of live pneumococcus, serotype 6B, with an average dose of 80,000 CFU/100µl leads to approximately 50% colonisation in healthy volunteers and its duration resembles that of natural colonisation. Pneumonia or invasive pneumococcal disease has never been reported in studies of more than 1500 inoculations and over 300 research bronchoscopies (Collins et al. 2014) done so far. Up to date the model has been utilised to predict efficacy of licenced vaccines, to test new vaccine protein-candidates, as a co-infection model, whereas recently has been used to evaluate transmission in adults. The endpoint of these clinical trials was the nasopharyngeal colonisation, with colonisation rates and pneumococcal density being the primary outcomes. The model has been also instrumental in studying natural protection, the dynamics of the nasal microbiome (de Steenhuijsen Piters et al. 2019), and systemic and mucosal immunity in the upper and low respiratory tracts, the sites where infection occurs. Given the scientific advances and the favourable safety profile of the pneumococcal colonisation model, the model has been expanded to explore the susceptibility of at-risk populations including people with asthma (ISRCTN16755478), smokers and the elderly (ISRCTN10948363).

1.8.1 The EHPC model as a method to predict vaccine efficacy

Using colonisation status as a surrogate for infection, the EHPC model can be used to assess the protective efficacy and herd protection potential of new vaccine. As proof-of-concept, a licenced pneumococcal conjugated vaccine, the PCV13, was tested

in healthy young adults in a randomised controlled clinical trial in protecting against nasopharyngeal colonisation (Collins et al. 2015). PCV13 was found to reduce significantly the rate of 6B colonisation and density in vaccinated healthy adults (18-50years of age), using classical culture. The protective effect of PCV remained robust after the re-assessment of colonisation status using molecular methods, although revealed an increased rate in low-density colonisation (German et al. 2019). The results of this RCT paved the way for the use of EHPC model for testing new protein-based vaccines, as it has proven safe, efficient and cost-effective in investigating the protective effects of the licensed vaccines.

The following year, a novel vaccine candidate, the GEN-004 vaccine, was tested in the EHPC model. The GEN-004 vaccine, consisting of three antigens identified following a proteomic screen for proteins that elicit robust Th17 cell responses (Streptococcus pneumoniae proteins SP0148, SP1912, and SP2108), was protective against pneumococcal carriage in murine models (Moffitt et al., 2014). This vaccine consistently reduced carriage acquisition by 18% to 36% versus placebo, but the differences were not statistically significant because the study was powered to detect 50% reduction (Skoberne et al. 2016). There was no evidence of reduced colonisation density or duration up to day 14 following inoculation. However, any Th17 cellular responses induced by the vaccine in this study have not yet been reported, which limits the conclusions one can draw from the study.

1.8.2 EHPC as a co-infection model

Primary viral infection has been associated with increased inflammation and higher risk of pneumococcal disease development. EHPC has been used as a virus-pneumococcal co-infection model and has provided important insights into how viruses effect acquisition of pneumococcal colonisation. Using the model, Glennie at al. showed that asymptomatic upper respiratory viral infection in the week preceding inoculation increases the risk of becoming colonised. Increased levels of nasal Factor H facilitated the adherence of pneumococcus to the human epithelium via PspC (Glennie et al. 2015). In another EHPC study, the dynamics of transient influenza virus-pneumococcal co-infection were investigated in the context of a transient influenza infection caused by vaccination with Live Attenuated Influenza Vaccine (LAIV) (Rylance et al. 2019). LAIV is

administered annually in children as a way to prevent seasonal influenza infection. The effect of LAIV vaccine on pneumococcal colonisation was tested in two subsequent RCT trial with inverted order of LAIV immunisation and pneumococcal challenge. Overall, the study showed that antecedent LAIV administration caused a significant, transient increase in pneumococcal density (1 log of CFU), whereas the inversed scenario was associated with reduced colonisation density and colonisation rates at Day 27, decreased AUC, and earlier bacterial clearance (Rylance et al. 2019). Further analysis of the immunological parameter demonstrated that inflammation induced by primary LAIV administration impaired the function of neutrophils and inhibited the monocytes infiltration in the nasal mucosa, resulting in the transient increase of pneumococcal density by hindering bacterial clearance (Jochems et al. 2018). On the other hand, pneumococcal precedence impaired acute inflammation post LAIV administration and affected LAIV immunogenicity (Carniel, Mitsi et al, 2019, unpublished data). Interactions of nasal microbiome and pneumococcus were also studied in the context LAIV, identifying microbiome profiles that are associated that are with low-density colonisation, in particular Corynebacterium/Dolosigranulum-dominated profiles (de Steenhuijsen Piters et al. 2019).

1.8.3 Advances in immunity against *S. pneumoniae* and correlates of protection

Findings derived from studies conducted in the EHPC model have identified humoral correlates of protection against nasopharyngeal pneumococcal colonisation. Natural exposure to serotype 6B resulted in greater levels of capsular polysaccharide, IgG secreting memory B cells to the homologous strain, and was associated with protection against acquisition of colonisation (Pennington et al. 2016). In the context of PCV vaccination, elevated levels of mucosal IgG to CPS-6B post vaccination blocked acquisition of colonisation through bacterial agglutination (Mitsi et al. 2017). In absence of vaccination, naturally acquired baseline levels to CPS-6B antibodies do not correlate with protection in adults. Establishment of colonisation post the bacterial challenge elicits capsular specific antibodies both systemically and in the nasal mucosa, (Ferreira et al. 2013, Mitsi et al. 2017, Pennington et al. 2016), and it has been characterised as a protective event against re-acquisition of the homologous pneumococcal serotype (Ferreira et al. 2013). Colonisation also elicits antibody responses against some

pneumococcal proteins, such as PspA, PspC and pluA (Ferreira et al. 2013), but their role in colonisation protection has not been elucidated yet in humans. The current findings suggest that naturally acquired humoral Immunity to the capsule is superior to pneumococcal proteins in protecting against carriage.

Furthermore, the model has revealed innate and adaptive cellular responses that are implicated in acquisition or clearance of colonisation. It was observed that in the human nose neutrophils are already present in high numbers and that pneumococcal colonisation leads to only a modest further recruitment (Jochems et al. 2018). Instead, colonisation triggered monocytes recruitment to the nasal lumen. Degranulation of pre-existing neutrophils in the nasal cavity was important for the initial control of carriage, whereas monocytes promoted clearance of nasopharyngeal colonisation (Jochems et al. 2018). The nasal mucosa cellular populations were further studied in the model, using advanced resolution techniques, such as CyTOF, and larger scale nasal sampling by taking nasal biopsies (Jochems et al. 2019). This study showed that increased responses of blood MAIT cells against *in vitro* stimulation with pneumococcus before the pneumococcal challenge associated with protection against establishment of colonisation and with increased mucosal MAIT cell populations, suggesting that MAIT cells may contribute in the protection against pneumococcal colonisation (Jochems et al. 2019).

Conventional $CD4^+$ T cells have also been investigated in the model. Experimental pneumococcal colonisation mediated the induction of pneumococcal-specific, Th17 $CD4^+$ T cells, which were elevated in both blood and the lung (Wright et al. 2013). This is an important observation but one of the few reports of lung immunity against *S. pneumoniae* derived from the model. The effect of pneumococcal colonisation on other cellular populations, with crucial role in guarding the lungs from infection has been poorly described. Therefore, the research work presented here has focused on the relationship between nasopharyngeal pneumococcal colonisation and lung immune responses in healthy adults, examining alterations in innate and adaptive lung immune responses and cytokine milieu of alveolar microenvironment. The findings of this work highlight the role of the model as a platform for testing mucosal administered vaccines.

1.9 Project Aims and Objectives

The overall scope of this research project was to investigate whether and to what expend nasopharyngeal pneumococcal colonisation influences lung immune responses to *S. pneumoniae.* To address this overarching research question, the project was designed around the following aims:

1. To investigate alveolar macrophage polarisation, shift and other phenotypic changes post pneumococcal colonisation
    - Define human AM phenotype and polarisation profile in the steady state
    - Validate and compare findings with an age-matching cohort from different setting
    - Measure expression levels of key activation markers and scavenger receptors on AM surface

2. To test the hypothesis of pneumococcal aspiration in the lungs during nasopharyngeal colonisation
    - Search for evidences of pneumococcal presence in the lungs, in the form of intact cells, fragments or pneumococcal DNA
    - Correlate nasal pneumococcal density with levels of pneumococcal DNA detected in the kung

3. To investigate the effect of colonisation on lung innate immune responses
    - Assess AM opsonophagocytic capacity post *in vivo* exposure to pneumococci
    - Elucidate the mechanism that augments AM opsonophagocytic capacity, if present, and assess the duration of this effect
    - Assess whether colonisation initiates inflammatory responses in the lung, resulting in neutrophils or monocytes infiltration

4. To investigate the effect of colonisation on lung adaptive immune responses
    - Assess alterations on the levels of CD4+ T cell subsets post colonisation

- Validate the findings of a previous EHPC study relative to pneumococcal-specific CD4+ Th17 elevated levels in lung post colonisation
- Define whether similar pneumococcal specific responses can be detected in other conventional T cells subsets or TCR γδ T cells
- Compare levels of anti-CPS-6B IgG in bronchoalveolar lavages across carriage status.

Chapter 2

Material and Methods

This chapter contains all the methodological approaches used throughout this project to generate the data presented in the following chapters.

2.1 Ethical Approval

All the experimental human pneumococcal challenge (EHPC) studies were in compliance with the Good Clinical Practice (GCP) and with the principles set out in the Declaration of Helsinki (World Medical, 2013). All studies where approved by local Research Ethics Committees (RECs), which are part of the United Kingdom National Research Ethics Service (NRES) (Table 2.1).

Table 2.1. List of experimental human pneumococcal challenge (EHPC) studies related to the project and theirs REC reference.

Title of EHPC study	REC Reference	Primary objective
Mucosal sampling pilot study	15/NW/0146	Assessing the impact of serial nasal scraping on carriage rates
The Effect of Live Attenuated Influenza Vaccine (LAIV) on EHPC – **Study A**	14/NW/1460	To assess the impact of Antecedent LAIV administration on carriage rates
The Effect of Live Attenuated Influenza Vaccine (LAIV) on EHPC – **Study B**		To assess the impact of Concurrent LAIV administration on carriage rates
New strains study- Re challenge phase	15/NW/0931	To establish a new colonisation model with a new strain. A non-vaccine strain 15B strain was tested in the model, following re-challenge with Spn6B at 6 to 12 after the first inoculation.

2.2 Studies design

The results described at the following chapters have been obtained by EHPC studies, where *S. pneumoniae*, serotype 6B (Spn6B) was used in achieving colonisation of the nasopharynx. These studies included lung sampling through the technique of research bronchoscopy.

2.2.1 Study design of EHPC

Healthy, non-smoking, adult volunteers aged from 18-50 years, enrolled in one of the EHPC studies (Gritzfeld et al. 2013) which conducted in Liverpool between 2015-2018. All volunteers had a baseline (Day -5) nasal wash sample, whereby we assessed naturally acquired colonisation. On Day 0, volunteers were challenged intranasally with a safe dose (80,000 CFU per naris) of live pneumococci, serotype 6B. Participants were followed up for 2 to 4 weeks post the challenge (Figure 2.1). Pneumococcal colonisation was detected by classical microbiology methods and individuals were defined as Spn colonised (carriage+) if any nasal wash culture following experimental challenge grew *S. pneumoniae,* serotype 6B. A subset of Spn colonised and non-colonised participants underwent a single time point research bronchoscopy, at any day between 21 to 203 days post the intranasal pneumococcal inoculation. Spn colonised individuals received 3 doses of amoxicillin at the end of the clinical trial (at day 14 or 27 or 29) prior to the bronchoscopy (Figure 2.1).

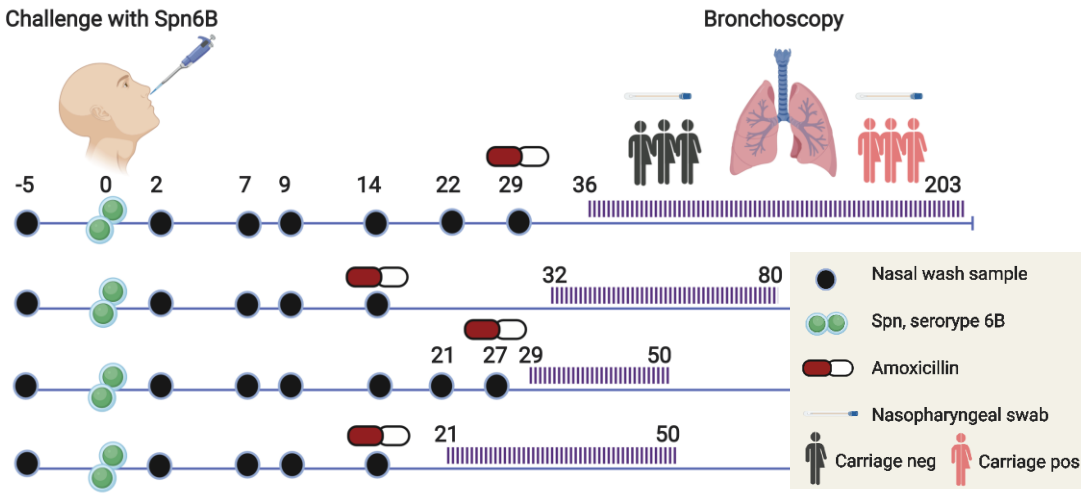


Figure 2.1. Study design of EHPC studies which included lung sampling through research bronchoscopy. Intranasal pneumococcal challenge at D0 with follow-up visits. Bronchoscopy were performed after the end of each trial and antibiotic treatment in the carriage positive volunteers.

2.3 Ethics statement

All volunteers gave written informed consent and research was conducted in compliance with all relevant ethical regulations. Ethical approval was given by the North-west National Health Service Research Ethics Committee (REC). Ethics Committee reference numbers: 15/NW/0146, 14/NW/1460 and 15/NW/0931 and Human Tissue Authority licensing number: 12548.

2.4 Clinical procedures

The intranasal pneumococcal challenge and the samples collection during the clinical trials involves a number of procedures, which are described in detail below.

2.4.1 Nasopharyngeal inoculation

Intra-nasal inoculation with *S. pneumoniae* was carried out by a trained clinical staff. The inoculation was performed while the volunteer was seated comfortably in a semi-recumbent position. With the head tilted back slightly, the tip of a P200 pipette was inserted just inside the nasal cavity and 100 µl of the bacterial inoculum was slowly dispersed across the nasal mucosa in a circular motion (Figure 2.2).

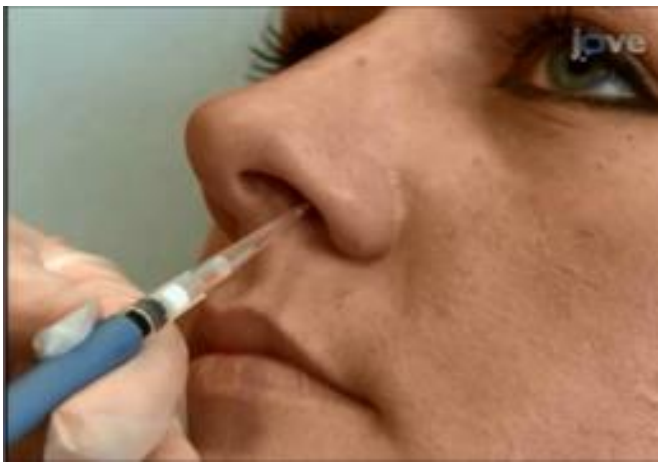


Figure 2.2. Inoculation of the nasal mucosa with live *S. pneumoniae.* Live pneumococci were installed in the nasal cavity of each volunteer with the use of pipette and both nostrils were inoculated with the same bacterial dose. Picture reproduced with consent of the volunteer.

During the inoculation, it was important that the pipette tip did not come in contact with the nasal mucosa, as a disruption in the integrity of the epithelium could result in bacteria entering the bloodstream. It was also important that the inoculum was not expelled too far back in the nasal cavity as it would run down the throat and be swallowed instead of remaining in the nasopharynx. The inoculation was repeated for the other nostril and then the volunteer remained in the semi-recumbent position for 10 minutes without sniffing or blowing the nose.

2.4.2 Nasal wash sampling method

Nasal wash was used to sample the nasopharynx of volunteers. Samples were collected one week before inoculation and at several time points post inoculation (Figure 2.1). A volunteer was seated comfortably, and the head was tilted back 30° from the vertical. The volunteer was then asked to take a deep breath in and hold their breath whilst pushing their tongue up and backwards against the roof of the mouth. A syringe filled with 20 ml of sterile saline was inserted into the anterior nasal space and 5 ml of saline was expelled. The volunteer then leaned forward immediately and expelled the fluid by exhaling rapidly through the nose into a foil bowl (Figure 2.3). The procedure was repeated three more times so that each naris had been washed twice and a total of 20 ml was used. Following collection, the samples were pooled in a centrifuge tube and transported to the laboratory at room temperature.

Figure 2.3. Nasal wash sample collection. A syringe filled with 20 ml of sterile saline was inserted into the anterior nasal space and 5 ml of saline was expelled. The volunteer then leaned forward and expelled the saline by exhaling rapidly through the nose into a foil bowl. Picture reproduced with permission of Dr. J. Rylance.

2.4.3 Nasopharyngeal sample collection and processing

After the treatment with antibiotics (only the pneumococcal colonised group) and during the preparation for the bronchoscopy, a nasopharyngeal swab (NPS) and an oropharyngeal swab (OPS) was collected in a medium containing skim milk, tryptone, glucose and glycerine (STGG) per volunteer to assess the clearance of pneumococcal colonisation. As, pneumococci usually reside the posterior of the human nasopharynx, this was the most suitable sampling method.

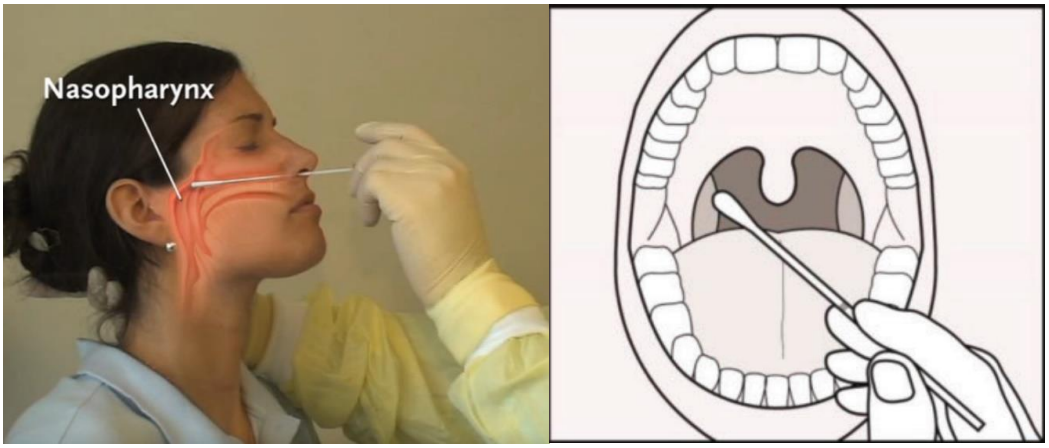


Figure 2.4. Collection of NPS and OPS prior the bronchoscopy. NPS and OPS were collected to assess clearance of pneumococcal colonisation in the nasopharynx of the volunteers on the day of the bronchoscopy. Picture that demonstrates collection of NPS was adapted form NEJM Procedure (Collection of Nasopharyngeal Specimens with the Swab Technique).

2.4.4 Bronchoscopy

In preparation for the bronchoscopy, participants received local anaesthesia using topical lidocaine gel and spray, with further 4% lidocaine administered to the larynx and 2% lidocaine to the bronchial tree via the scope. Using a single use, flexible bronchoscope (Figure 2.5), warmed 0.9% saline was instilled to the right middle lobe in sequential aliquots (40-50 ml). Bronchoalveolar lavage fluid (BAL) was aspirated into a sterile syringe using gentle manual suction. BAL yields were recorded, and the samples were transported immediately to the laboratory on ice (Figure 2.5). The volunteers' heart rate, blood pressure and oxygen saturations were continuously monitored during the procedure, with supplemental oxygen given by nasal cannula. All procedures were carried out in a research ward by a senior bronchoscopists, experienced in obtaining BAL for research purposes.

Figure 2.5. Bronchoscopy and bronchoalveolar lavage sample collection. Bronchoscope was inserted through the nasal route and warm saline was instilled into the right middle lobe. BAL fluid was collected in sterile tubes and transferred on ice.

2.5 Laboratory procedures

Bacterial stocks for either inoculation or *in vitro* assays and BAL sample processing where the core laboratories procedures used in this study.

2.5.1 Pneumococcal growth conditions for *in vivo* administration

A clinical pneumococcal isolate of serotype 6B, strain BHN418 (a gift of Prof. P Hermans, Radboud University Nijmegen, Netherlands) grew under aseptic conditions and was used for intranasal challenge in healthy, non-smoking adults, aged 18 to 50 years. The strain was initially cultured on Columbia Blood Agar with horse blood (Oxoid) and incubated at 37°C overnight in 5% CO_2.The following day a "parent" bead stock was made by scraping all the overnight growth from the blood agar plate and was added into a cryovial containing ceramic beads and cryo-preservative fluid (Technical Service Consultants Ltd.). Vials were stored at -80°C and were tested for purity and colony uniformity before being used to prepare the inoculum stocks. These "parent" stocks (also called as seed stock) were used to prepare all subsequent inoculation stocks.

2.5.2 Inoculum stock preparation

Preparation of inoculum stocks was carried out in a fumehood, using a dedicated incubator and set of pipettes. A loopful from the "parent" bead stock was streaked for heavy growth onto a blood plate, ensuring that the entire plate was covered. The plate was then incubated overnight at 37°C, 5% CO_2, following liquid culture the next day. A

vegetable-based growth medium- Vegitone Infusion broth (Fluka, Sigma-Aldrich, Missouri, USA) was use for the liquid growth phase of the inoculum, as it was crucial that no animal products were administered to the volunteers. The bacterial culture was incubated at 37°C, 5% CO_2 (no shaking) until mid-log phase was reached (0.30-0.35 OD measured at 600nm), following centrifugation at 3345g for 15min. Supernatant was discarded, whereas the bacterial pellet was resuspended in fresh Vegitone broth plus 20% v/v glycerol (Sigma-Aldrich, glycerol for molecular biology). After thorough mixing, 1ml aliquots were prepared and stored at -80°C. Within a week after freezing, the concentration of each stock was quantified by applying the Miles and Misra method on two to three stock aliquots (M&M, see section 2.5.4) to ensure reproducibility. Each inoculum stock (1ml vial was used per inoculum session) was diluted in sterile phosphate buffered saline (PBS) from known concentration to the administrated inoculum dose, which was fixed at 80,000 CFU/100ul per nostril across the above mentioned EHPC studies (Table 2.1).

2.5.3 Bacterial strains and growth conditions for *in vitro* assays

The challenge strain (serotype 6B, BHN41) was also used in *in vitro* assays, such as alveolar macrophages opsonophagocytic killing assay (OPA) and cell stimulation assays in a heat-inactivated form. For the purposes of these experimental assays, the pneumococcal strain was cultured as described above but in Todd Hewitt broth supplemented with 0.5% yeast extract (THY) until early log phase. Other bacteria used in the OPA were two gram positive (*Streptococcus pyogenes* and *Staphylococcus aureus*) and one gram negative (*Escherichia coli*). *S. pyogenes* (MGA315) was cultured from a single colony in Brain Heart Infusion (BHI) broth overnight at 37^{o}C with 5% CO_2. *S. aureus* (human isolate from EHPC trial) and *E. coli* (NCTC86) were cultured in BHI and Luria Broth (LB), respectively, from a single colony overnight, on a shaking rotor at 37^{o}C with aeration. All bacterial stocks were grown till the early log phase. After addition of 20% v/v glycerol, they were aliquoted to 1ml vials and stored at stored at -80^{o}C until further use. The concentration of each bacterial stock was quantified by Miles and Misra method.

2.5.4 Quantification of bacterial stock – Miles and Misra method

A modification of the Miles & Misra method was used to perform viability counts on the bacterial stocks prior use in the experimental assays or on the inoculation Spn6B stocks (Miles, Misra and Irwin 1938). Blood or LB agar plates were divided into six sections, which were labelled from 1 to 6 (Figure 2.6). In a 96 well U-bottom plate (Corning Inc.), 20 µl of bacteria was added to 180 µl of sterile PBS and serially diluted (10-fold) until a dilution of 10^6 was reached. Three drops of 10 µl from each dilution were placed on the corresponding section of the plate and allowed to dry. The plate was then inverted and incubated overnight at 37°C, 5% CO_2. The next day, visible colonies in each dilution section were counted and recorded (Figure 2.6).

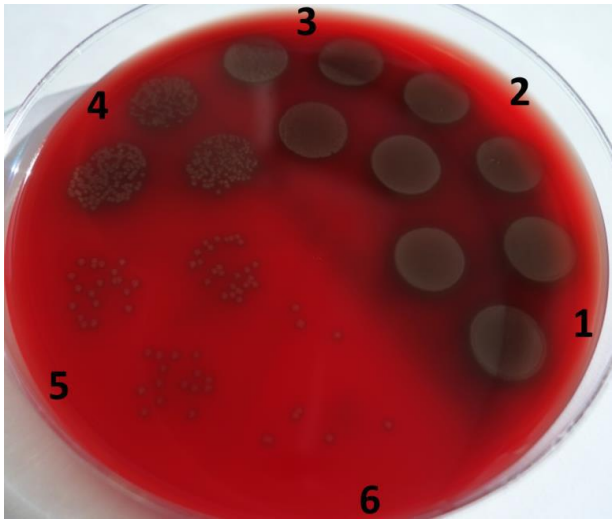
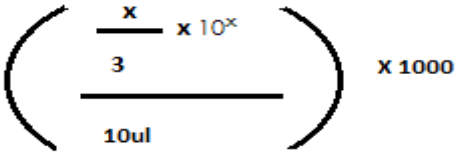


Figure 2.6. Miles and Misra bacterial quantification. Three 10 µl drops from a serial dilution were placed on the corresponding dilution section of the plate. The plate was then inverted and incubated overnight at 37°C, 5% CO_2. Individual colonies can be counted in the 5^{th} and 6^{th} dilution.

The final concentration of the bacterial stock was calculated using the following equation. (x= number of total colonies counted in a dilution section, 10^x = dilution factor). Bacterial concentration was expressed as CFU/ml.



2.5.5 Nasal wash sample processing

Nasal wash samples were processed using our published method (Gritzfeld et al. 2013). The collected samples were transferred to the laboratory within 1 hour after collection and centrifuged at 4,000g for 10min. The pellet was separated from the supernatant and was resuspended in 100µl of STGG. 20µl of suspension were plated directly on a horse blood agar plate supplemented with 4µg/ml gentamycin (Sigma). Additionally, 10µl were serially diluted 4 times in a 96-well plate and Miles & Misra quantification method was performed on a gentamycin-enriched blood agar plate to determine the pneumococcal density (section 2.5.4).

2.5.6 Nasopharyngeal and Oropharyngeal swab sample processing

The collected nasopharyngeal and oropharyngeal samples were plated directly on a gentamycin-enriched blood agar plate. The swab was placed back in the tube and the sample was frozen at -80^{o}C until DNA extraction.

2.5.7 Bronchoalveolar lavage processing

BAL samples were processed as previously described (Mitsi et al. 2018, Zaidi et al. 2017). During the bronchoscopic procedure, the BAL fluid was collected in several sterile, plastic, non-adhering, 50ml tubes and stored on ice. BAL samples were transferred to the lab and were processed within 30min from the collection time. The whole BAL sample was filtered using a 70µM filter (Thermofisher) placed on the top of a 250ml sterile conical flask. This enabled the combination of subsequent BAL fractions, generating a homogeneous lavage fluid. Then, the combined BAL fluid was re-distributed to 50ml falcon tubes and centrifuged at 400g for 10 min at 4°C. The supernatant was removed and kept at -80^{o}C till further use. The cell pellets were resuspended in cold RPMI (Gibco™ RPMI 1640 medium) containing 1% antibiotic cocktail (Penicillin,

Neomycin and Streptomycin- PNS, Sigma-Aldrich) and combined in one tube. The centrifugation step was repeated once, and the final cell pellet was resuspended in cold RPMI, plus 1% PNS and 10% heat-inactivated, fetal bovine serum (HI-FBS, Gibco) (hereafter referred to as complete RPMI). Viability cells count was performed in each BAL sample, using trypan blue in raw material and a haemocytometer. This method allowed us to differentially count alveolar macrophages (AM) and lymphocytes (Figure 2.7). BAL samples yielded a median 5.81 x 10^6 cells/ml (IQR: 5.81 x 10^6 – 7.81 x 10^6 cells/ml), from which approximately 80% was constituted by AM (median: 4.5 x 10^6 AM/ml, IQR: 2.65 x 10^6 - 6.5 x 10^6 AM/ml). Following counting, the cell concentration was adjusted to 5 x 10^5 AM/ml, following seeding of 2.5 x 10^5 AMs per well in a 24-well plate (Corning) and incubated overnight. In BAL samples with high cell yield, a faction of cells was spun down at 400g for 10 min and the cell pellet was resuspended in cryo-preservative medium (CryoABC, Immunospot), following gradual freezer in -80^{o}C and liquid nitrogen tank for further use.

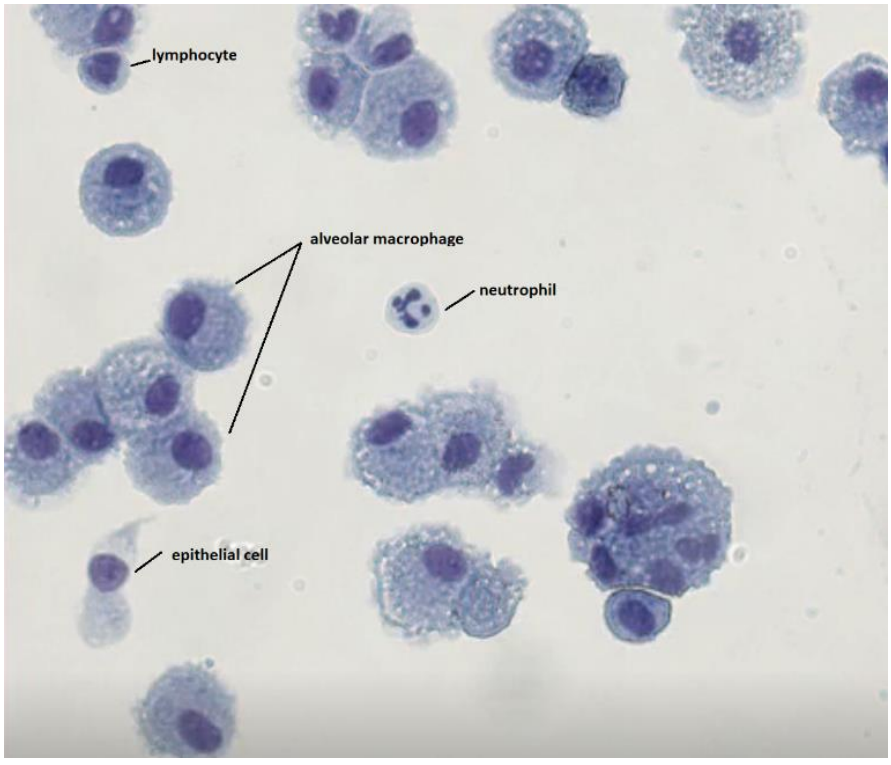


Figure 2.7. Microscope image of whole BAL differential staining. Alveolar macrophages represent the most abundant lung cellular population (adherent cells), whereas lymphocytes (non-adherent) are commonly found in lower proportion. Neutrophils are immune cells

occasionally present in the healthy human lung. Additionally, the presence of bronchial epithelial cells in the BAL sample is not very common. Image source: (Collins et al. 2014)

2.5.8 Alveolar macrophages isolation

AMs were routinely separated from other cell populations by seeding and adherence on 24-well plate (Greiner Bio-One, Kremsmünster, Austria) (Wright et al. 2013). After 4 hours adherence step, the non-adherent fraction was removed. AM remained adherent on the 24-well plate and the culture was replenished with warm complete RPMI, following overnight incubation at 37^{o}C with 5% CO_2. A spare well of seeded AM was used for assessment of their viability prior to functional assays. 250ul of 2.5mM EDTA in cold RPMI was added in the well to accelerate AM detachment from the plastic surface, following viability assessment by Trypan blue staining. In experiments where a highly pure AM population (95% purity based on the cell sorter counts) was required, AMs were purified from the whole BAL sample (half BAL cell pellet used) through cell sorting (FACS ARIAIII cell sorter, BD Biosciences), using a combination of cell surface antibody markers for a range of lung cell population (Table 2.2). Gating strategy followed for AM sorting is illustrated on Figure 2.8. In addition, the characteristic size and granularity of AM further enabled the identification of this major lung population and resulted in a highly pure AM extract after the cell sorting. The isolated AMs were counted under the microscope (median: 1.1 x 10^6 AM/ml, IQR: 0.8 x10^6 – 1.7 x10^6 AM/ml) and seeded on 96-well plate at the concentration of 2.5 x10^4 per well, following overnight incubation at 37^{o}C, 5% CO_2.

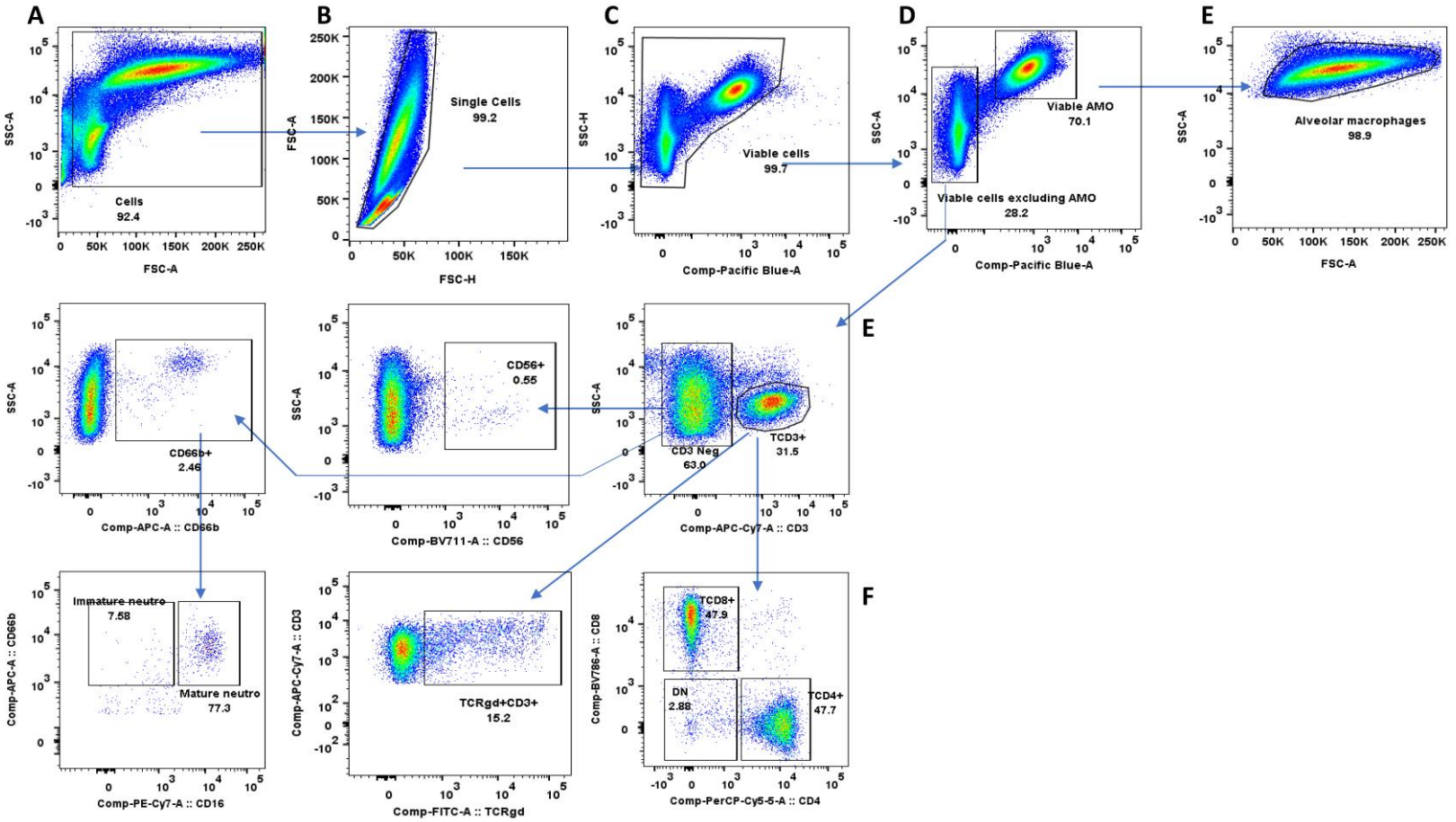


Figure 2.8. Gating strategy of alveolar macrophage sorting. Alveolar macrophages were sorted through negative selection from other lung cell populations utilising their (A) district size and granularity and (D) autofluorescence in the violet channels.

2.5.9 BAL lymphocytes isolation

BAL lymphocytes were routinely separated from AMs post the 4 hours adherence step. Then, the cells were centrifuged at 400g for 5min, resuspended in complete RPMI and seeded in 96-well plates at equal concentrations of 600,000 to 1 million cells. In some experiments, isolated $CD4^+$ T cells were used. $CD4^+$ T cells were isolated from fresh whole BAL sample post cell staining with a combination of key cell surface antibody markers (Table 2.2) and following sorting using a FACS ARIAIII cell sorter.

Table 2.2. Conjugated antibodies used for sorting of human alveolar macrophages and $CD4^+$ T cells from whole BAL sample. This panel was used in results of Chapter 4.

Marker	Fluorochrome	Clone	Reference
TCR-γδ	FITC	11F4	304006
CD4	PerCPCy5.5	SK3	344608
CD16	PECy7	3G8	302016
CD66b	APC	G10F5	305118
CD3	APC-Cy7	SK7	344818
Live and Dead	BV510	NA	L34957
CD56	BV711	NCAM	563169
CD8	BV785	SK1	344734

2.6 Experimental assays

The functional, molecular, flow cytometric assays and immunoassays used in this project are described in more details below.

2.6.1 Alveolar macrophage opsonophagocytic assays (OPA)

AMs opsonophagocytic capacity was evaluated as previously described with minor modifications (Wright et al. 2012). Live *S. pneumoniae* serotype 6B (inoculation strain) or *S. pyogenes* or *S. aureus* or *E. coli* were opsonized in a 1:16 final dilution of human intravenous immunoglobulin (IVIG, Gamunex, Grifols Inc, Spain) in HBSS +/+ (with $Ca2^+$ $Mg2^+$) at 37°C for 15min. AMs were washed twice with pre-warmed plain RPMI and were

incubated with an opsonised bacterial strain in Opsonisation Buffer B (HBSS +/+ plus 1% gelatine solution and 5% FBS) and 10% baby rabbit complement (Mast Group, UK) at 37^{o}C on a shaking rotor for 60min. Following incubation, 10 µl of reaction mixture was plated, in triplicate, onto blood agar (Oxoid) or Luria-broth agar plates and incubated at 37°C, 5% CO_2 overnight. Colony forming units (CFUs) from cell supernatants were counted the following day. Multiplicity of infection (MOI) used was 1 :100 for all the gram-positive bacteria. Opsonophagocytic killing assay for the gram-negative (*E. coli*) was modified as described elsewhere (MOI= 1:20 for 30min and 7% baby rabbit complement) (Abbanat et al. 2017).

In the assays where cell-sorted AMs were infected with opsonised Spn6B, the MOI was modified to 1:20 due to increased loss of cells during the high-throughput cell sorting. These experiments included a condition, in which purified AMs were co-cultured with autologous $CD4^+$ T cells (isolated from the same BAL sample through cell sorting). The $CD4^+$ T cells were added on the top of AMs, just before the uptake assay, in 1:5 ratio (CD4: AM).

In some experiments AMs were stimulated with 2ng/ml, 20ng/ml, 200ng/ml and 2,000ng/ml of recombinant IFN-γ (Bio-techne). The different concentrations of IFN-γ were added to the cells during the preparatory steps of opsonophagocytic assays. No cells pre-treatment with recombinant IFN-γ occurred. Additionally, in the experiments, the IFN-γ receptor 1 (IFN-γR1) was neutralised, using anti-human IFN-γR1 (Bio-techne). The monoclonal antibody was added in AMs culture at the concentration of 0.15µg/ml in the presence of up to 2ng/ml recombinant IFN-γ (Neutralization Dose as per manufacturer instructions) 30min prior to IFN-γ addition.

Due to the lack of reading of lysosome acidification within AMs, the term pneumococcal/bacterial uptake rather than killing was used. The assays results were calculated using the equation: “Bacterial uptake (%) = 100 – [(100*Condition)/Control] “, where ‘Condition’ represents the average of CFUs recovered from each experimental condition and ‘Control’ represents the average CFU recovered from the bacterial dose (received only bacteria, OBB and complement but no cells).

2.6.2 Bacterial DNA extraction from BAL samples and quantification of pneumococcal DNA by qPCR

The extraction of bacterial DNA from the BAL, OPS and NP swab samples was performed, using our published protocol with minor modifications (Connor et al. 2018). 15ml of uncentrifuged BAL or 200ul of NP and OP sample were separated from total sample and centrifuged at full speed for 15min. Following centrifugation, the supernatant was discarded, and DNA was extracted from the pellet using the Agowa kit for bacterial DNA extraction. The extracted DNA was eluted in a volume of 63ul of elution buffer. DNA purity and quality were assessed by a spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific).

Presence of pneumococcal DNA in BAL samples was determined using primers and probe specifically designed for 6B serotype, targeting on a capsular polysaccharide gene known as *wciP*, the rhamnosyl transferase gene. The primers and probe sequences were: forward primer 5'- GCTAGAGATGGTTCCTTCAGTTGAT- 3'; reverse primer 5'- CATACTCTAGTGCAAACTTTGCAAAAT- 3' and probe 5'- [FAM] ACT GTC TCA TGA TAA TT [MGBEQ] -3' as previously published (Tarrago et al. 2008). Primers and probe used in their optimised concentrations, 900nM primers and 200nM TaqMan MGB probe per reaction. A non-template control and a negative control per DNA extraction were included in every run. DNA was amplified with the real-time PCR System (Agilent Technologies, Statagene Mx3005P) by using the following cycling parameters: 95^{o}C for 10 min, followed by 40 cycles at 95^{o}C for 15 sec and 60^{o}C for 1 min. A standard curve of a 10-fold dilution series of genomic DNA extracted from Spn6B was used. The genomic DNA was extracted using the Qiagen Genomic-tip 20/G Kit (Qiagen) and quantified by nanodrop. The conversion from weight pneumococcal DNA to number of DNA copies *S. pneumoniae* was based on the weight of one genome copy TIGR4 calculated by the genome length in base pairs times the weight of a DNA base pair (650 Dalton). The lower limit of detection (LLOD) of the method was set at 40 cycles. Amplification values >40 were considered negative. A sample was considered positive if at least two of three yielded a positive result within the <40-cycle cut-off. Data were analysed using MxPro software.

2.6.3 Confocal microscopy assays

Confocal microscopy was used for visualisation of pneumococci is association with AMs. In these experiments, fresh lung cells were stained *in vitro*, fixed on slides and observed under the microscope "in house", whereas frozen cells were sent to an independent laboratory.

Fresh BAL cells were washed and stained for surface markers (anti-CD14 Texas Red and CD45-magenda). Cells were permeabilised and incubated with anti-6B pneumococcal antisera or anti-23F pneumococcal antisera (negative control) for 30 minutes on ice and then secondary-conjugated antibody (anti-rabbit 488) for 30 more minutes. After washing, cells were cytospun onto microscope slides and allowed to air dry. DAPI solution was applied directly on the spun cells for 5 minutes. After washing, samples were mounted using Aqua PolyMount (VWR International) with a coverslip onto the microslide. The entire cytospin for each sample was manually viewed by microscopy for detection of pneumococci. Multiple fields of view (>3) were imaged for each sample. Images were captured using an inverted Tissue FAXS Zeiss Confocal Microscope. Z stacks were recorded at 1µm intervals at either 40x oil or 63x oil objectives. The confocal microscope operator was blinded to the colonisation status of the volunteer at the time of sampling.

Thawed BAL samples were fixed in phosphate-buffered saline (PBS) containing 4% v/v EM grade formaldehyde (Sigma) for 20 min at room temperature (RT) before incubating 10 min with PBS containing 0.1% v/v TritonX-100 to permeabilise the cells. Readily, samples were incubated for 30 min in blocking solution (PBS containing 5% v/v goat serum) and then, incubated for 1 h with primary antibodies diluted in blocking solution, washed three times with PBS and incubated again for 45 min with secondary antibody solution. Then, samples were washed three times with PBS and once with water before adding the mounting medium containing DAPI (Thermoscientific ProLong Gold Antifade Mountant) and closing the slides with coverslips. Anti-pneumococcal capsule anti-6B serum (Statens Serum Institute) was used to stain bacteria. Macrophages were labelled using anti-human CD169 (Thermoscientific). Combinations of Alexafluor conjugated antibodies from Thermoscientific were used as secondary antibodies (488 and 568nm with different host specificity). An Olympus FV1000 confocal laser scanning microscope was used to acquire the images using 40x objectives, and the free software

ImageJ (http://imagej.nih.gov/ij/) was used for image processing. For the bacterial localisation studies, Alexafluor 633-conjugated Wheat germ agglutinin was used prior to membrane permeabilization. Z-stack was created from microscope images, elaborated using Huygens Essential deconvolution software version 16 (Scientific Volume Imaging, Netherlands) and viewed in Imaris 3D reconstruction software 9.4 (Bitplane, Switzerland).

2.6.4 Flow cytometry assays

In each flow cytometry assay, the corresponding cell population was stained with predetermined optimal concentration of fluorochrome-conjugated monoclonal antibodies against human cell surface proteins or intracellular cytokines.

2.6.4.1 AM, monocytes and neutrophil immunophenotyping

Myeloid lineage cells were immunophenotyped using monoclonal antibodies for key surface markers. Whole BAL cells (1 x 10^6 cells) were stained with 1:50 diluted Aqua Viability dye (LIVE/DEAD® Fixable Dead Cell Stain kit, Invitrogen, UK) for 15 min on ice, following staining with a combination of antibodies- anti-CD45 FITC, anti-CD80 APC-H7, anti-CD86 PE, anti-CD206 PE-CF594, anti-CD14 PerCP Cy5.5, anti-CD16 PE Cy7, anti-CD163 APC, anti-CD11b AF700, anti-CD11c PB, anti-CD64 BV605 and anti-HLADR BV785 (Table 2.3)- for additional 15min on ice in the absence of light. Then the cells were washed with 2ml of cold PBS and centrifuged at 400g for 10min at 4^{o}C. The cell pellet was resuspended in 200ul cold PBS and the samples were acquired on a FACS ARIA III sorter/cytometer (BD Biosciences) and analysed using Flowjo version 10 (Treestar). BAL samples with macroscopically visual red blood cell contamination were excluded from monocytes and neutrophils analysis.

Table 2.3. Conjugated antibodies and viability dye used for alveolar macrophage and monocyte immunophenotyping in BAL samples. This panel was used in results of Chapter 3 and 4.

Marker	Fluorochrome	Clone	Provider	Reference
CD45	FITC	HI30	Biolegend	304006
CD86	PE	IT2.2	Biolegend	305438
CD206	PE-CF594	19.2	BD Biosciences	564063
CD14	PerCPCy5.5	MΦP9	BD Biosciences	562692
CD16	PECy7	3G8	Biolegend	302016
CD163	APC	GHI/61	Biolegend	333610
CD80	APC-H7	L307.4	BD Biosciences	561134
CD11b	Alexa Fluor 700	ICRF44	BD Biosciences	557918
CD11c	BV421	Bu15	Biolegend	337212
Live and Dead	BV510	NA	Fisher Scientific	L34957
CD64	BV605	10.1	Biolegend	305034
HLADR	BV785	L243	Biolegend	307642

2.6.4.2 AM stimulation with HI-Spn6B and IFN-γ

In this assay, AM macrophages were treated with 10-fold increased recombinant IFN-γ (Bio-techne), plus 5µg/mL heat inactivated Spn6B (HI-Spn6B). The latter was prepared from Spn6B bacterial stock 1ml aliquots, which were heated at 56^{o}C for 30min. The assays include an unstimulated condition, where cells were left unstimulated, as well as a condition that include stimulation with Spn6B but not IFN-γ. In more details, 1 million of BAL cells per condition, resuspended in complete RPMI, were added in 24-well plate and incubated overnight at 37^{o}C, 5% CO_2. Non-adherent cells were removed, and AMs were washed 3x with pre-warmed plain RPMI, following stimulation with 10x increased concentration of IFN-γ (2ng/ml, 20ng/ml, 200ng/ml and 2,000ng/ml) for 30min. Post the cytokine stimulation, cells received 5µg/ml of HI-Spn6B and were incubated for 2 hours. Non-cytokine/non-Spn treated and non-cytokine/Spn treated

controls were included per volunteer. Cytokines were retained within alveolar macrophages by the addition of the GolgiPlug (BD Biosciences)- a protein transport inhibitor, containing Brefeldin A- and were incubated at 37^{o}C, 5% CO_2 for 2 more hours.

Cell Permeabilisation and fixation step: Post incubation time, AMs were washed with PBS and detached from the wells by adding of 2.5mM EDTA solution. Cells were collected in FACS tubes and pelleted (400g for 10 min centrifugation) and resuspended in 1ml Perm/Fix buffer, following 30min incubation in the dark. Then the cells were washed twice with 2mls of Perm wash, following staining for human AM surface markers - anti-CD169-PE, anti-CD14 PerCyP5.5, CD206 PE-CF594 and CD45 Pacific Orange, whereas for intracellular staining anti-TNF-α BV605 was used (Table 2.4) for 45min. Post staining step incubation time, the samples were washed with 3mls of PBS, spun down at 400g for 10min and then the cell pellet was resuspended in 200ul PBS. All the samples were acquired on a LSRII flow cytometer (BD Biosciences) and analysed using Flowjo version 10 (Treestar).

Table 2.4. Conjugated antibodies used for intracellular cytokine staining after treatment with recombinant IFN-γ and stimulation with HI-Spn6B. This panel was used in results of Chapter 4.

Marker	Fluorochrome	Clone	Provider	Reference
CD169	PE	7-239	Biolegend	346004
CD14	PerCPCy5.5	MΦP9	BD Biosciences	562692
CD206	PE-CF594	19.2	BD Biosciences	564063
CD45	BV510	HI30	Life Technologies	MHCD4530
TNF-α	BV605	MAb11	Biolegend	502936

2.6.4.3 Transcription factors analysis

1 million of BAL cells were washed with 3 mL of PBS and stained with 1:50 diluted Aqua Viability dye (LIVE/DEAD Fixable Dead Cell Stain kit, Invitrogen, UK) for 15min, following addition of antibody cocktail against the following surface markers CD3-APC.cy7, CD4–PerCP5.5, CD8–AF700, CD69–BV650, anti-CD25-PETexasRed and CD45-BV711 (Table 2.5) for additional 15min on ice in the absence of light. After incubation, the cells were washed with 2mls of cold PBS and centrifuged at 400g for 10min at 4^{o}C.

For permeabilization and fixation, Foxp3/Transcription Factor Staining Buffer Set (eBiosciences, San Diego, CA) was used as described at section 2.6.4.2, without the prior addition of EDTA. Permeabilised/fixed cells were stained intracellularly for transcription factors analysis with anti-T-bet-APC, anti-Gata-3-PE and anti-Foxp3-FITC (table 2.5) for 30min on ice in the absence of light. All samples were acquired on a LSRII flow cytometer (BD Biosciences).

Table 2.5. Conjugated antibodies and viability dye used for analysis of transcription factors within T cell subsets. This panel was used in results of Chapter 4.

Marker	Fluorochrome	Clone	Provider	Reference
CD25	PE/Dazzle	M-A251	Biolegend	356126
CD4	PerCPCy5.5	SK3	Biolegend	344608
CD3	APC-Cy7	SK7	Biolegend	344818
CD8	Alexa Fluor 700	SK1	Biolegend	344724
Live and Dead	BV510	NA	Fisher Scientific	L34957
CD69	BV650	FN50	Biolegend	310934
CD45	BV711	HI30	Biolegend	304050
FOXP3	Alexa Fluor 488	259D	Biolegend	320212
GATA-3	PE	16E10A23	Biolegend	653803
T-bet	APC	4B10	Biolegend	644814

2.6.4.4 INF-γ, TNF-α and IL-17 producing BAL lymphocytes post Spn stimulation

In these assays, cells were harvested, stained and analysed as previously described, with minor modifications (Wright et al., 2013; Wright et al., 2012) to ensure accurate reproducibility of previous findings. The previous antibody panel was expanded to include tissue resident cell markers, as well as a surface marker for TCR-γδ T cells. Therefore, non-adherent cells were collected from the BAL samples post an adherence step, centrifuged at 400g for 5min, resuspended in complete RPMI and seeded in 96-well plates at equal concentrations of 600,000 to 1 million cells per condition. Cells were stimulated with 5µg/ml of HI-Spn6B and incubated for 2 hours at 37^{o}C, following addition of GolgiPlug (BD Biosciences) and overnight incubation at 37^{o}C, 5% CO2. A non-stimulated with Spn6B (mock) cell condition was included per volunteer. After 16 hours, the cells were washed with PBS and stained with Violet Viability dye (LIVE/DEAD Fixable Dead Cell Stain kit, Invitrogen, UK) and anti-CD3-APCH7, anti-TCR-γδ –PECy7 (BD Biosciences, USA), anti-CD4–PerCP5.5, anti-CD8–AF700, anti-CD69–BV650, anti-CD25-PE.TxsRed, anti-CD103–BV605, anti-CD49a-APC (Biolegend, San Diego, CA). For the assessment of intracellular cytokine production, after permeabilization and fixation, the cells were stained with the following markers: anti-IFN-γ-PE, anti-IL17A–BV510 and TNF-α–BV711 (BD Biosciences). All samples were acquired on a LSRII flow cytometer (BD Biosciences).

Table 2.6. Conjugated antibodies and dyes used for analysis of intracellular cytokine production in T cell subsets. This panel was used in results of Chapter 4 and 5.

Marker	Fluorochrome	Clone	Provider	Reference
CD4	PerCPCy5.5	SK3	Biolegend	344608
CD3	APC-H7	SK7	BD Biosciences	560176
CD8	Alexa Fluor 700	SK1	Biolegend	344724
TCR-γδ	PECy7	11F2	BD Biosciences	655410
CD49a	APC	TS2/7	Biolegend	328314
CD25	PE/Dazzle	M-A251	Biolegend	356126
CD69	BV650	FN50	Biolegend	310934
CD103	BV605	Ber-ACT8	Biolegend	350218
Live and Dead	BV421	NA	Fisher Scientific	L34964
IL17A	BV510	N49-653	BD Biosciences	563295
TNF-α	BV711	MAb11	Biolegend	502936
INF-γ	PE	45.B3	Biolegend	502509

2.6.5 Luminex analysis of Bronchoalveolar lavage fluid

The acellular BAL fluid (BAL supernatant) was collected post centrifugation of whole BAL sample (400g for 10min at 4^{o}C), divided to 1ml aliquots and stored at -80^{o}C until analysis. On the day of the analysis frozen BAL fluid samples were concentrated x10 (1ml of BAL concentrated to 100ul using vacuum concentrator RVC2-18) and acquired using a 30-plex magnetic Luminex human cytokine kit (ThermoFisher), following manufacturer's instructions. Analytes measured by the kit are presented on table 2.7. Particularly, 30µL of the concentrated BAL fluid was placed on a 96-well plate (U-bottom) followed by addition of 30µL of Assay diluent and 30µL of incubation buffer to each well. Standard serum sample was serially diluted 1:10 in assay diluent. 60µl of each dilution was mixed with 30ul of incubation buffer in the working plate. 15µl of antibody beads were added into each well (including standard, sample and blank wells). 45µl of total reaction per condition was transferred to the Luminex plate in duplicates. The plate was incubated overnight at 4^{o}C under agitation on an orbital shaker (500 rpm agitation).

On the following day, the antibody beads were washed twice with 200µl Wash Solution while the Luminex plate was attached to a magnet when the washing buffer was discarded. Following washing and air drying, 25µL Biotinylated detector antibody was added to each well and the plate was incubated for one hour at RT, placed on an orbital plate shaker. After the incubation, the beads were washed twice with wash buffer and 25µL Streptavidin-RPE solution was added to each assay well. The samples were incubated for 30 minutes at RT on an orbital plate shaker. After the final incubation step, the beads were washed 3 times with wash solution, following addition of 105µl wash solution in each well. Samples were acquired and analysed on a LX200 with xPonent3.1 software following manufacturer's instructions. Samples were analysed in duplicates and BAL samples with a CV > 50 % were excluded.

Table 2.7. Cytokine human magnetic 30-Plex panel. Luminex platform designed for quantifying cytokines, chemokines and growth factors in a range of human samples.

Cytokines	Chemokines	Growth Factors
G-CSF	Eotaxin	EGF
GM-CSF	IP-10	FGF-basic
IFN-α	MCP-1	HGF
IFN-γ	MIG	VEGF
IL-1β	MIP-1α	
IL-1RA	MIP-1β	
IL-2	RANTES	
IL-2R		
IL-4		
IL-5		
IL-6		
IL-7		
IL-8		
IL-10		
IL-12 (p40/p70)		
IL-13		
IL-15		
IL-17		
TNF-α		

2.6.6 Alveolar macrophage gene analysis using Nanostring platform

AM gene expression was assessed by using Nanostring platform based on our previously published protocol (Jochems et al. 2018). Alveolar macrophages from Spn colonised and non-colonised volunteers were sorted by FACS ARIAIII cell sorter, as described at section 2.26, and stored in Qiazol (Qiagen) at -80C until RNA extraction.

2.6.6.1 RNA extraction from alveolar macrophages

RNA extraction was performed using the RNEasy micro kit (Qiagen) with on column DNA digestion. On the day of RNA extraction, samples were thawed on ice and 1ml of Qiazol was added to them. The samples were split equally to two tubes and 100ul 1-Bromo-3-chloropropane was added to each tube. Homogenates were shaken vigorously for 15 sec, following centrifugation at 12,000g for 15 min at 4°C. After centrifugation the upper aqueous phase was aspirated and transferred to a new sterile, RNase-free tube and 1 volume of 70% ethanol in RNase-free water was added in. Prior to sample (homogenate with ethanol) loading on the RNeasy Micro spin column, the column was treated with 5µl of carrier RNA (4ng/µl). Loaded sample on the column was centrifuge for 15 sec at 8,000g. The flow-through was collected and this step was repeated 3 times. The column washed twice with RW1 buffer, while both DNA and RNA were bound to the membrane. DNA was digested by application of DNAse I mix directly on the column membrane. Digested DNA was removed through washing steps. The RNA was eluted from the column, when RNase-free water was added upon the membrane for 5min and RNA collected to a sterile, RNase-free tube after 1min centrifugation at 12,000g. Extracted RNA was quantified using using Qubit® Fluorometer and nanodrop. In addition, extracted RNA was quantified by qPCR targeting B2M gene (Bioanalyzer, Agilent).

2.6.6.2 Preparation of cDNA library and Nanostring platform

The synthesis of first-strand cDNA was done using VILO superscript. The extracted RNA was diluted in RNase-free water to correspond to 100 cells. 4µl (up to 2.5µg) of diluted RNA were mixed with 1µL SuperScript™ VILO™ MasterMix and transferred to a thermocycler, applying the following program parameters: 25^{o}C for 10min, 42^{o}C for 120min and 85^{o}C for 5min.

The single cell immunology v2 kit (Nanostring) was used with 20-pre-amplification cycles for all samples. Hybridised samples were prepared on a Prep Station, according to hybridization protocol for nCounter XT CodeSet Gene Expression and scanned on a nCounter® MAX (Nanostring). Hybridization reaction took place at room temperature for each sample with the following components: 3 µl of Reporter CodeSet, 5 µl of hybridization buffer, up to 8 µl (25–100 ng) of sample RNA, and 2 µl of Capture ProbeSet. Immediately after the addition of Capture ProbeSet, the Capture ProbeSet tubes were spun down briefly and placed into the pre-heated 65°C thermal cycler and incubated for 16 hours, following 4^{o}C step. The following day nanostring plate was scanned by nCounter® MAX (Nanostring).

Raw counts were analysed using the R/Bioconductor package DESeq2 for internal normalisation, which gave lower variance than normalising to included housekeeping genes. Differentially Expressed Genes (DEG) were identified using a model matrix correcting for repeated individual measurements. Log CPM from raw counts were calculated using the 'edgeR' package. 2logFold chances were further analysed by the 'fgsea' package, through BMT pathways gene set enrichment analysis.

2.6.7 Levels of IgG to Spn6B capsular polysaccharide in the BAL fluid

Levels of IgG to CPS-Spn6B were measured in BAL fluid using the standardised ELISA method and reagents, as previously described (Goldblatt et al. 2011, Mitsi et al. 2017). 96-well plates (NUNC maxisorp) were coated with 5 µg/ml of purified 6B capsular polysaccharide (Statens Serum Institute) and incubated overnight at 4°C. The following day, plates were washed three times with PBS-Tween 0.05% between each step, using a microplate washer (HydroFlex microplate washer, Tecan).

Acellular, unconcentrated BAL samples were pre-incubated with 10µg/ml cell wall polysaccharide mixture (CWPS Multi, Statens Serum Institute) for 2h before they were transferred to pre-coated plates, following 2h incubation at room temperature. 89-SF reference serum (U.S. Food and Drug Administration) was used as a standard. Bound antibodies were detected using goat anti-human IgG conjugated with alkaline phosphatase (Sigma-Aldrich) in 1:4000 for 2 hours at room temperature. 0.5 mg/ml of p-nitrophenyl phosphate (PNPP) was added as a substrate. Optical densities were measured at 405 nm using a FLUOstar Omega microplate reader (BMG Labtech, UK). All samples were run in duplicates and samples with CV >20% were excluded. Results were expressed as ng/ml calculated using the assigned IgG concentrations in reference serum 89-SF.

Chapter 3

Phenotype and Polarisation Profile of Human Alveolar Macrophages

3.1 Introduction

The healthy human alveoli are dominated by alveolar macrophages (AM) (Wissinger, Goulding and Hussell 2009). These cells play a critical role in regulating immune responses (Gordon and Read 2002) and maintaining homeostasis in the lung (Wissinger et al. 2009, Snelgrove, Godlee and Hussell 2011). Both functions are of great importance in preventing infection, disease and extensive lung tissue damage. However, each function requires plasticity within the lung resident macrophage population so that pro-inflammatory responses to tissue debris or to innocuous antigens are inhibited, but effective immune responses to pathogenic microorganisms are not compromised with the local microenvironment to tailor them to the task required. Alveolar macrophage plasticity to adapt to changes in their microenvironment is fundamental in retaining the lung health (Stout and Suttles 2004).

Macrophages have been classified into classically activated (M1 mode polarised) and alternatively activated (M2 mode polarised) (Mosser and Edwards 2008, Hussell and Bell 2014). Classically activated macrophages are generated in response to IFN-γ, TNF-α or activated by Toll Like Receptor (TLR) ligands. They have enhanced antimicrobial activity, produce cytokines that are important for host defence and interact with T cells to give rise to Th1 and Th17 cells, with the latter producing IL-17, a cytokine that is associated with increased neutrophil recruitment (Loke et al. 2007). M1 macrophages express the transcription factor IRF5 (Krausgruber et al. 2011), the costimulatory

molecules CD80 and CD86, and the FCγ receptor I, CD64 (Biswas and Mantovani 2010, Martinez and Gordon 2014). In contrast to classically activated macrophages, alternatively activated macrophages (M2 phenotype) have reduced antimicrobial capacity and promote tissue remodelling. IL-4 is an early signal released in response to Th2-mediated inflammation (Loke et al. 2007). The presence of IL-4 and IL-13 converts macrophages into alternatively activated macrophages, and this is amplified by IL-33 and IL-25 produced by epithelial cells, macrophages and Th2 cells (Gordon and Martinez 2010) in order to promote tissue remodelling (Loke et al. 2007). Alternatively activated macrophages produce fewer proinflammatory cytokines and are less efficient at killing intracellular pathogens such *Mycobacterium tuberculosis* (Mosser and Edwards 2008, Edwards et al. 2006). The surface-marker expression profile of M2 macrophages includes the scavenger receptor CD163 and the mannose receptor CD206 (Biswas and Mantovani 2010, Martinez and Gordon 2014). In addition, macrophages, generated in response to cytokines such as IL-10, produce anti-inflammatory cytokines (especially IL-10 and IL1-RA) to supress immune responses.

This categorisation is derived from murine models, and from *in vitro* polarisation of human macrophages using a combination of cytokines. However, whether alveolar macrophage polarisation in steady state fits the traditional M1/M2 dichotomy is still not well understood. Human studies using healthy alveolar macrophages are few or do not directly address the question to their polarisation. There is an unverified yet assumption that human alveolar macrophages do not neatly fit into the current macrophage classification by flow cytometric analysis (Habibzay, Weiss and Hussell 2013) (Hussell and Bell 2014).

Therefore, in this study we sought to describe the phenotype and polarisation profile of human alveolar macrophages. To pursue this purpose, we immunophenotyped human alveolar macrophages from adults living in two different geographical locations: UK and Malawi. The two setting were selected to represent a developed and low-income country, respectively. This allowed us to study human AM polarisation in steady state and ascertained the stability of the phenotype in two distinct geographical regions and following *in vivo* exposure to bacterial and viral stimuli.

3.2 Material and Methods

Bronchoalveolar lavage samples collected and analysed in the present study derived from two different human adult cohorts: a) UK and b) Malawi cohort. UK cohort were challenged intranasally with *S. pneumoniae* serotype 6B, whereas Malawi cohort was divided in two groups: i) healthy HIV-1-uninfected and ii) age-matched asymptomatic HIV-1-infected volunteers. The latter have never received any antiretroviral treatment (ART naïve).

Common clinical and experimental procedures applied in both cohorts are described in more details in Chapter 2 (Material and Methods). Both UK and Malawi demographic data and alveolar macrophages immunophenotyping are presenting in full details in the current methods section due to slightly differential approach.

3.2.1 UK Cohort: Experimental human pneumococcal intranasal challenge and carriage determination

Briefly, in the UK, healthy, non-smoking participants aged 18-50 years were recruited from an ongoing study of the experimental human pneumococcal challenge (EHPC) model (Chapter 2- Figure 2.1/EHPC study 2). At Day 0 volunteers were inoculated with 80,000 CFU of live pneumococci serotype 6B per naris. Pneumococcal colonisation was detected by classical microbiology methods and quantified by serial dilution from nasal washes performed at days 2, 7, 9 and 27 (Gritzfeld et al. 2013). A subset of 25 participants, 16 individuals who had become colonized (carriage +) with *Streptococcus pneumoniae*, and 9 who had not (carriage –) underwent a single time-point research bronchoscopy in the Clinical Research Unit (CRU) at the Royal Liverpool University Hospital (RLUH) **between 4 to 7 weeks after the bacterial challenge** (Zaidi et al. 2017). Demographic data of the UK and Malawi study participants are presented in table 3.1. Extensive description of following procedures and methods can be found in Chapter 2.

Table 3.1. Demographics of the EHPC study participants- UK. Abbreviations; CFU= colony forming units. A single time-point bronchoscopy was performed at any day between 4 to 7 weeks post the bacterial challenge.

UK Cohort	Pneumococcal non-colonised (n=9)	Pneumococcal colonised (n=16)	p value
Median age (range) -years	20 (19-25)	21 (18-31)	p>0.05
Sex (M:F)	4:5	7:9	
Median inoculation dose (range) - *CFU/nostril	82,500 (76667-89,000)	87,500 (77500-93,000)	p>0.05
Median duration of carriage (range)- days post inoculation	N/A	27.0 (14.0-27.0)	

3.2.2 Malawi Cohort- Study participants

The study was conducted at the Queen Elizabeth Central Hospital, a large teaching hospital in Blantyre, Malawi. Participants were recruited from the hospital's voluntary counselling and testing (VCT) and antiretroviral treatment (ART) clinics. They were adults aged ≥18 years comprising healthy HIV-1-uninfected and asymptomatic HIV-1-infected volunteers with no clinical evidence of active disease and willing to undergo bronchoscopy and bronchoalveolar lavage (BAL) for research purposes (Mtunthama et al. 2008). HIV testing was performed on whole blood using two commercial point-of-care rapid HIV test kits, Determine HIV 1/2 kit (Abbott Diagnostic Division) and Unigold HIV 1/2 kit (Trinity Biotech Inc.). A participant was considered HIV-uninfected if the test was negative by both kits or HIV-infected if the test was positive by both kits. If Determine and Unigold results were discordant, a third rapid test using Bioline HIV 1/2 kit (Standard Diagnostics Inc.) was performed to resolve the discordance. None of the participants were on ART at the time of recruitment to the study, but all initiated ART after sample collection according to the 'test and treat' Malawi national treatment guidelines. Exclusion criteria for the study were: current or history of smoking, use of immunosuppressive drugs, severe anaemia (Hb<8g/dl) and known or suspected pregnancy.

Table 3.2. Demographics of study participants – Malawi

Malawi Cohort	HIV-uninfected (n=10)	HIV-infected ART-naive (n=10)	p value
Median age (range) -years	33 (20-35)	33 (22-35)	p>0.05
Sex (M:F)	7:3	7:3	
Peripheral blood CD4 count (cells/µl), median (IQR)	664 (469-746)	251 (154-356)	0.0001
White blood cell count (cells/L), median (IQR)	4.4 (3.4-4.8)	4.7 (4.0-6.4)	0.4692
Haemoglobin (g/dL, median (IQR)	15.6 (14.3-16.5)	13.0 (11.3-13.8)	0.0121
Haematocrit (%), median (IQR)	47.4(41.8-49.7)	38.9(35.2-41.4)	0.0140

3.2.3: Intranasal pneumococcal challenge- UK Cohort

Full details can be found in section 2.4.1 (Nasopharyngeal inoculation)

3.2.4 Bronchoscopy- UK and Malawi cohorts

The same bronchoscopic procedure was applied in both UK and Malawi. Full details can be found in section 2.4.4.

3.2.5 Bronchoalveolar lavage sample processing- UK and Malawi cohorts

BAL samples collected in UK and Malawi were processed using the same methodology described in section 2.5.7.

3.2.6 Alveolar macrophage isolation- UK and Malawi cohorts

In both sites, alveolar macrophages were separated from the other cell populations via an adherence step. Full details can be found in section 2.5.8.

3.2.7 Alveolar macrophage immunophenotyping

Flow cytometry-based immunophenotyping was used to characterise AM phenotype obtained from BAL fluid. The expressional levels of key surface markers were measured immediately after sample collection. Whole BAL cells (1 x 10^6 cells) were stained with predetermined optimal concentration of flourochrome-conjugated monoclonal antibodies against human cell surface proteins.

At the UK site, cells were stained with Aqua Viability dye (LIVE/DEAD® Fixable Dead Cell Stain kit, Invitrogen, UK), anti-CD45 FITC, anti-CD80 APC-H7, anti-CD86 PE, anti-CD206 PE-CF594, anti-CD14 PerCP Cy5.5, anti-CD16 PE Cy7, anti-CD163 APC, anti-CD11b AF700, anti-CD11C PB, anti-CD64 BV605 and anti-HLADR BV785. All the samples were acquired on the FacsAria III sorter/cytometer (BD Biosciences).

At the Malawi site, the cells were stained with anti-CD45 Alexa Fluor 700, anti-CD206 APC, anti-CD66b FITC, anti-CD163 Brilliant Violet 421, and anti-CD86 PE Dazzle (All Biolegend, UK). Further details of the antibodies are included in Table 3.3. Flow cytometry data were analysed using FlowJo software (TreeStar, USA). Compensation was set using CompBeads (BD Biosciences). All samples were acquired on the BD LSRFortessa flow cytometer (BD Biosciences).

Flow cytometry data were analysed using FlowJo software (TreeStar, USA) (Figure 3.1 and 3.2). Expression of surface markers was calculated by subtracting the Median Fluorescent Intensity (MFI) of the surface marker on the AM population from the Median Fluorescent Intensity (MFI) of the surface marker on the Neutrophil population (negative control), divided by twice the robust standard deviation of the MFI of the surface marker on the neutrophil population.

Table 3.3. Summary of the panel composition at the UK site (no shading) and Malawi site (grey shading).

Marker	Fluorochrome	Clone	Provider	Filter	Isotype
CD45	FITC	HI30	Biolegend	530/30	IgG1
CD80	APC-H7	L307.4	BD Biosciences	780/60	IgG1
CD86	PE	IT2.2	Biolegend	585/42	IgG2b
CD206	PE-CF594	19.2	BD Biosciences	616/23	IgG1
CD14	PerCPCy5.5	MΘP9	BD Biosciences	695/40	IgG2b
CD16	PECy7	3G8	Biolegend	780/60	IgG1
CD163	APC	GHI/61	Biolegend	660/20	IgG1
CD11b	AF700	ICRF44	BD Biosciences	730/45	IgG1
CD11c	PB	Bu15	Biolegend	450/50	IgG1
CD64	BV605	10.1	Biolegend	610/20	IgG1
HLADR	BV785	L243	Biolegend	780/60	IgG2a
Live & Dead	BV510	NA	Invitrogen	510/50	NA
CD206	APC	15-2	Biolegend	660/20	IgG1
CD66b	FITC	G10FG	Biolegend	530/30	IgM
CD163	Bv421	GHI/61	Biolegend	450/50	IgG1
CD45	AF700	2D1	Biolegend	730/45	IgG1
CD86	PE DAZZLE	IT2.2	Biolegend	616/23	IgG2b

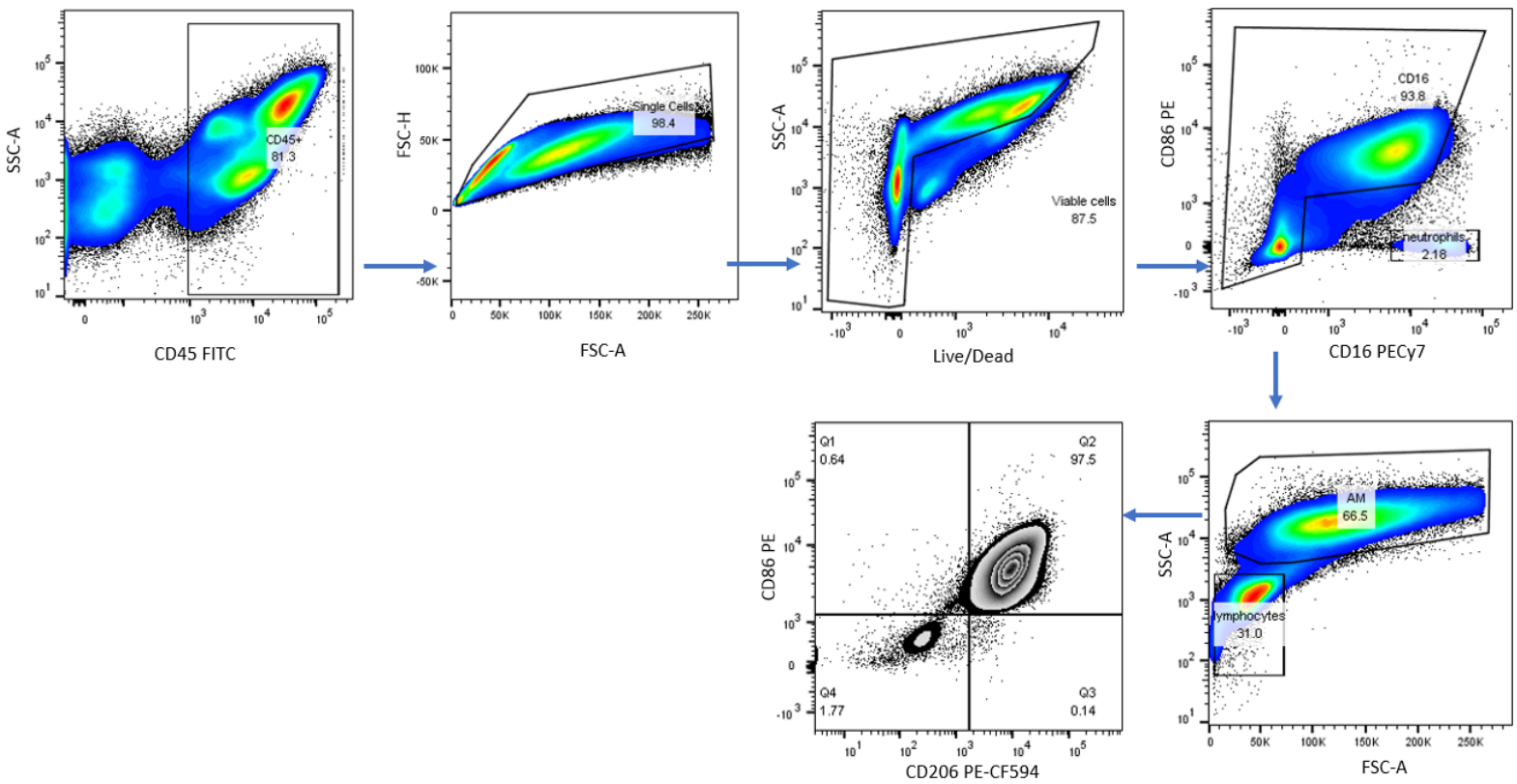


Figure 3.1. Gating strategy used to identify human alveolar macrophages in the UK Cohort. Leucocytes were identified as $CD45^{+}$, following exclusion of doublets and dead cells (precaution was taken not to gate out highly autofluorescent alveolar macrophages). Neutrophils were identified as CD45+CD16+CD86- cells. Alveolar macrophage (AM) and lymphocyte populations were identified by their forward scatter (FSC) and side scatter (SSC) characteristic properties. Using CD206 and CD86 we separated AM to four subpopulations: $CD206^{lo}CD86^{hi}$, $CD206^{hi}CD86^{hi}$, $CD206^{hi}CD86^{lo}$ and $CD206^{lo}CD86^{lo}$. Neutrophils or lymphocytes were used as the negative population to calculate the degree of expression of AM surface markers CD163, HLA-DR, CD64 and CD80, respectively.

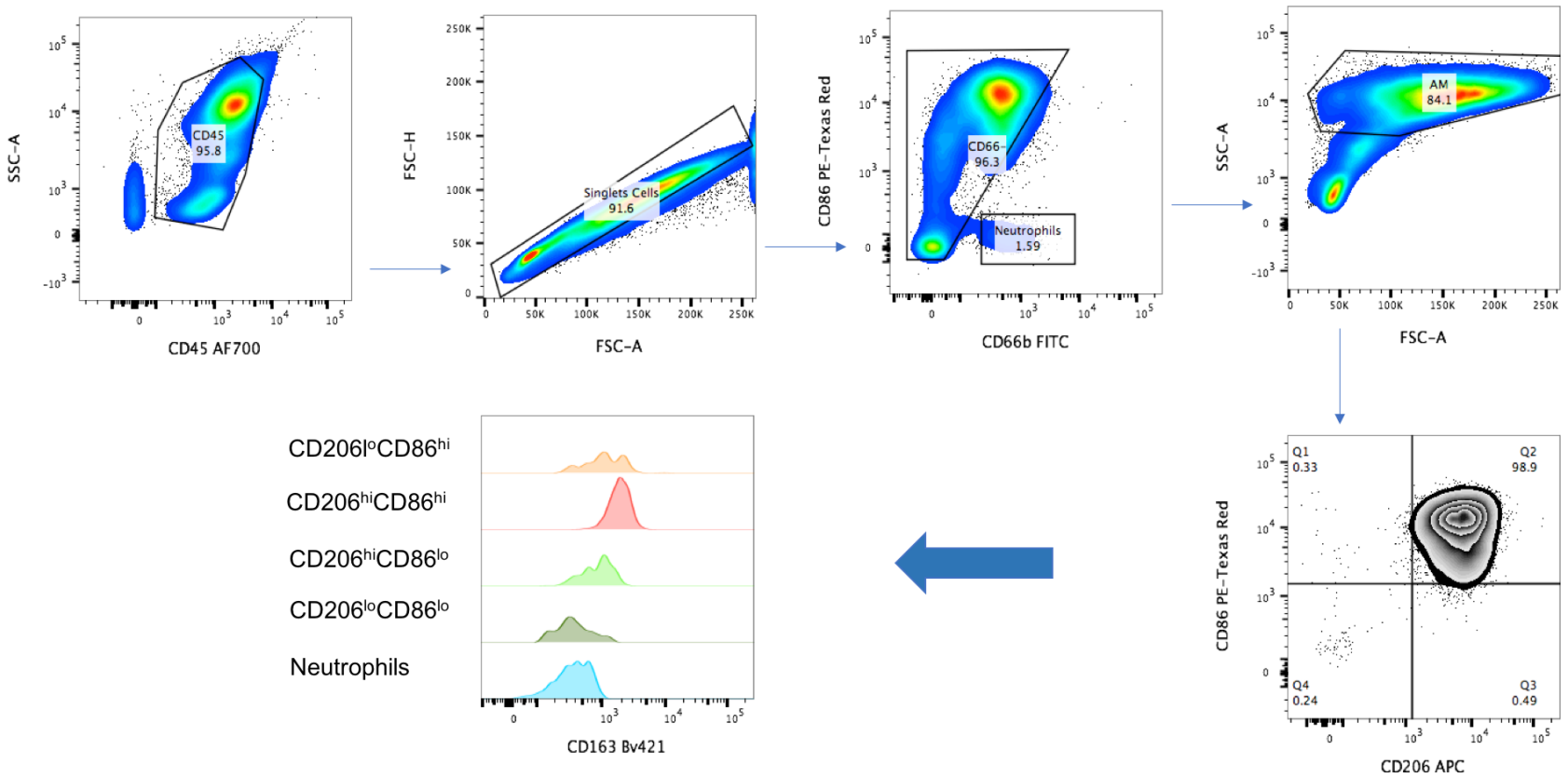


Figure 3.2. Gating strategy used to identify human alveolar macrophages in the Malawi Cohort. The same gating strategy applied on the Malawi cohort, with a difference of a neutrophil marker. Here we used CD66b to identify neutrophils as CD45+CD66b+CD86-. AM gated as described above and the degree of CD163 expression on the surface of AM subsets calculated using neutrophils as the negative population.

3.2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism (Version 6, GraphPad Software, La Jolla, CA). Two-tailed statistical tests were used throughout the study. Non-parametric data were not logarithmically transformed, therefore when two non-parametric groups were compared, a Mann-Whitney or Wilcoxon test was used for unpaired and paired groups respectively. Differences were considered significant at $p \leq 0.05$ (*p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001).

3.3 Results

3.3.1 Human alveolar macrophages simultaneously express M1 and M2 markers in steady state

Data from the two human cohorts from the different geographical locations, were collected independently as part of different ongoing research studies, in which the human lung was sampled. The comparison of immunophenotyping data of only the healthy adults revealed an identical pattern of surface marker expression by human AM. In both cohorts, the majority of AM were characterised as $CD206^{hi}CD86^{hi}$ (Figure 3.3A and 3.3C). M1-like phenotype ($CD206^{lo}CD86^{hi}$) and M2-like phenotype ($CD206^{hi}CD86^{lo}$) subsets represented a small proportion (less than 1%) of the total AM population (Figure 3.3A and 3.3C).

When we looked at the expression levels of the M2 marker, CD163, in each AM subset in both cohorts a similar pattern was revealed. The $CD206^{hi}CD86^{hi}$ subset expressed greater levels of CD163, compared to the M1-like and M2-like subpopulations (Figure 3.3B and 3.3D). UK samples were further analysed for additional M1 markers (CD80, CD64) and an activation marker (HLADR). In these, the $CD206^{hi}CD86^{hi}$ subset expressed the highest levels of CD80, CD64 and HLADR expression in comparison to the other AM subsets (Figure 3.4A-C).

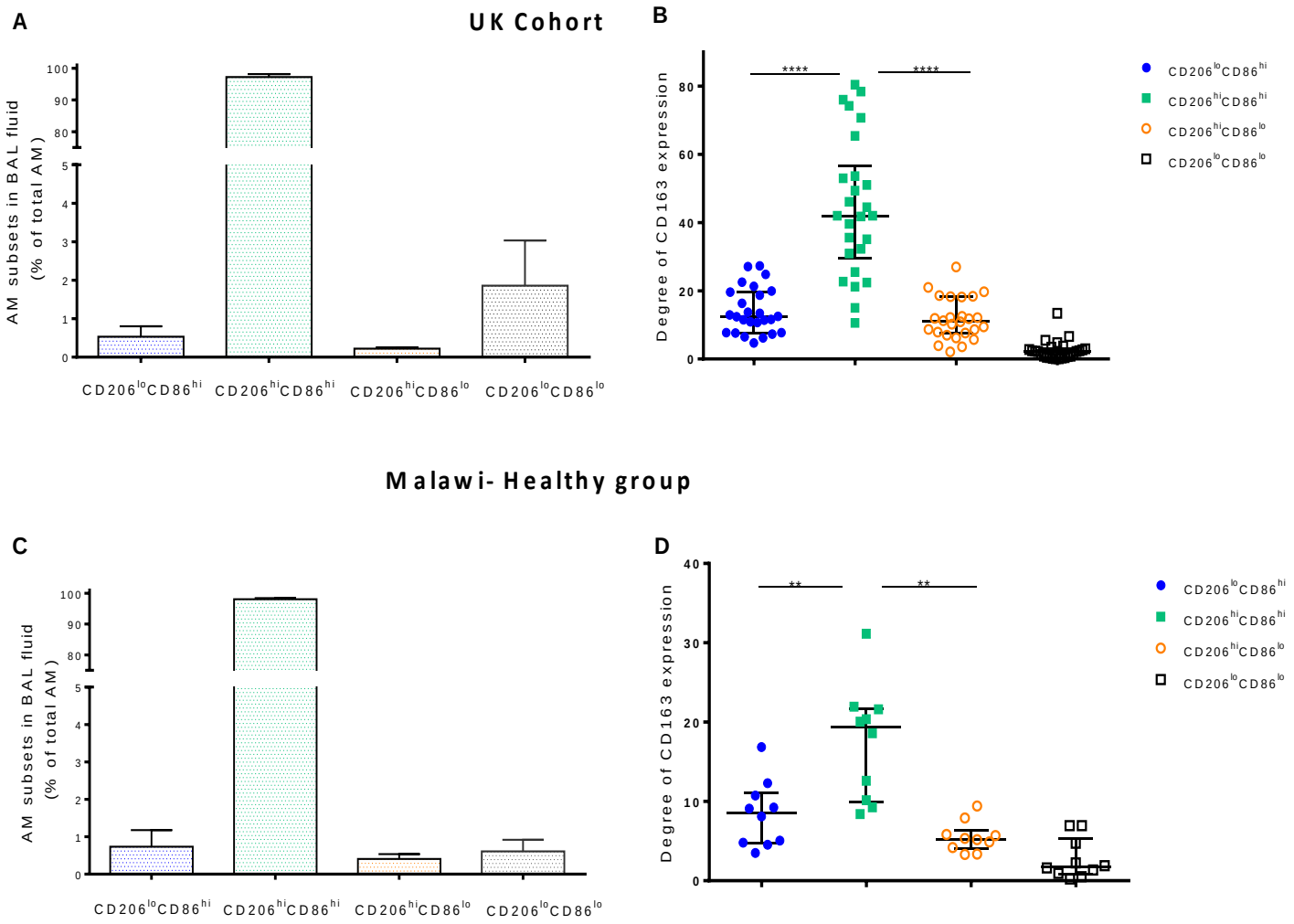


Figure 3.3. Human AM express simultaneously M1 and M2 markers. A) Percentages of AM subsets gated for CD206 and CD86 and the **B)** the degree of CD163 expression in each of the subsets (UK cohort, n=25). $CD206^{hi}CD86^{hi}$ subset occupies the highest proportion and expresses the highest levels of CD163 when compared with the other three. **C-D)** The same pattern was observed on the AM derived from the Malawi cohort, n = 10. Black error bars represent median with IQRs. Wilcoxon test was used for the comparison between AM subsets from the same cohort. ****p<0.0001 and **p<0.01

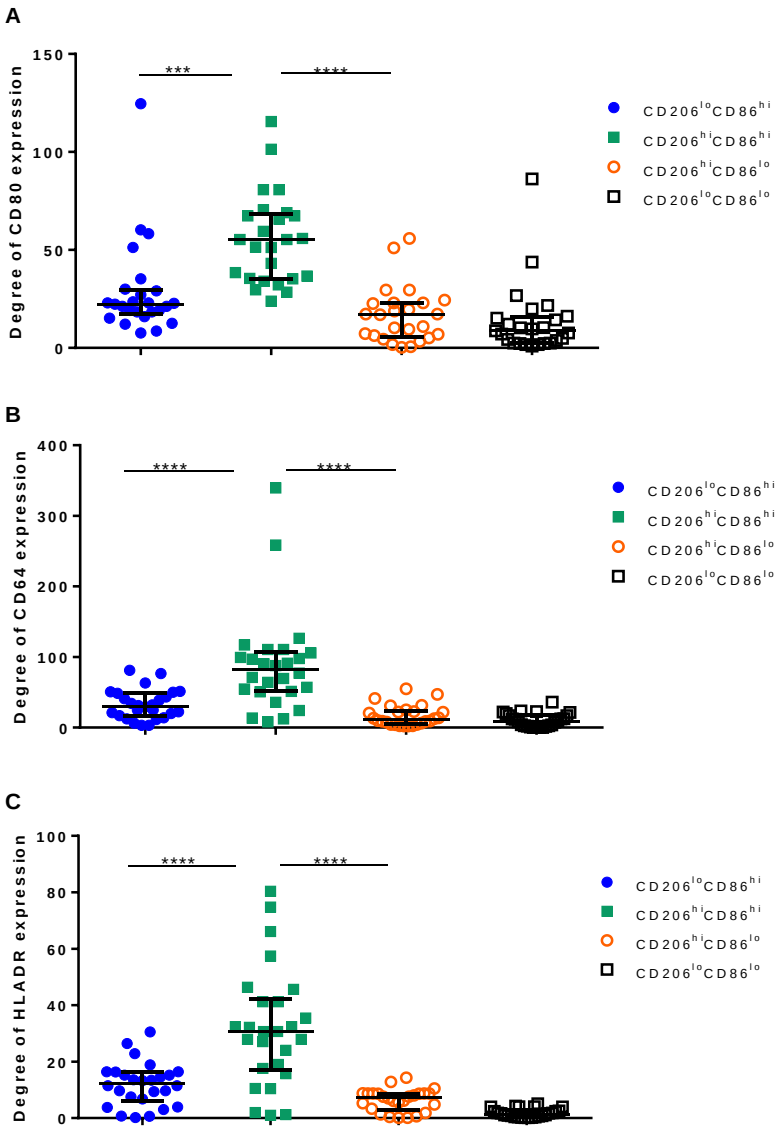


Figure 3.4. Expressional levels of CD80, CD64 and HLADR on the AM subsets isolated from the UK healthy individuals. **A)** The degree of CD80, **B)** CD64 and **C)** HLADR expression was compared between the different AM subsets (n=25). These surface markers were highly expressed in the $CD206^{hi}CD86^{hi}$ subset. Black error bars represent median with IQRs. Wilcoxon test was used in the comparison between AM subsets from the same cohort. ****p<0.0001, ***p<0.001 and **p<0.01

3.3.2 Colonisation of the human nasopharynx with *S. pneumoniae* does not alter the AM phenotype

We have previously shown that live intranasal pneumococcal challenge alters the alveolar environment by increasing the levels of pneumococcal-specific memory $CD4^+$ Th17 cells in the human lung (Wright et al. 2013). We therefore investigated whether *in vivo* exposure to intranasal pneumococcal challenge could alter the AM phenotype. We compared experimentally pneumococcal-colonised individuals (Spn colonised following inoculation with *S. pneumoniae*) with non-colonised individuals (cleared the bacterial immediately) and we found no difference in M1- or M2-like phenotype between the two experimental groups (Figure 3.5). Specifically, the expression of CD163, CD80, CD64 and HLADR measured in the four AM subsets remain at the same levels between non-colonised and Spn-colonised individuals (Figure 3.6A-D).

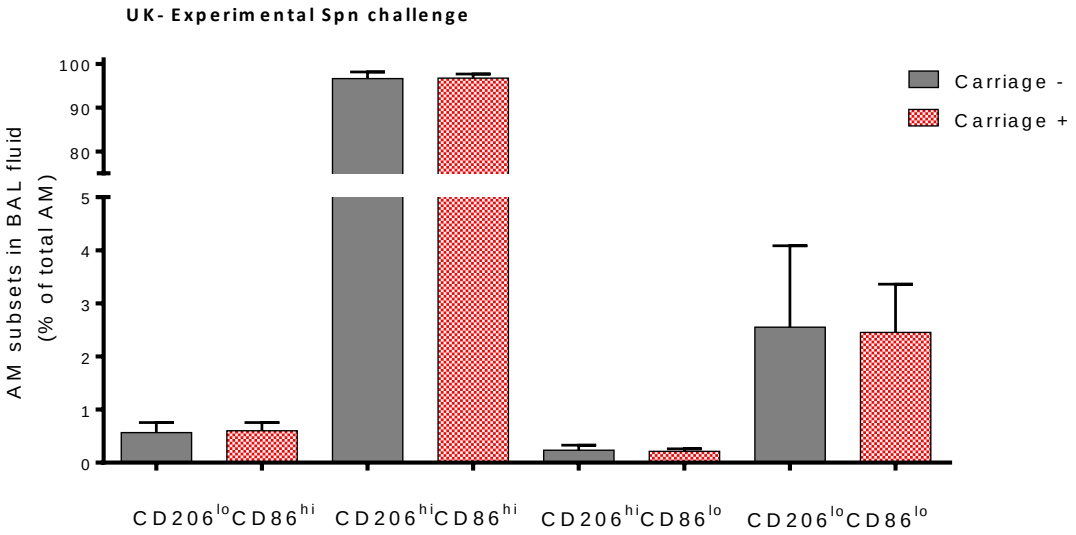


Figure 3.5. Pneumococcal colonisation does not alter the AM phenotype. AM subsets were compared between non-colonised (n=9) and Spn colonised (n= 16) post experimental nasal pneumococcal inoculation. The data shows that there was no alteration of AM phenotype mediated by nasal pneumococcal carriage. $CD206^{hi}CD86^{hi}$ was the dominant subset with the highest CD163 expression amongst the rest. Error bars represent median with IQRs. Data were analysed using Mann Whitney U test.

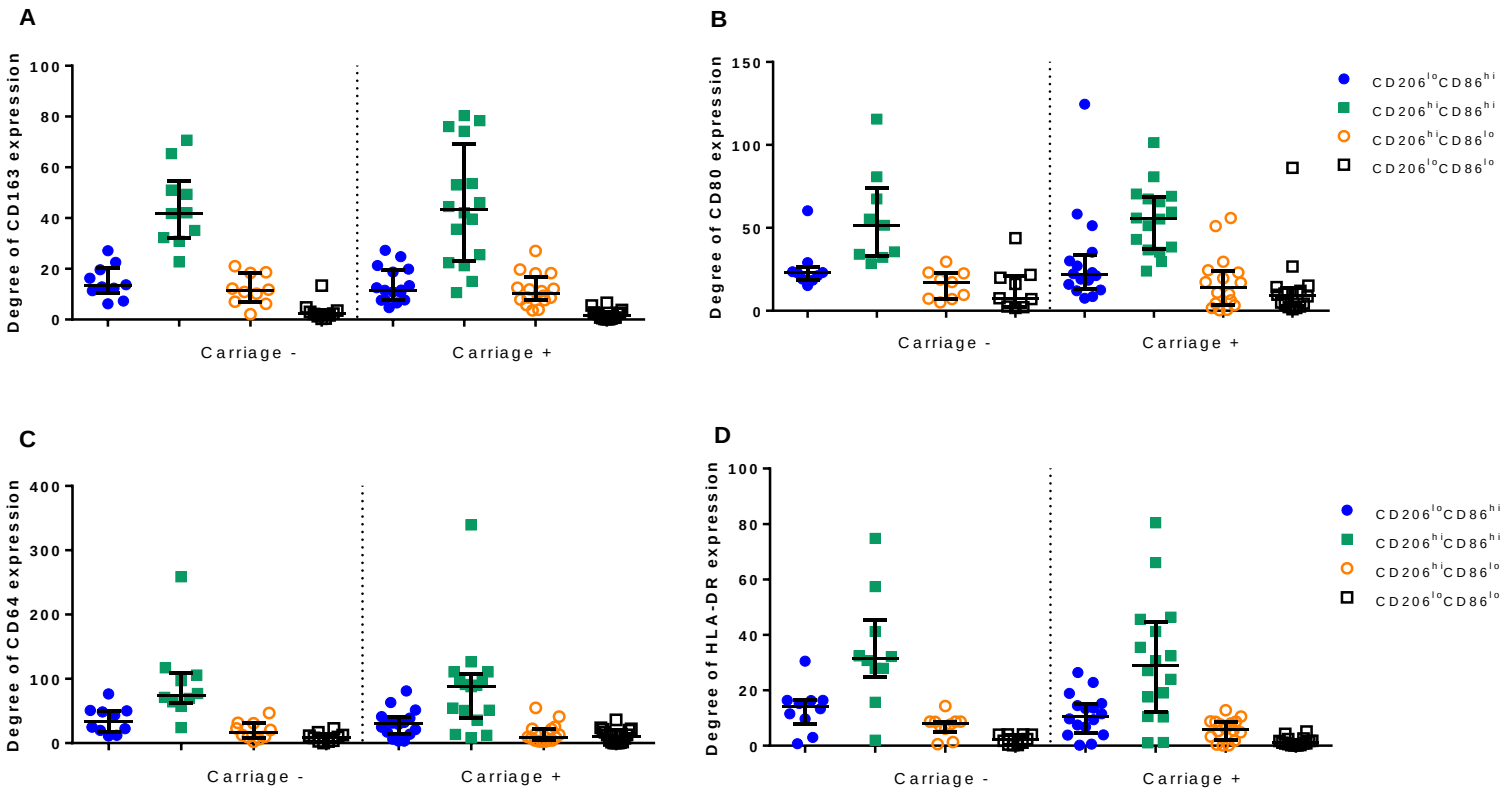


Figure 3.6. Nasal pneumococcal colonisation does not induce polarisation or higher activation of AM. A-D) Levels of CD163, CD80, CD64 and HLADR expression respectively between carriage negative (n=9) and carriage positive subjects (n=16). There is no significant difference on levels of CD80, CD64 and HLADR expression mediated by nasopharynx pneumococcal colonisation. Black error bars represent median with IQRs. Mann Whitney U test was used.

3.3.3 Chronic HIV infection is associated with a lower expression of CD163 on CD206^{lo}CD86^{hi} AM

Infection with HIV virus disrupts the alveolar cytokine microenvironment (Jambo et al. 2017). Here we looked at whether HIV infection- a chronic condition that mainly affects the T cell lung population- could polarise and alter the AM phenotype. We compared asymptomatic ART-naïve HIV-infected adults with age-matched HIV-uninfected healthy individuals. Interestingly, in the Malawian population, AM subsets from HIV-infected individuals followed the same pattern of subdivision described in healthy volunteers. No difference was found in the proportion of the investigated AM subsets between the ART-naïve HIV-infected adults and HIV-uninfected healthy individuals (Figure 3.7A). However, the expression of CD163 was significantly reduced in the CD206^{lo}CD86^{hi} subset in HIV-infected individuals (median 0.47x, IQR: 0.42x- 0.61x) (p=0.015) (Figure 3.7B). In the predominant CD206^{hi}CD86^{hi} subset, there was a moderate reduction (median 0.66x) of CD163 expression in the HIV-infected group, which however was not statistically significant when compared with the healthy age-matched group (Figure 3.7B).

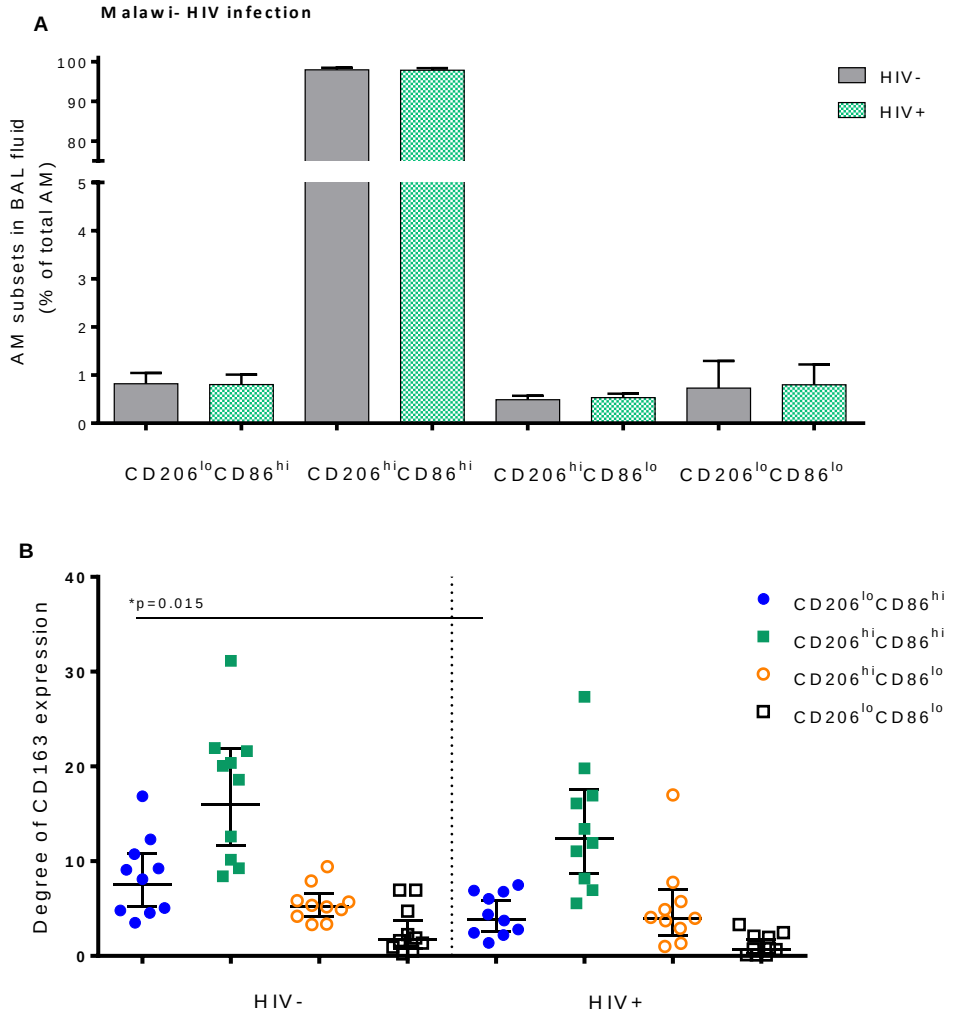


Figure 3.7. HIV infection reduces the levels of CD163 expression in the CD206^{lo}CD86^{hi} A) AM subsets are compared between healthy (n=10) and HIV-infected individuals (n=10). AM collected from HIV infected subjects follow the same pattern with healthy individuals when gated for CD206 and CD86. **B)** The HIV infection regresses the expression of CD163 in the CD206^{lo}CD86^{hi} subset. Black error bars represent median with IQRs. Mann Whitney U test was used.

3.4 Discussion

In this study, we compared the polarisation of AM isolated from human volunteers from two distinct geographical locations, UK and Malawi. In the UK cohort, which was experimentally challenged with *S. pneumoniae* intranasally, a single time-point bronchoscopy was performed at any day between 4 to 7 weeks post the bacterial challenge. We report that in healthy human pulmonary mucosa, AM adopt a hybrid phenotype, which shares features of both M1 (CD80, CD86, CD64) and M2 (CD206 and CD163) polarised macrophages. The same phenotype has been previously reported on the decidual macrophages (resident macrophages of the uterine lining), which exhibit characteristics of both pro-inflammatory and tolerogenic macrophages (Quillay et al. 2015).

We also found that nasal pneumococcal colonisation, even though it alters $CD4^+$ T cell responses in the alveoli (Wright et al. 2013), does not alter the expression of key M1 and M2 polarisation markers on the surface of AM. This leads to the speculation that a nasal pneumococcal exposure episode is either incompetent to modulate AM polarisation or its effect is transient and quickly irreversible due to macrophages high plasticity (Stout and Suttles 2004). By contrast, chronic HIV infection did affect the expression of CD163 on the M1-like AM subset ($CD206^{lo}CD86^{hi}$), with a lower expression of CD163 observed in HIV-infected individuals than HIV-uninfected controls. This finding is intriguing and may be due to active HIV infection of this alveolar macrophage subset (Jambo et al. 2014) which has been shown to strongly repress CD163 expression on infected-macrophages (Porcheray et al. 2006). Furthermore, CD163 is shed during activation as soluble CD163 (sCD163)(Burdo et al. 2011) and this might in part explain the reduction of CD163 on the surface M1-like AM in HIV-infected individuals. However, even with *in vivo* exposure to pneumococci or HIV, the $CD206^{hi}CD86^{hi}$ AM subset remained the major population, and this ascertains the stability of this phenotype.

A potential weakness of the current study is the small number of markers used in both cohorts, which may have inhibited the identification of more stark changes associated with AM polarisation. Studies have highlighted a number of factors that may affect polarisation of human macrophages *in vivo* and have suggested the development of a standardised way of assessing human alveolar macrophage polarisation(Murray et al. 2014). Inclusion of surface markers, such as MARCO and CD200R, intracellular

markers for cytokines and transcription factors expression (IL6, IL1b, IL8, TGF-b1 IR5, pSTAT1) and markers for monocyte-deriving macrophages (CD209 or VSIG4) would benefit the study by increasing resolution and enabling the identification of additional macrophage sub-population in the human lung. Future studies that will involve in depth phenotyping of the human alveolar macrophages would assess the complexity of this unique type of human macrophages and how they respond to cues provided by the local tissue microenvironment in the lung. It is currently unknown whether the time-point interval between pneumococcal challenge and bronchoscopy have influenced the findings of the current study. Future studies that will aim to sample the human lung immediately after the completion of the follow-up visits and antibiotic treatment may allow us to detect more profound and dynamic alterations in alveolar macrophage phenotype and polarisation profile.

Our data provides strong evidence suggesting that not all macrophages fall into M1 and M2 subsets. The major AM population in steady state expresses a duo M1/M2 phenotype. This phenotype is present in individuals from two distinct geographical location and is stable even after exposure to stimuli known to alter the alveolar environment. This is consistent with recent call for a rethink of the M1/M2 macrophage paradigm (Hussell and Bell 2014). It is possible that the lack of stark polarisation is helpful in maintaining a healthy balance between immune tolerance and protective immunity in the alveolar space. However, whether other tissue macrophages, beyond decidual and alveolar macrophages, exhibit similar phenotypes *in vivo* warrants further investigation. As the clear majority of alveolar macrophages combine M1 and M2 features in steady state, such a phenotype that may allow brisk and adaptive responsiveness to multiple elements in the local milieu.

Chapter 4

Heightened human alveolar macrophage responsiveness to Bacterial Pathogens post nasopharyngeal pneumococcal colonisation

4.1 Introduction

Human and murine studies have mainly focused on examining the effect of nasopharyngeal pneumococcal colonisation on adaptive immune responses of lung mucosa, due to the potential application of findings on new vaccine designs against pneumococcal pneumonia. However, limited data exist on the impact that pneumococcal colonisation may have on lung innate immune cells, in particularly alveolar macrophages, the predominant lung cell population.

The alveolar macrophage (AM) – an innate type resident lung cell- is an integral component of lung immunity and its long-lifespan aids function (Marriott and Dockrell 2007). Alveolar macrophages are the first cells that combat pneumococci during early infection and the main cell population that mediates mucosal responses in the lower airways (Gordon and Read 2002). They also play a key role in shaping the adaptive immunity through their effects on dendritic cells and T cells (Lambrecht 2006). In murine models, it has been shown that AMs are mainly self-maintained, although during lung insult or as a result of ageing (Morales-Nebreda et al. 2015) differentiation of peripheral monocytes in a macrophage colony-stimulating factor (M-CSF) and granulocyte

macrophage (GM)-CSF-dependent manner contributes to their replenishment (Hashimoto et al. 2013, Guilliams et al. 2013).

A clear understanding of the mechanisms that underlie brisk but controlled lung immune responses at early stages of the infection is essential to inform us why high rates of pneumonia persists in the high-risk groups (infants, elderly and immunocompromised). Although, current immunisation strategies to pneumococcal disease target exclusively B cell dependent immunity, the recently described memory properties of innate cells, such as natural killer (NK) cells, monocytes (Netea et al. 2016, Quintin et al. 2014, Cheng et al. 2014) and most recently murine alveolar macrophages (Yao et al. 2018), indicate that innate immunity could be considered as a promising alternative or complementary vaccine target.

To investigate the association between nasopharyngeal pneumococcal colonisation and lung innate mucosal immune responses, we utilised our safe and reproducible experimental human pneumococcal challenge model. This model has been successfully used in the past to identify immune responses and correlates of protection against pneumococcal colonisation (Mitsi et al. 2017, Pennington et al. 2016, Ferreira et al. 2013), and also as a surrogate of protection and carriage density for testing novel pneumococcal vaccines. We showed for the first time that nasal human colonisation in absence of disease leads to pneumococcal aspiration to the lower respiratory tract, which enhanced AM opsonophagocytic capacity against a range of bacterial respiratory pathogens likely through an interferon-γ (IFN-γ)-mediated mechanism.

4.2 Material and Methods

Innate cellular responses to pneumococcus were measured in healthy, non-smoking, adults aged from 18-50 years old who participated in one of the three consecutive experimental human pneumococcal clinical trials conducting in Liverpool between 2015 and 2018 (Figure 4.1). Colonisation status was assessed by classical microbiology and molecular methods (lytA qPCR) on nasal wash samples. At the end of the follow-up visits (either D14 or D29 depending the EHPC study) Spn colonised individuals receive three courses of amoxicillin. In total 72 healthy young adults- 35 non-colonised and 37 Spn colonised- gave written consent and underwent a single-time point

research bronchoscopy at any day between Day 29 to Day 203 after the bacterial challenge (Day 0) (Figure 4.1). This procedure allowed us to sample the lung mucosa and compare AM function, cross-talk between AM and $CD4^+$ T cells, as well as cytokine milieu in both experimental groups. The time frame of BAL samples collection was deliberately selected to differ between studies, as this enabled us to investigate the duration of pneumococcal-mediated effects on the lung immunity (Figure 4.1).

Full details of the methods used to produce the data presented within this chapter are given in Chapter 2. Some method details more relevant to this chapter have been included in for ease of reference.

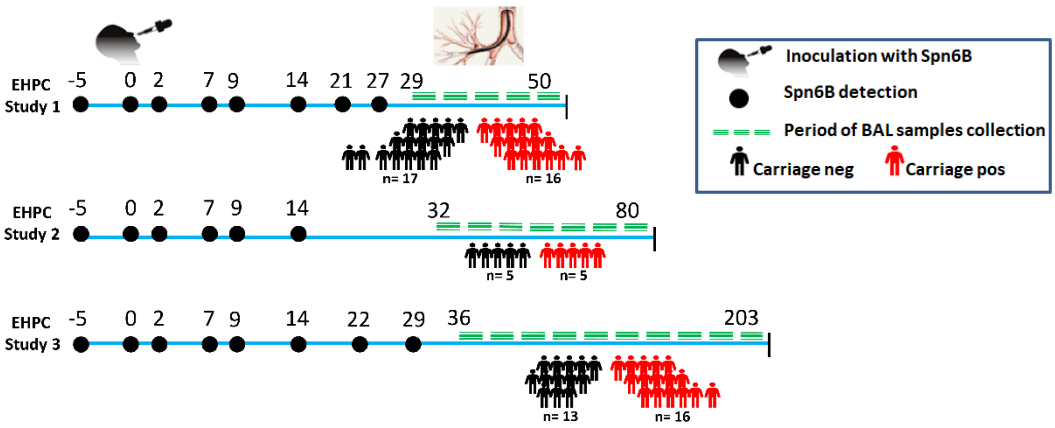


Figure 4.1. Study design. Defined time-period of BAL samples collection from Spn colonised (carriage+) and non-colonised (carriage-) individuals within three independent experimental human pneumococcal challenge (EHPC) studies. Individuals were purposively sampled according to colonisation state. After the final nasal wash (day 27, 17 or 29 based on study), Spn colonised individuals received a three-day course of antibiotics. Total BAL sample size, n= 72 (35 carriage negative and 37 carriage positive individuals).

4.2.1 Ethical approval and BAL consent

All volunteers gave written informed consent for the bronchoscopy and research was conducted in compliance with all relevant ethical regulations. Ethical approval was given by the North-west National Health Service Research Ethics Committee (REC). Ethics Committee reference numbers: 15/NW/0146 and 14/NW/1460.

4.2.2 Inoculum stock preparation and inoculation

All volunteers received intranasally a dose of 80,000 CFU/100µl of live Spn6B per nostril at D0 (Figure 4.1). Full details of inoculum stock preparation and inoculation procedure can be found in sections 2.5.2 and 2.4.1. respectively.

4.2.3 Nasal wash processing

Full details of nasal wash sample processing can be found in section 2.5.5.

4.2.4 Nasopharyngeal and oropharyngeal samples processing

Full details of NPS and OPS samples processing can be found in section 2.5.6.

4.2.5 Bronchoalveolar lavage processing

Full details BAL sample processing can be found in section 2.5.7.

4.2.6 Alveolar macrophages isolation

Alveolar macrophages used to generate the results presented in this chapter were isolated either using an adherence step or were purified from the whole BAL sample through cell sorting (FACS ARIAIII cell sorter, BD Biosciences). Full details can be found in section 2.5.8.

4.2.7 Lung lymphocytes isolation

Lymphocytes from BAL samples were separated from alveolar macrophages after adherence of the latter on a 24-well plate or were purified from the whole BAL sample through cell sorting (FACS ARIAIII cell sorter, BD Biosciences). Full details can be found in section 2.5.9.

4.2.8 Bacterial strains and growth conditions for *in vitro* assays

A live stock of *S. pneumoniae,* serotype 6B (the challenge strain) was used in uptake assays, whereas a heat-inactivated stock was used in lymphocyte or alveolar macrophage stimulation assays. Live stocks of a *Staphylococcus aureus, Streptococcus*

pyogenes and *Escherichia Coli* strain were also used in uptake assays. Full details of bacterial strain and their growth conditions can be found in section 2.5.3.

4.2.9 Opsonophagocytic Assays (OPA) using alveolar macrophages

All the versions of OPA assays used to generate the results presented in this chapter are described in full details in section 2.6.1.

4.2.10 Bacterial DNA extraction and qPCR

DNA was extracted from whole BAL, OPS and NPS samples, following qPCR analysis for the detection and quantification of pneumococcal DNA in these samples. The optimised qPCR protocol was specifically designed for the detection of the challenge strain (spn6B), targeting on a capsular polysaccharide gene known as *wciP*, the rhamnosyl transferase gene. Full details can be found in section 2.6.2.

4.2.11 Confocal microscopy assays

Full details of the confocal microscopy assays used to generate the results presented in this chapter can be found in section 2.6.3.

4.2.12 Flow cytometry-based assays

Full details of the different flow cytometry-based assay, as well as the combination of conjugated antibodies used per assay can be found in section 2.6.4.

4.2.13 Luminex analysis of BAL fluid

Full details of cytokine analysis in BAL fluid can be found in section 2.6.5.

4.2.14 Alveolar macrophage gene analysis using Nasostring platform

Full details of Nanostring method can be found in section 2.6.6.

4.2.15 Statistical analysis

Statistical analyses were performed using GraphPad Prism (Version 6, GraphPad Software, La Jolla, CA) and R software (version 3.5.1), including Bioconductor packages. Two-tailed statistical tests were used throughout the study. If two parametric groups were compared, a two-tailed t test was used for unpaired and paired groups. If two non-parametric groups were compared, a Mann-Whitney or Wilcoxon test was used for unpaired and paired groups respectively. If three or more non-parametric paired groups were compared, a Friedman test was used. For gene expression and Luminex analysis p values were corrected by applying multiple correction testing (Benjamin-Hochberg). To quantify association between groups, Pearson or Spearman correlation test was used for parametric or non-parametric groups, respectively. Differences were considered significant at $p \leq 0.05$ (*p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001).

4.3 Results

Here we combined the experimental human pneumococcal challenge model with the use of research bronchoscopy to investigate whether and how nasopharyngeal pneumococcal colonisation affects the alveolar macrophage function in healthy adults.

4.3.1 Experimental nasal pneumococcal colonisation enhanced alveolar macrophage responsiveness to respiratory pathogens

Antecedent pneumococcal colonisation was associated with 11.4% increase in alveolar macrophage (AM) capacity to take up pneumococci *in vitro.* AM opsonophagocytic capacity (OPA) ranged from 58.5% to 81.6% in the non-colonised group (carriage-) and from 69.7% to 90.7% in the Spn colonised (carriage+) (p=0.005, Figure 4.2A). The increased AM OPA in the carriage+ group was reproducible between studies and persisted for approximately 3 months following the intranasal pneumococcal inoculation (Figure 4.2B). Later after challenge (120-203 days), uptake was similar between the two groups, although we were insufficiently powered to detect any statistical differences (n=4 per group).

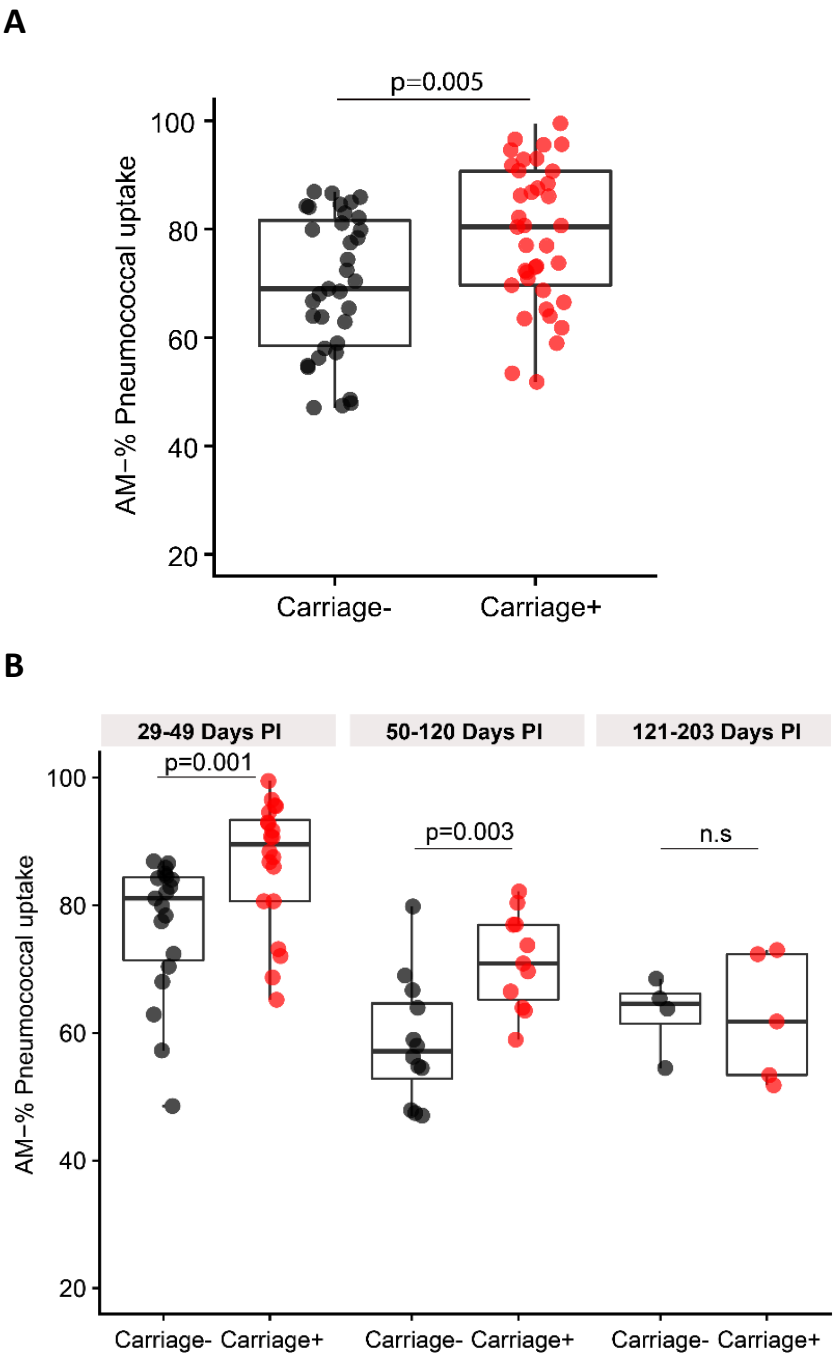


Figure 4.2. Enhanced alveolar macrophage opsonophagocytic capacity post nasal pneumococcal colonisation. A) Percentage of pneumococcal uptake by AM post *in vitro* infection in carriage- (n=35) and carriage+ (n=37) group. **p=0.005 by Mann-Whitney test. Multiplicity of infection (MOI) used was 1: 100. **B)** Chronological representation of all BAL samples (n=72) collected from one to six months post intranasal pneumococcal inoculation divided into three consecutive time periods. T1: ***p= 0.001, T2: **p= 0.003 and T3: p= 0.82 by Mann-Whitney test.

We also sought to examine whether this enhanced activity was specific to pneumococcus or whether AM responses to other pathogens were similarly increased. AMs from Spn colonised individuals had greater capacity to take up the respiratory pathogens *Streptococcus pyogenes* and *Staphylococcus aureus* (increased by 18% and 11%, respectively) when compared with AMs isolated from non-colonised individuals (p=0.009 and p=0.038 respectively, Figure 4.3). For the gram-negative bacterium *Escherichia coli* there was a non-significant increase in AM OPA in the carriage+ group (median: 20.6% increase, p=0.067, Figure 4.3).

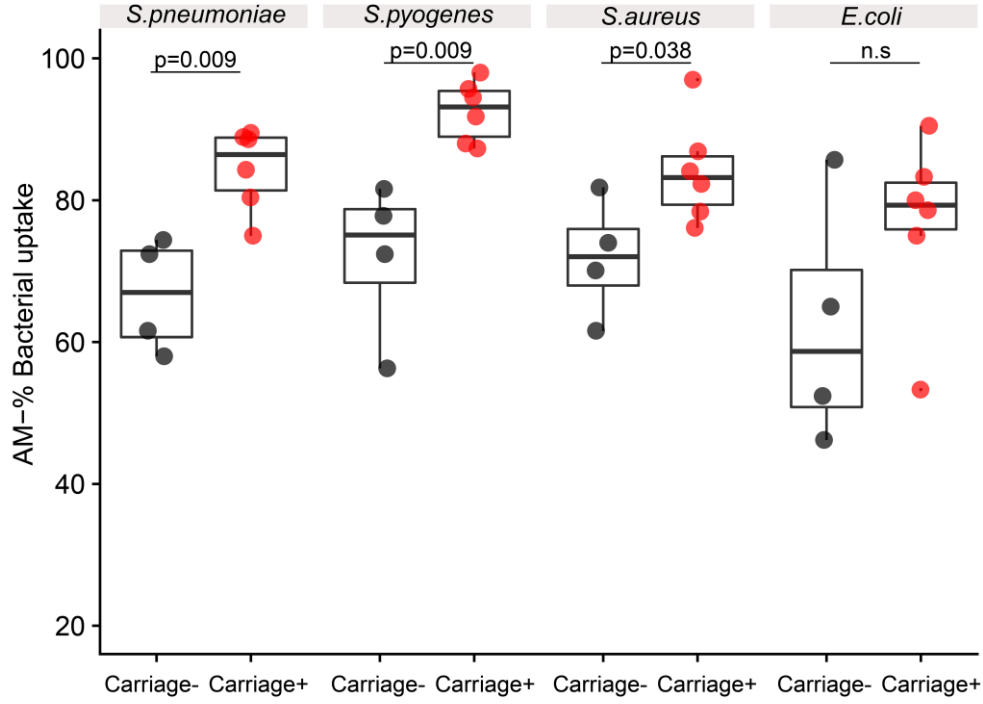


Figure 4.3. Alveolar macrophages increase responsiveness to take up other bacteria post nasal pneumococcal colonisation. Percentage of bacterial uptake by AM post *in vitro* infection with *Spn6B* or *S. aureus* or *S. pyogenes* or *E. coli*. **p= 0.009, **p= 0.009, *p= 0.038 and p=0.067, respectively by Mann-Whitney test. Boxplots and individual subjects are depicted with Carriage- in black dots and Carriage+ in red dots. BAL samples were collected between 29 to 49 days post challenge.

4.3.2 Micro-aspiration and evidences of pneumococcal presence in the lungs

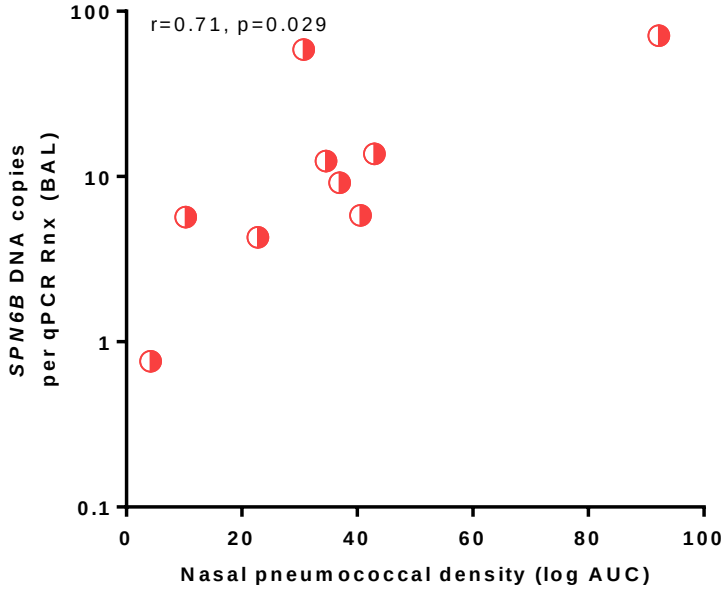
It is widely assumed that pneumococcal aspiration occurs and leads to bacterial entrance in the lower airways, with the potential to develop subsequent pneumonia in the high-risk groups. While pneumococcal aspiration has been linked with nasal pneumococcal density in pneumonia cases (Albrich et al. 2012), it has never been previously described in the absence of disease. To investigate whether pneumococcus is the stimulus of the enhanced AM responses in the pulmonary mucosa post nasal colonisation, we sought to find evidence of presence of the pneumococcal challenge strain (6B serotype or *Spn6B*) in the alveolar spaces.

For the detection of pneumococcus in the BAL samples, we utilised both classical microbiology and molecular methods targeting a capsular polysaccharide gene specific to Spn6B ***(wciP*- the rhamnosyl transferase gene)**. Whole DNA was extracted from 43 EHPC participants who had research bronchoscopy within a short period (29-50 days) post their nasal pneumococcal challenge. Spn6B DNA was detected in the BAL of 41% (9/22) of Spn colonised individuals (Table 4.1), 1 to 3 weeks following the clearance of nasal colonisation. None of the non-colonised individuals had detectable Spn6B DNA in their BAL sample. Additionally, BAL samples collected from volunteers between 50-120 days post challenge were negative for presence of Spn6B DNA, irrespective of colonisation status. Nasal pneumococcal density positively correlated with the copies of Spn6B DNA detected in BAL samples (Figure 4.4A). Spn colonised individuals differed in both density and duration of the colonisation episode (Figure 4.4B). In addition, sampling of the nasopharynx by nasopharyngeal (NP) swabs was performed in a subset of subjects prior to the bronchoscopy, in order to ensure that colonisation had been interrupted the day of the lung sampling. Whereas no live SPN6B was detected in any NP sample after culturing and qPCR against Spn6B capsule, two participants had SPN6B growth on agar plate after platting of BAL supernatant (Table 4.1). qPCR data confirmed the presence of 6B DNA in the whole BAL of the same two participants. Moreover, sorted AM capacity to take up pneumococci correlated positively with nasal pneumococcal density (Figure 4.5).

Methods of spn6B DNA detection in BAL and NP	Carriage pos.	Carriage neg.
Spn6B detected in the BAL by qPCR	9/22 (41%)	0/21 (0%)
Live Spn6B detected in BAL by culturing	2/16 (12.5%)	0/10 (0%)
Spn6B DNA detected in the NP and OP swabs by qPCR	0/12 (0%)	0/10 (0%)
Live Spn6B detected in NP and OP swabs by culturing	0/12 (0%)	0/10 (0%)

Table 4.1. Methods of Spn6B detection in lung and nose the day of research bronchoscopy. Spn6B DNA was detected in 41% of carriage positive volunteers (9 in 22 carriers) by qPCR targeting a Spn6B specific capsular polysaccharide gene. Spn6B DNA was not detected in any (0/21) non-colonised subjects. BAL samples were plated on blood agar plate and pneumococcal growth was observed in 12.5% (2/16) carriage positive volunteers, whereas there was no growth for any the carriage negatives (0/10). Nasopharyngeal (NP) and oropharyngeal (OP) swabs were taken prior to the bronchoscopy from 22 participants. SPN6B was not detected in any NPS or OPS sample after culturing or qPCR analysis. BAL samples were collected between 29 to 49 days post challenge.

A



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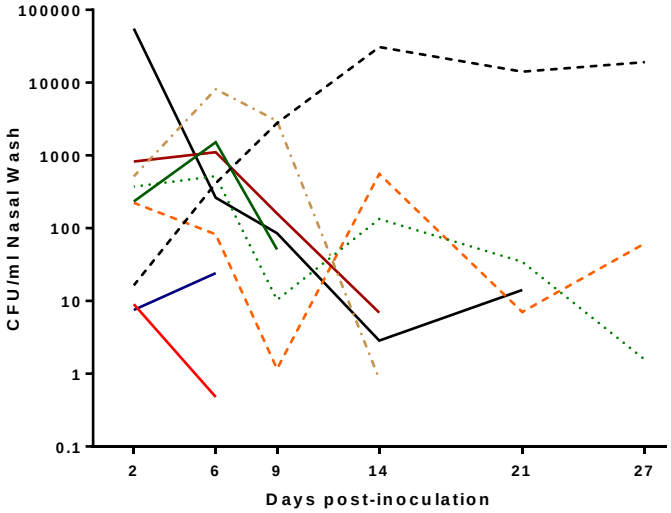


Figure 4.4. Evidences of pneumococcal presence in the lung of nasopharyngeal Spn colonised individuals. A) Positive correlation between the nasal pneumococcal density, expressed as the Area Under the Curve (log AUC) and the copies of pneumococcal DNA (Spn6B) detected in the BAL fluid of carriage+ individuals. r= 0.71, *p=0.029 by Pearson correlation test. **B)** Duration and density of nasal colonisation per individual with detected Spn6B DNA in the BAL fluid (9 in 22 Spn colonised). The end of each coloured line indicates the time point that the individual cleared colonisation, as assessed by classical microbiology methods.

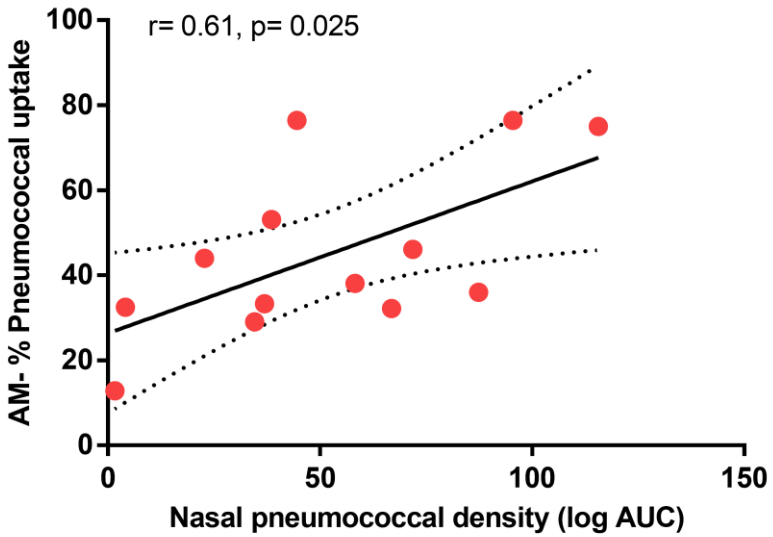


Figure 4.5. Nasal pneumococcal density is associated with increased AM capacity to take up pneumococci. Positive correlation between the nasal pneumococcal density (log AUC) and sorted AMs opsonophagocytic activity (n=13). Pearson correlation test results and linear regression line with 95% confidence interval are shown.

Utilising confocal microscopy and anti-sera against the polysaccharide capsule of serotype 6B, we confirmed the relationship between nasal colonisation and presence of pneumococcal particles in the lung. Pneumococcal cells were found associated with the surface of AMs or internalised by them; a phenomenon only observed in Spn colonised volunteers (Figure 4.6A-C).

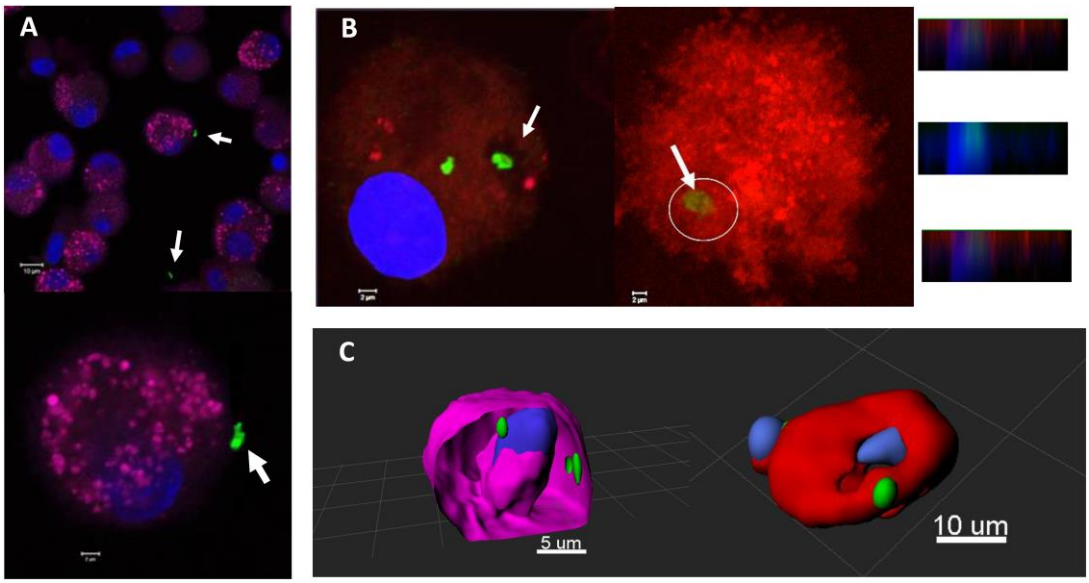


Figure 4.6. *S. pneumoniae* in association with alveolar macrophages. A-C) Representative images taken by confocal microscope showing: **A)** pneumococci around AMs and **B)** internalised pneumococci by AMs derived from Spn colonised individuals. CD45-magenda or CD14-red, nucleus-blue (DAPI) and Spn6 capsule-green. Scale bar= 2µm. Inset panels represent Z-stack images of the cell with internalised pneumococcus. **C)** 3D reconstruction of deconvolved Z-stack confocal images of alveolar macrophages. The samples were stained with either wheat germ agglutinin (on the left; magenta) or anti-CD169 monoclonal antibody (on the right; red). Spn6 in green and nuclei in blue (DAPI).

4.3.3. $CD4^+$ Th1 skewed responses rapidly prime AMs

To investigate whether the observed augmented AM capacity to take up pneumococci *in vitro* was dependent on lung lymphocytes, we co-incubated AMs with autologous $CD3^+CD4^+$ T cells during *in vitro* infection with *Spn6B*. The presence of $CD3^+CD4^+$ T cells enhanced the basal AM opsonophagocytic capacity in both non-colonised (1.6-fold, $p<0.0001$) and Spn colonised individuals (1.8-fold, $p<0.0001$) (Figure 4.7). AM opsonophagocytic capacity differed between the two groups at baseline (prior to lung-derived autologous $CD3^+CD4^+$ T cell addition) and remained higher in Spn-colonised when autologous $CD4^+$ T cells were present (Figure 4.7).

To elucidate the mechanism underlying this increased boosting of AM function by $CD4^+$ T cells from Spn colonised individuals, we stained lung lymphocytes intracellularly for T-box transcription factor expressed in T-cells (T-bet), GATA-binding protein-3 (GATA-3) and Forkhead box P3 (FoxP3) transcription factors. The gating strategy used to identify the proportion of $CD4^+$T-bet^+, $CD4^+$GATA-3^+ and $CD4^+$FoxP3^+ T cells among the total lung $CD4^+$ T cells is shown on Figure 4.8.

In the Spn colonised group the levels of $CD4^+$ T-bet expressing cells were twice as high than in the non-colonised group ($p= 0.003$), indicating Th1-polarisation (Figure 4.9). There were no significant differences in the levels of neither $CD4^+$ GATA-3 expressing nor $CD4^+$ FoxP3 expressing T cells between the two groups (Figure 4.9).

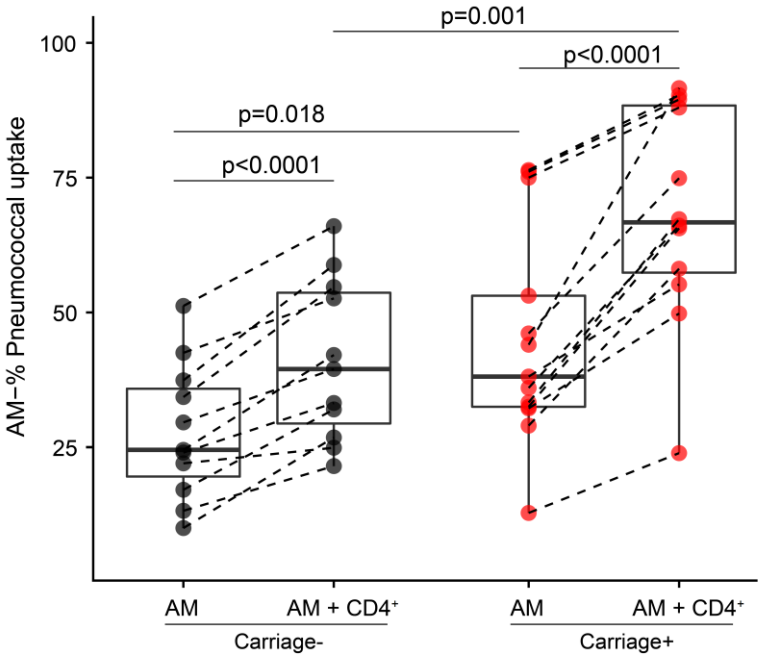


Figure 4.7. AMs crosstalk and priming by autologous CD4$^+$ T subsets. Comparison of phagocytic activity between sorted AM and sorted AM plus autologous BAL isolated CD3$^+$CD4$^+$ T cells from both carriage- (n=11) and carriage+ (n= 13). MOI=1:20. AM and CD4$^+$ T cells were used in a 10:1 ratio. ****p< 0.0001 in both groups by paired t-test. Comparison of AM basal opsonophagocytic activity between the two groups. *p= 0.018 by unpaired t-test with Welch's correction. Comparison of AM opsonophagocytic activity in the presence of lung CD4$^+$ T cells between carriage- and carriage+ group. **p= 0.001 by unpaired t-test with Welch's corrections. Boxplots and individual subjects are depicted with carriage- in black carriage+ in red, with paired samples connected by dashed line.

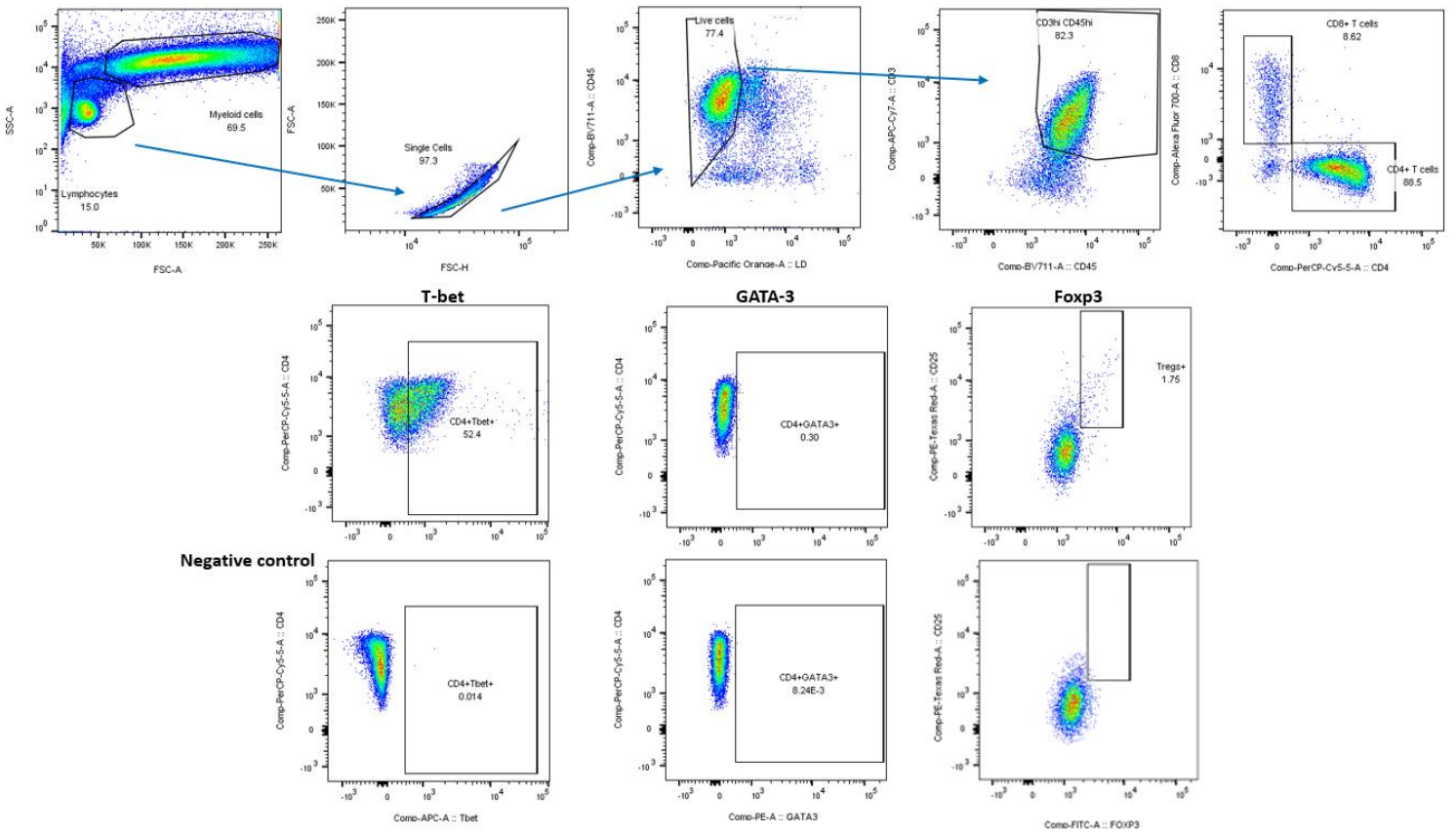


Figure 4.8 Transcription factors expression in CD4+ T cells. Gating strategy of $CD3^{+}CD4^{+}$ T cells for the transcription factors T-bet, GATA-3 and FoxP3 expression for one representative volunteer. Fluorescence Minus One (FMO) controls were used to verify flow cytometric data.

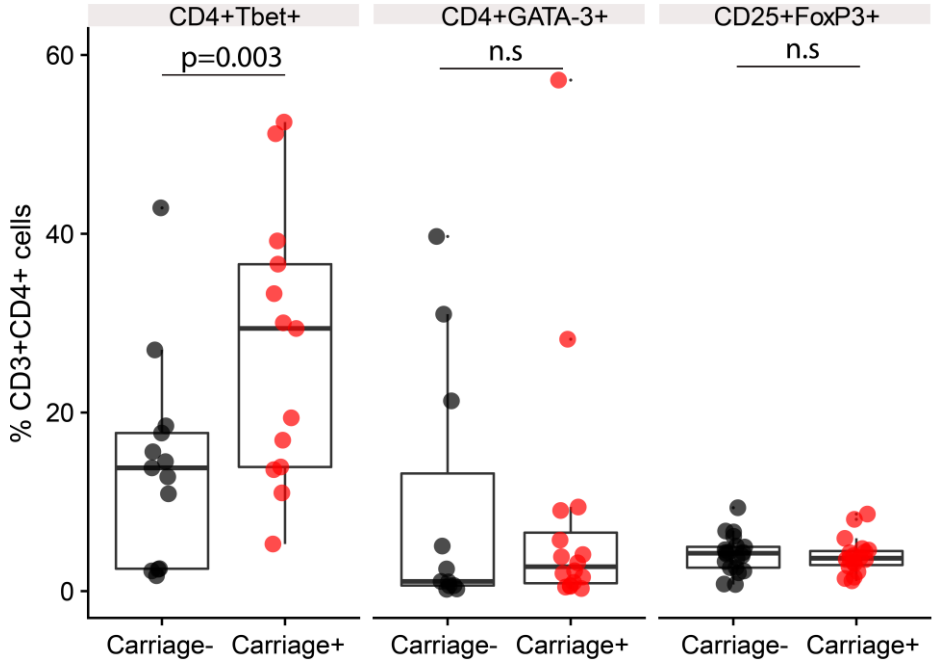


Figure 4.9. Th1 polarisation of lung CD4⁺ T cells in the pneumococcal colonised group. Intracellular staining of CD4⁺ T cells for T-bet, GATA-3 and FoxP3 transcription factors, expressed as percentage of CD3⁺CD4⁺ BAL lymphocytes. **p= 0.003, p= 0.85, p= 0.33 respectively by unpaired t-test with Welch correction test. Boxplots and individual subjects are depicted with carriage- in black dots and carriage+ in red dots.

In parallel, lymphocytes from both Spn colonised and non-colonised volunteers were stimulated with pneumococcal antigen (Heat Inactivated-Spn6B). Cytokine (IFN-γ, Tumour necrosis factor a [TNF-α] or Interleukin 17A [IL-17A]) producing CD4⁺ T-cells were subsequently detected by flow cytometry (Figure 4.10). Alveolar macrophages OPA correlated with cytokine producing CD4⁺ T cells, classified as spontaneous (unstimulated) or pneumococcal-responding cells (Figure 4.11). Increased levels of IFN-γ producing CD4⁺ T cells, both pneumococcal-specific and spontaneous responding, positively correlated with AMs ability to take up live pneumococci *in vitro*. (Figure 4.11A). On the other hand, AMs OPA correlated positively with only the pneumococcal-specific TNF-α producing CD4⁺ T cells (Figure 4.11B), whereas IL-17A producing CD4⁺ T cells did not correlate with AMs OPA in any condition (Figure 4.11C).

Together, these results indicate that skewing of CD4⁺ T cells and production of IFN-γ associated with the increased capacity of AM to take up bacteria.

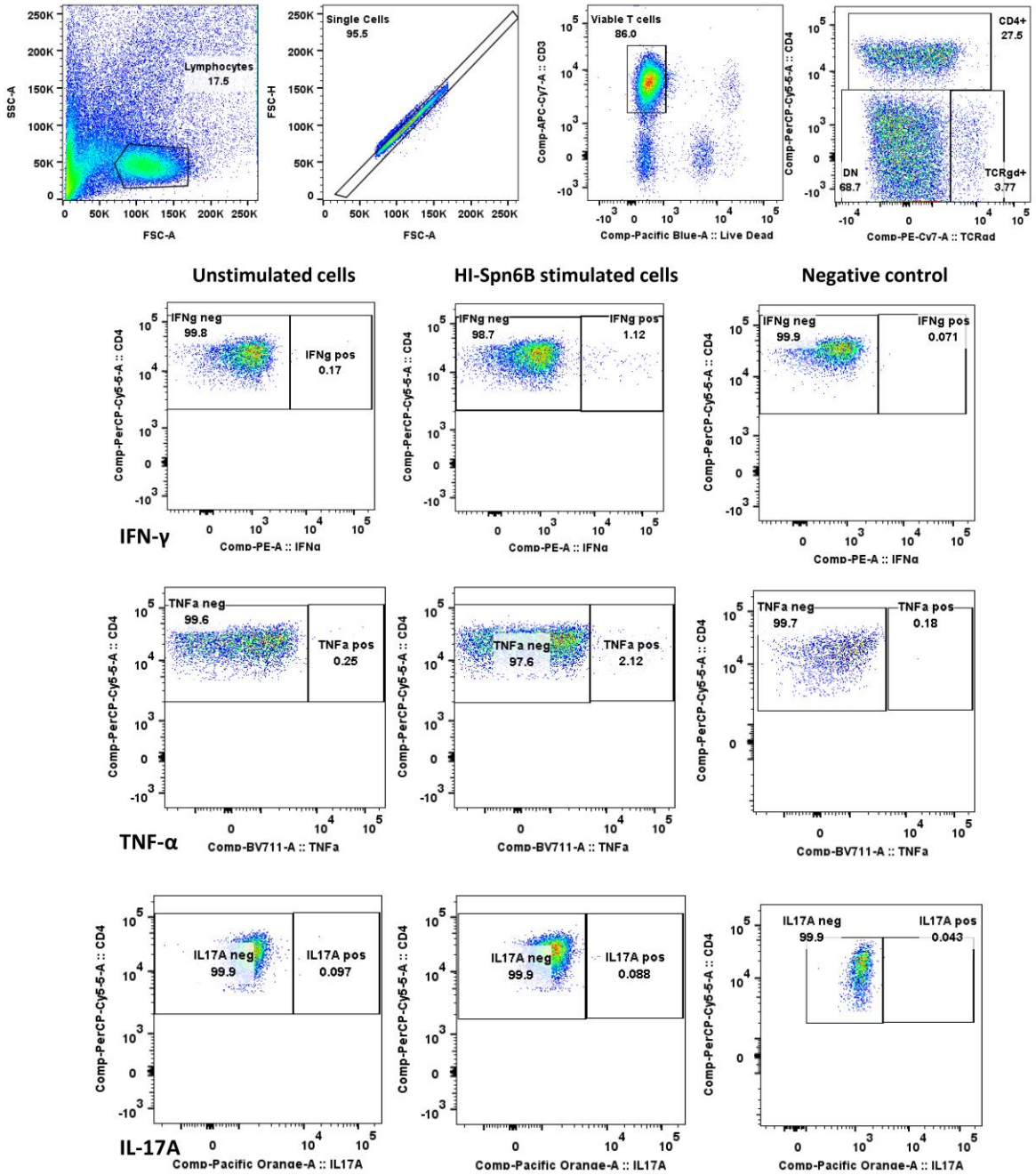


Figure 4.10. Gating strategy of cytokine (INF-γ, TNF-α and IL-17A) producing T cells following overnight stimulation with HI-Spn6B. Cytokines production were defined by intracellular staining. Gates from one representative volunteer are shown**.** Fluorescence Minus One (FMO) controls were used to verify flow cytometric data.

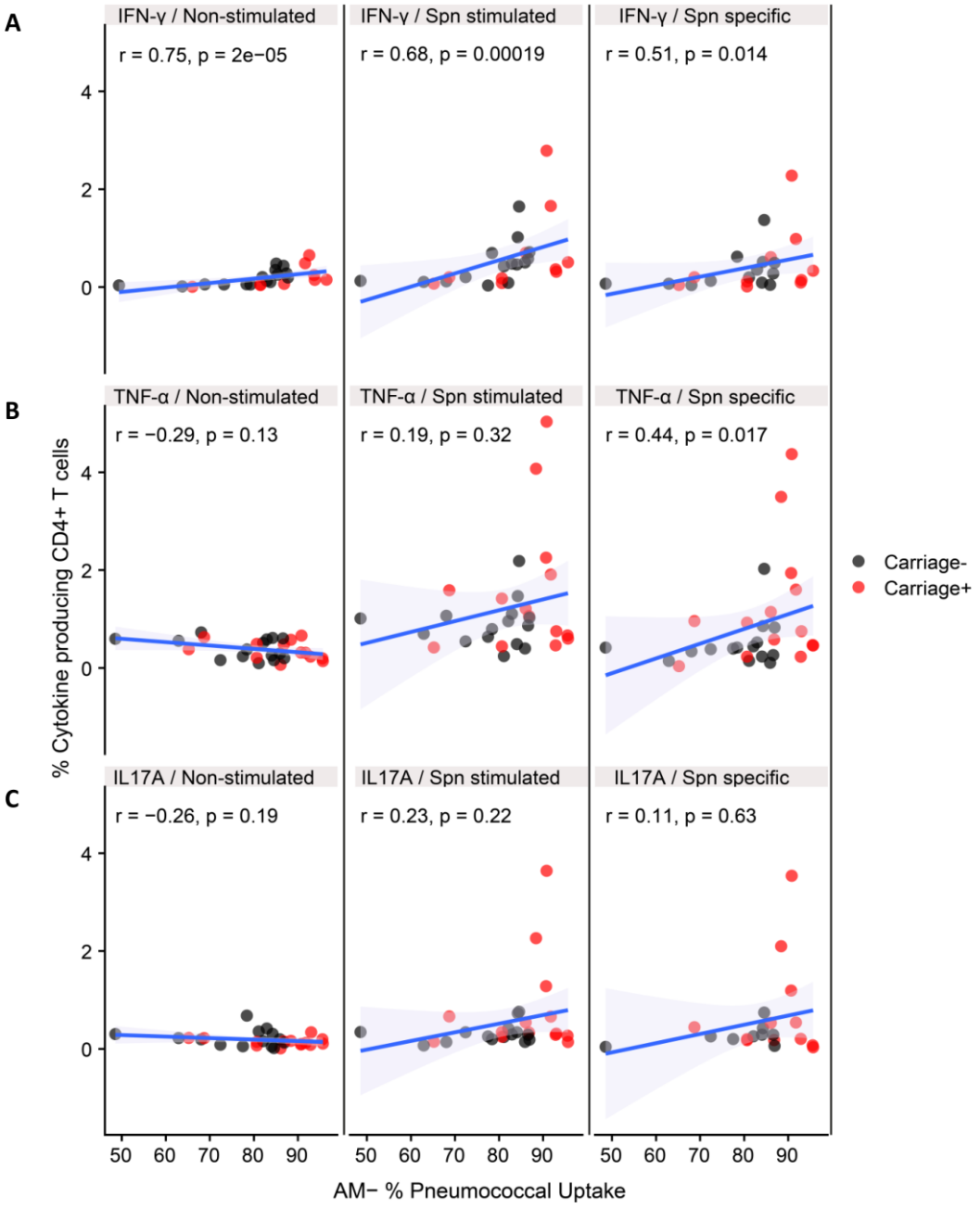


Figure 4.11. Correlations of AM opsonophagocytic activity with CD4⁺ Th1 and Th17 responses. A) From left to right are illustrated significant correlations between the levels of IFN-γ expressing CD4⁺ T cells at baseline (non-stimulated), total IFN-γ expressing CD4⁺ T cells post stimulation with HI-Spn6B and the Spn-specific responding CD4⁺ T cells (non-stimulated condition subtracted from Spn-stimulated condition) with alveolar macrophage OPA. Spearman Rho and p values are shown. **B)** Significant correlation of Spn-specific, TNF-α expressing CD4⁺ T cells with AM OPA. Spearman Rho and p value are shown. **C)** From left to right are illustrated the levels of IL-17A expressing CD4⁺ T cells at baseline and the levels of total and Spn-specific, IL-17A expressing CD4⁺ T cells in association with AM OPA. No significant correlations. Spearman correlation test results and linear regression line with 95% confidence interval (purple shedding) interval and are shown.

4.3.4 Alterations of lung cytokine milieu post nasal colonization and the effect of IFN-γ on AM opsonophagocytic capacity

The alveolar microenvironment is crucial for cell signalling, shaping how local cells respond to different stimuli (Hussell and Bell 2014). To assess alterations of the alveolar cytokine milieu induced by nasal pneumococcal colonisation, we measured levels of 30 cytokines and chemokines in the BAL fluid retrieved from both Spn colonised and non-colonised individuals (Figure 4.12A). Three cytokines had higher detectable levels in the BAL fluid of Spn colonised group: GM-CSF (p=0.032) and the pro-inflammatory cytokines IFN-γ (p=0.047) and IFN-α (p=0.043) (Figure 4.12B), although statistical significance was lost upon correcting for multiple testing. Full list of the 30 cytokines compared between the two groups will be found in Appendix (Table I).

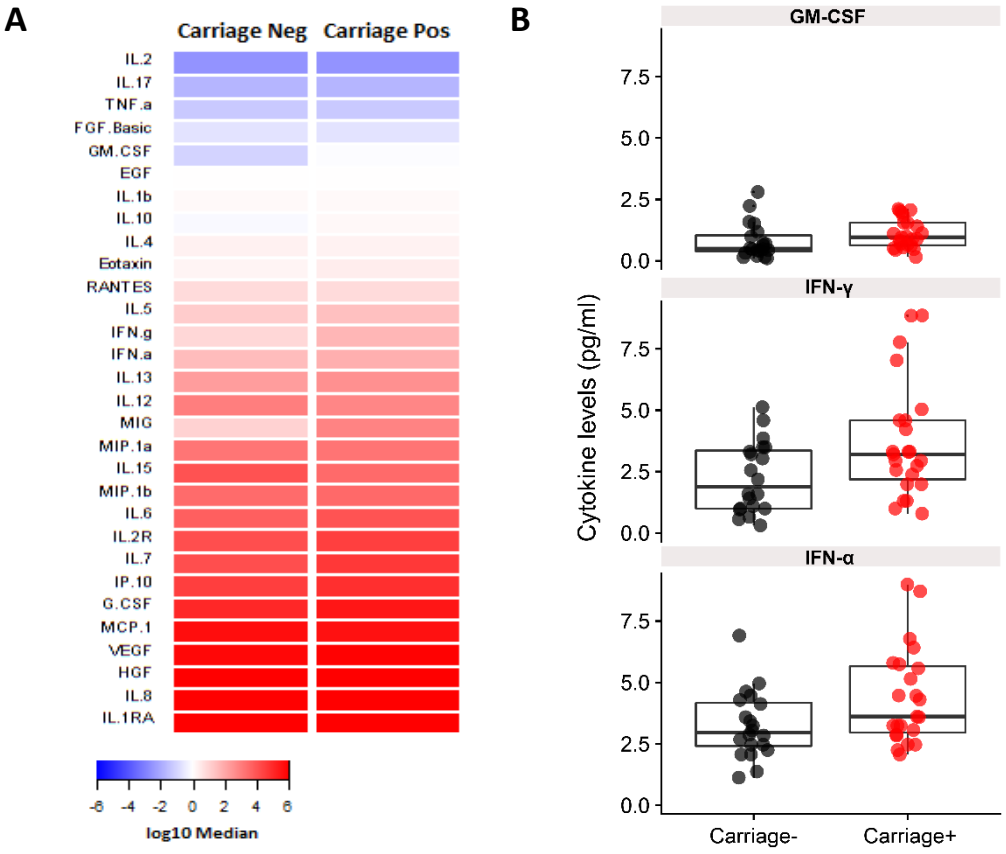


Figure 4.12. Lung cytokine milieu and alterations mediated by nasal colonisation. A) Heatmap of the 30 cytokines levels, expressed as log10 median (pg/mL), measured in the BAL fluid (carriage-; n=20 and carriage+; n=22). **B)** Levels of significantly different cytokines between the two groups, expressed as pg/ml. GM-CSF, IFN-γ and IFN-a with *p= 0.032, *p=0.047 and *p=0.043 respectively, analysed by Mann-Whitney test. Boxplots and individual subjects are depicted with carriage- in black dots and carriage+ in red dots.

To address the role of increased secretion of IFN-γ, a prototypic Th1 cytokine, in the pulmonary airspaces and its effect on AM function, we primarily neutralized the IFN-γ receptor 1 (IFN-γR1), a subunit of the heterodimeric IFN-γ receptor found on macrophages. IFN-γ binds directly on IFN-γR1, with latter to play an essential role in the IFN-γ pathway that is required for the cellular response to infectious agents (van de Wetering et al. 2010). Directly before the OPA, AMs were stimulated with exogenous IFN-γ, either with a concentration that would reflect better the average *in vivo* levels of IFN-γ in the lung lining fluid of Spn colonised group (0.4ng/ml) or with 2ng/ml. Sole addition of IFN-γ enhanced significantly the AM capacity to take up pneumococci in both concentrations (median: 1.37-fold change using 0.4ng/ml and 1.45-fold change using 2ng/ml of recombinant IFN-γ). Blocking of IFN-γ binding receptor prior to IFN-γ addition inhibited the stimulatory function of the cytokine upon AM (Figure 4.13). We investigated further the role of IFN-γ upon AM activation by stimulating them with 10-fold increasing concentrations of exogenous IFN-γ. The lowest tested titres of IFN-γ (2 and 20ng/ml) augmented AMs OPA, resulting both in 1.5-fold increase (1.5x median; IQR:1.2x- 2.1x) in AM pneumococcal uptake, whereas no significant increase was seen with the highest used concentrations (200 and 2000ng/ml) (Figure 4.14A). These results were verified when AM response was assessed using a flow cytometric cytokine production assay (Figure 4.15). AMs produced increased levels of TNF-α in response to stimulation with HI-*Spn6B* only at the lower pre-stimulation doses of IFN-γ (Figure 4.14B). The mechanism seems to have a threshold, as demonstrated by the data, with IFN-γ signalling being beneficial for AM function at lower doses *in vitro*, but not at higher concentration.

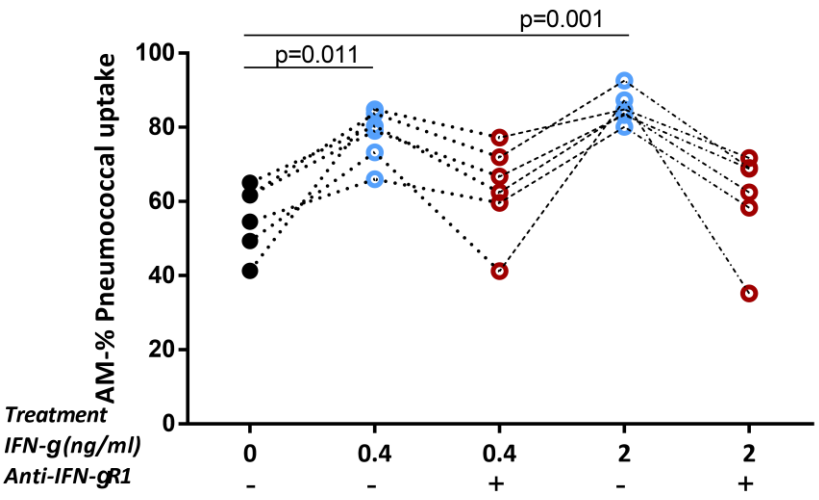
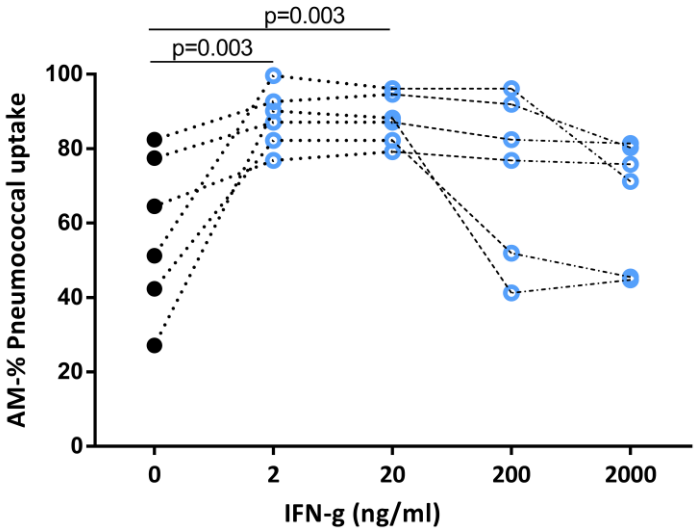


Figure 4.13. IFN-γ enhances alveolar macrophage capacity to uptake pneumococci *in vitro.* AM stimulated with 0.4 or 2ng/ml of recombinant IFN-γ (light blue dots) in presence or absence of anti-human antibodies against IFN-γR1 (brown dots). Unstimulated AMs used as control condition (black dots). *p< 0.011, *** p< 0.001 by Friedman test followed by Dunn's multiple comparison.

A



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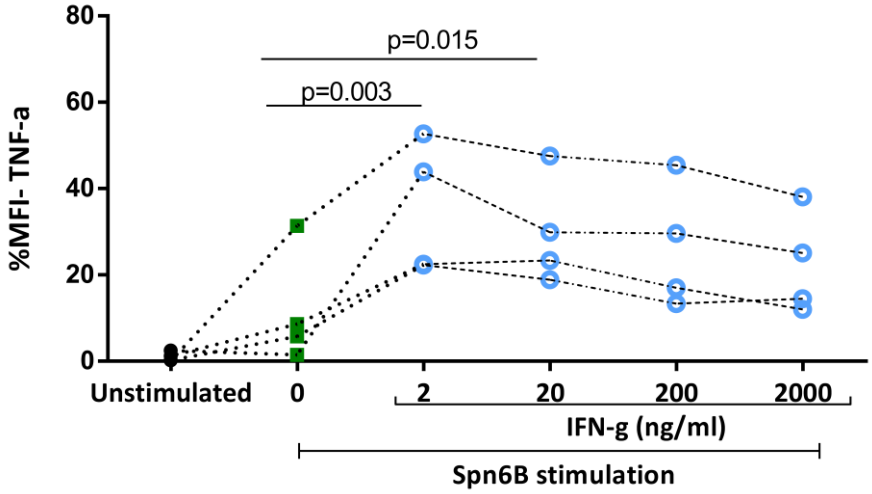


Figure 4.14. The effect of graduate increase of IFN-γ concentration on alveolar macrophage function. A) The effect of 10-fold increasing doses of exogenous IFN-γ (2-2000ng/ml) on the capacity of AM to uptake pneumococcus (live Spn6B used, MOI= 1:100). AM isolated from 6 non-challenged subjects. Individuals samples are depicted and connected by dashed lines. ** p< 0.003 by Friedman test followed by Dunn's multiple comparison. **B)** TNF-α production from AMs, pre-treated or not with exogenous IFN-γ (2-2000ng/ml), following stimulation with HI-Spn6B. AM isolated from 4 non-challenged subjects. Individuals samples are depicted and connected by dashed lines. *p< 0.015, ** p< 0.003 by Friedman test followed by Dunn's multiple comparison.

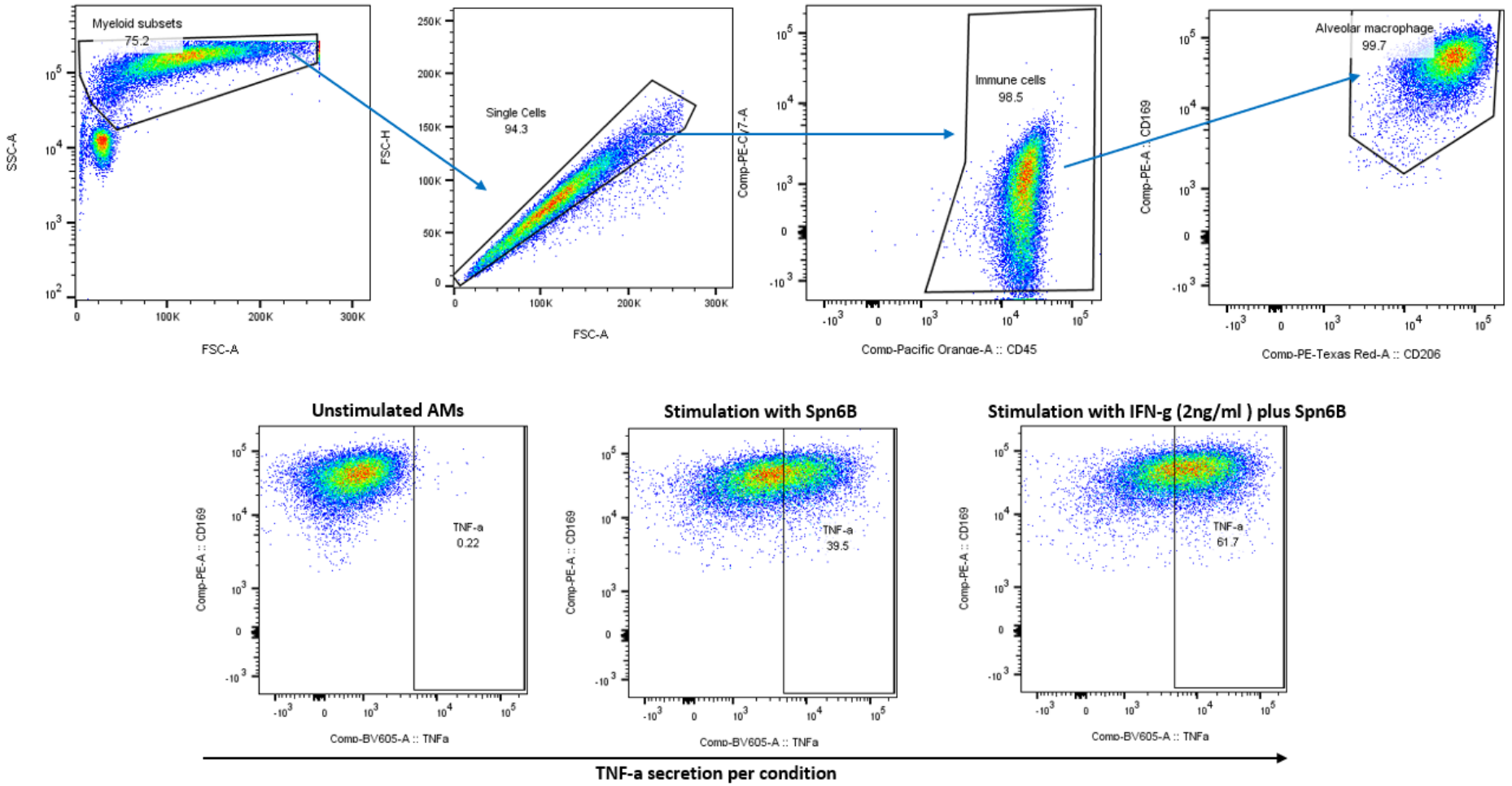


Figure 4.15. Gating strategy followed for cultured alveolar macrophages. AMs were stimulated with HI-Spn6B or HI-Spn6B plus IFN-γ or unstimulated to assess TNF-α secretion per experimental condition.

4.3.5 Pneumococcal colonisation may promote monocyte-to-macrophage differentiation in the alveolar spaces

Previously, we have demonstrated that AMs are not altered phenotypically by the presence of pneumococcus in the nasopharynx, as defined by classical monocyte polarisation surface markers (Mitsi et al. 2018). However, given the increased capacity of AM to take up pneumococcus, we extended our assessment to other lung myeloid cell populations (monocytes and neutrophils), in order to determine whether recent pneumococcal carriage alters the distribution of these cells in the airway. Identification of monocytes in the BAL samples using flow cytometry is shown on Figure 4.16, whereas the gating strategy followed for neutrophils was previously presented in Chapter 3 (Figure 3.1).

Spn colonised individuals displayed significantly greater AM levels (1.2-fold increase, p=0.04) and higher AMs/monocytes ratio (2.3-fold increase, p=0.04) in the lung compared to non-colonised individuals (Figure 4.17A). On the other hand, monocyte levels, both total and $CD14^{hi}CD16^{lo}$ and $CD14^{hi}CD16^{hi}$ subsets, had no significant difference between the two groups, despite their trend for increased presence in the non-colonised group (Figure 4.17B). Similarly, no difference in neutrophil levels was observed between the two groups (Figure 4.17B), indicating that nasal carriage in absence of disease does not lead to neutrophil recruitment to the lung.

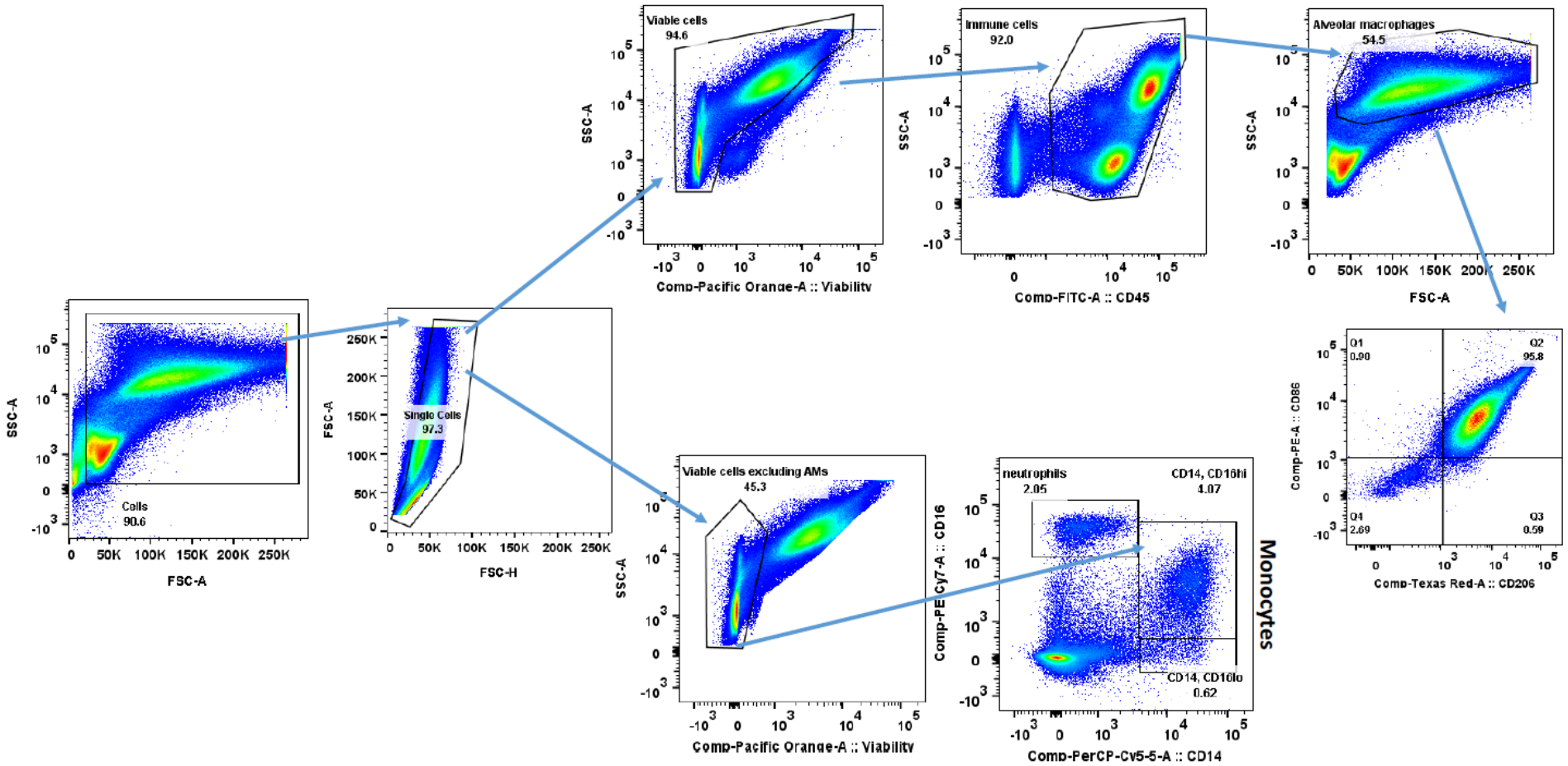


Figure 4.16. Gating strategy followed for monocytes. Monocytes in the BAL samples were defined as two subsets: $CD14^{hi}CD16^{lo}$ and $CD14^{hi}CD16^{hi}$.

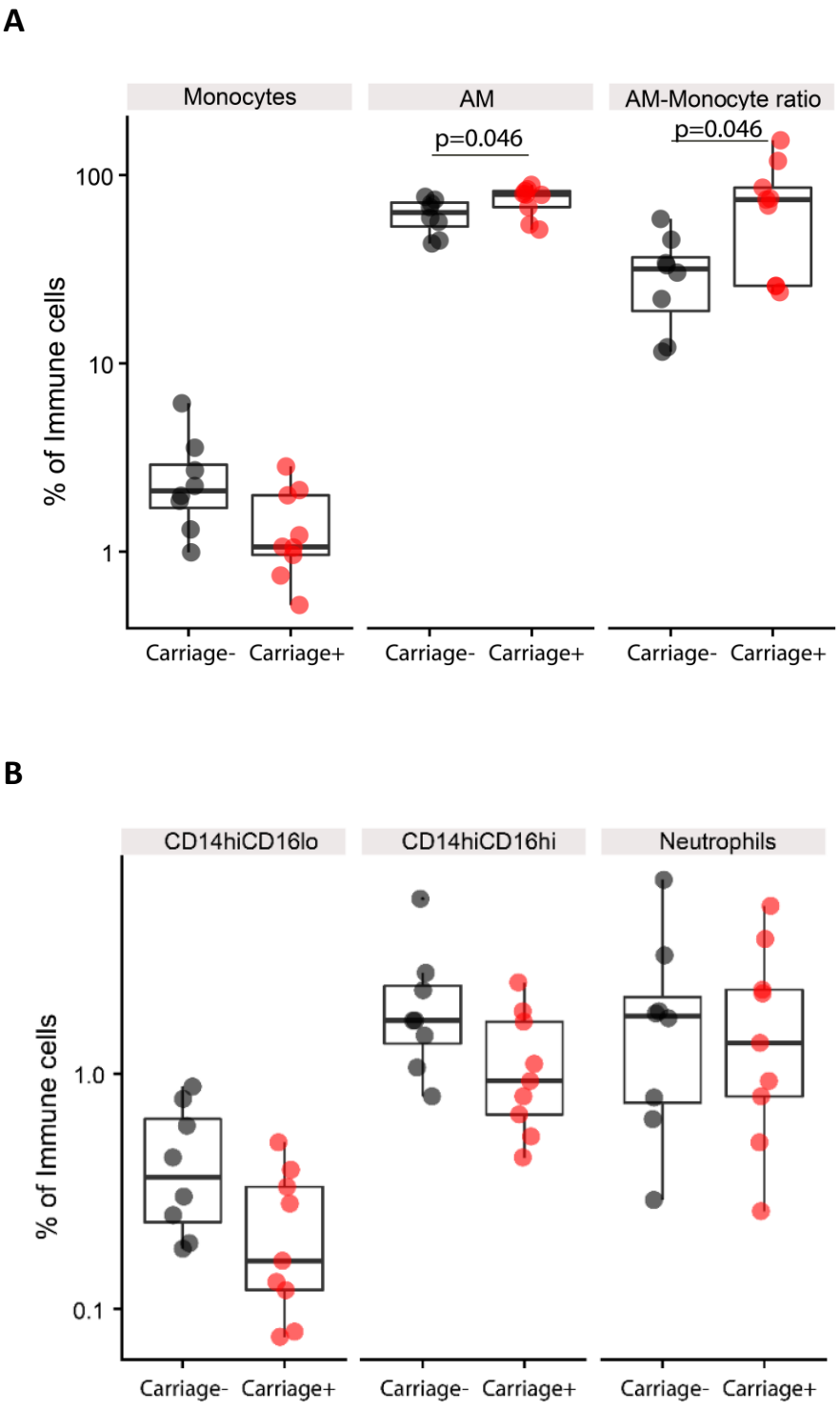


Figure 4.17. Pneumococcal colonisation may promote monocyte-to-macrophage differentiation. A) Levels of monocytes and AMs in the BAL of carriage- (n=8) and carriage+ (n=9), expressed as percentage of $CD45^{+}$ cells. Significant comparison of AM levels and AM: Monocytes ratio between the two study groups, * p=0.046 by Mann-Whitney test. Boxplots and individual subjects are depicted with Carriage- in black and Carriage+ in red. **B)** Monocytes and neutrophils analysed based on their CD14, CD16 expression. In monocytes, CD16 expressional levels divided them to two subsets, CD14hiCD16lo and CD14hiCD16hi. Boxplots and individual subjects are depicted with carriage- in black and carriage+ in red.

To test whether antecedent pneumococcal colonisation led to monocyte differentiation and AM activation, we sought to identify the differential gene signatures of Spn colonised and non-colonised volunteers. We isolated AMs by cell sorting from a subset of BAL samples and performed NanoString expression analysis of 594 immunological genes. Gene set enrichment analysis was performed on all genes, ranked from high to low expressed in the Spn colonised group compared to the non-colonised group, using published blood transcriptional modules (Appendix- Table II) (Li et al., 2014). Purified alveolar macrophages from Spn colonised individuals showed an enrichment in pathways of cell differentiation and function, revealing under-presentation of monocytes surface markers and over-presentation of antigen-presentation markers in the Spn colonised group (Figure 4.18). Some of the genes in these sets that were most increased or decreased in Spn colonized individuals included: *HLA-DQA1, CLEC7A, LY96 and HLA-DPB1* (antigen presentation module M5.0) and *PTAFR*, *FCGRT* and *CD4* (monocyte surface signature-module S4). This finding complements the previous observation that nasal colonisation may lead to monocyte-alveolar macrophage differentiation. We then looked whether individual AM gene expression was associated with colonisation and/or function. Between, the two groups 34 genes were altered, although none remained significant after correction for multiple testing (potentially due to small sample size of 5 per group) (Appendix-Table III).When the AM OPA per individual was compared with gene expression (log counts per million [CPM]) measured for each of the 594 genes, 34 genes were positively correlated with AM function to take-up the bacteria (Appendix- Table IV). Only four genes were both significantly correlated with AM OPA and significantly increased in Spn colonised individuals: T-box 21 (TBX21), ecto-5'-nucleotidase (NT5E), Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) and Toll like receptor 8 (TLR8) (Figure 4.19).

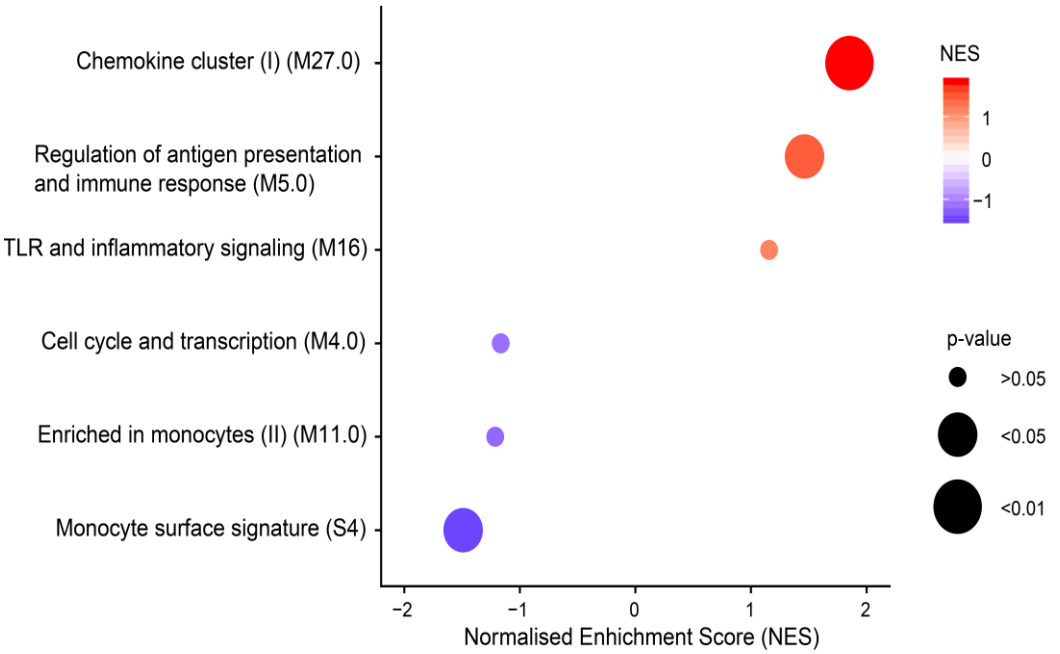


Figure 4.18. Enrichment in pathways of differentiation and function in Spn colonised group. Top pathways after gene set enrichment analysis for pathways and function applied on 2logFC (n= 5 subjects per group). NES presented in gradient colour. Red shades indicate pathways over-presented, whereas blue shades pathways under-presented in the carriage positive group. **p <0.01 and *p<0.05.

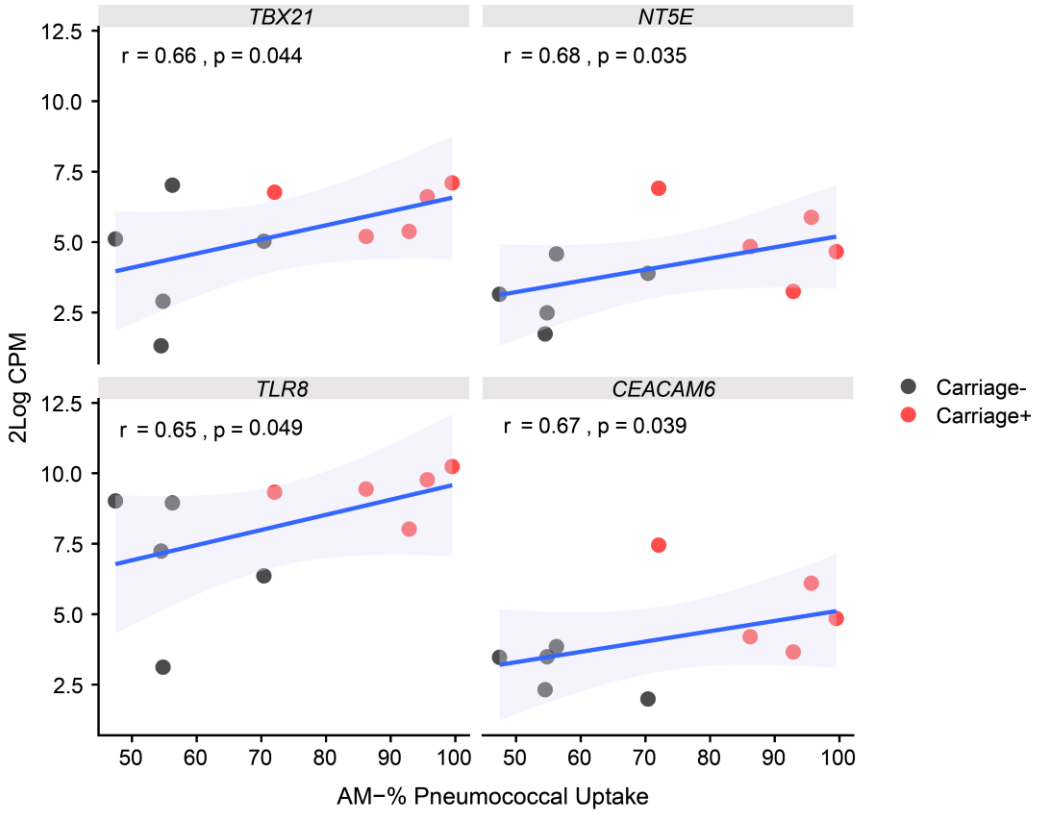


Figure 4.19. Increased AM opsonophagocytic activity is associated with M1 polarisation in AM based on gene expression analysis. Correlations between alveolar macrophage OPA and 2log CPM of TBX21, NT5E, TLR8 and CEACAM6. Spearman correlation test results and linear regression line with 95% confidence interval (purple shedding) interval and are shown.

4.4 Discussion

In this study we aimed to determine the effect of nasopharyngeal colonisation on lung immune responses, focusing on alveolar macrophages and their cross-talk with cells of adaptive immunity. Using our experimental human pneumococcal challenge model, we demonstrated that prior nasopharyngeal pneumococcal colonisation results in bacterial aspiration to the lower airspaces, leading to increased AM opsonophagocytic capacity against both pneumococcus and other bacterial pathogens. Aspirated pneumococci most likely act as the stimulus that leads to enhanced AM responsiveness. We observed that autologous lung derived$CD4^+$ T cells cross-talk enhanced AM basal capacity to take up pneumococci *in vitro* and that higher proportion of Th1 CD4+ T cells was found in the BAL samples of Spn-colonised group. Combined with the increased Th1 cytokine secretion measured in the lung of Spn colonised group, we propose that CD4+ T cells affect AM antibacterial function via an IFN-γ dependent manner. Systemic effects of colonisation may have also contributed to the heightened lung immune responses. However, the systemic profile of immune cells usually does not reflect the mucosal sites, due to compartmentalisation (Sathaliyawala et al. 2013). Also, in the past we described that the effect of pneumococcal colonisation on cognate T cells is more pronounced in the lung than in blood (Wright et al. 2013). Despite temporary influences from blood, the human lung exhibits a unique microenvironment that adapts to environmental challenges, and so do AM adapt to accommodate the ever-changing needs of the tissue (Hussell and Bell 2014).

The lung mucosa is not the sterile environment previously thought (Charlson et al. 2011, Man, de Steenhuijsen Piters and Bogaert 2017). By employing classical microbiology, molecular and visualisation methods, we demonstrated that pneumococcal aspiration occurs during nasal pneumococcal colonisation, a phenomenon that was previously observed only in pneumonia cases (Albrich et al. 2012, Greenberg et al. 2011). The positive correlation between AM opsonophagocytic activity and nasal pneumococcal density suggested pneumococcal cell trafficking from the nasopharynx to the lung airways. Spn-colonised volunteers received amoxicillin treatment prior the bronchoscopy to clear pneumococcal colonisation. This was a pre-requisite in sampling the human lung without artificially introducing pneumococci, however it is possible that bacterial fragments generated during the antibiotic treatment

might trigger host responses both systemically and at the respiratory mucosal sites. Although, it is difficult to assess the direct effect of this intervention on the AM responsiveness, our findings indicate that AM function is equally increased in those Spn-colonised volunteers who had naturally cleared colonisation 1 to 2 weeks prior the antibiotic treatment.

Interestingly, pneumococci were detected inside AMs or isolated alive from the BAL sample of Spn colonised individuals when colonisation had cleared a few days prior to the bronchoscopy. This observation merits of further investigation of pneumococcal survival inside AMs, analytical characterisation of the AM subsets in the health human lung and molecular signatures that drives differential AM function. This findings together with the recent report that pneumococcus has the ability to survive and replicate within murine splenic macrophages (Ercoli et al. 2018), points out for the need to analyse human AM to single cell levels. AM subpopulations that retain pneumococci or internalise them utilising receptors that could lead to insufficient bacterial lysis could stand as potential pneumococcal reservoir.

The increased opsonophagocytic capacity displayed by AMs was a non-specific response to pneumococcal stimulus. AM responded with equal efficacy to both Spn and other gram-positive respiratory pathogens *in vitro*. By contrast, we did not see significant enhancement of AM opsonophagocytic activity (OPA) against *E. coli,* although the small sample size used might have limited the detection of a less pronounced difference between the two experimental groups.

Our observation shares some similarities with the findings of emerging studies on "trained immunity" (termed also as innate immune memory) (Cheng et al. 2014, Saeed et al. 2014), which have shown the increased responsiveness of innate immune cells to microbial stimuli, caused by epigenetic changes post their activation by varying stimuli (e.g. *Candida albicans* infection, Bacille Calmette-Guerin [BCG] or measles vaccination). Similar to our observation, this augmented functional state persisted for weeks to months, and moreover it conferred resistance to reinfection or heterologous infection (Netea et al. 2016, Kleinnijenhuis et al. 2012, Netea, Quintin and van der Meer 2011, Quintin et al. 2014). Further controlled human infection studies, including pre- and post-pneumococcal challenge BAL sampling and focusing on AM epigenetic and metabolic changes, will be able to address whether human alveolar macrophages acquire a "trained immunity" phenotype as response to live pneumococcus. However, the

increased levels of IFN-γ detected in the BAL supernatant of Spn-colonised volunteers that had cleared nasal pneumococcal colonisation is a confounder that should be taken under consideration when evaluating potential AM reprogramming post exposure to pneumococcus. It is possible that that *S. pneumoniae* can persist within the human lung even after colonisation has been cleared, creating potentially a reservoir of bacteria that could cause ongoing immune stimulation.

Our findings on $CD4^+$ Th1 skewed responses and exogenous IFN-γ effect on AM antimicrobial function support the idea that Th1 type responses and interferons are crucial in controlling bacteria at the early stages of infection. Increased rates of pneumococcal colonisation in children and clinical cases of pneumonia in adults have been associated with a reduction in systemic circulating Th-1 (IFN-γ secreting) $CD4^+$ T-cells (Zhang et al. 2007, Kemp et al. 2002). Polymorphisms in the adaptor MAL, which regulates IFN-γ signalling (Ni Cheallaigh et al. 2016), have been associated with altered susceptibility to a number of infectious diseases including severe pneumococcal disease (Khor et al. 2007). Moreover, we observed a rapid priming of AMs when co-cultured with autologous lung derived $CD4^+$ T cells *in vitro*. A very recent study in mice described a similar mechanistic link between adaptive and innate immune system, suggesting that effector $CD8^+$ T cells, in the context of respiratory adenoviral infection, are able to prime AMs and render innate memory via IFN-γ (Yao et al. 2018).

Our study highlighted that IFN-γ has a dose-dependent effect on human AM function, which offers an explanation to the contradictory reports around this topic. For instance, in a murine model high production of IFN-γ during influenza infection impaired phagocytosis and killing of *S. pneumoniae* by alveolar macrophages (Mina, Brown and Klugman 2015, Sun and Metzger 2008). In contrast, many other evidences suggest that induction of IFN-γ secretion, related to non-acute viral infection, is beneficial for innate immune cells, promoting a range of antimicrobial functions, plus macrophages polarization and activation (Matsuzawa, Fujiwara and Washi 2014, MacMicking 2012, Yao et al. 2018). The dose-dependent effect of IFN-γ on AM OPA could also explain why HIV-infected adults are still at an increased risk of developing pneumococcal pneumonia, despite the preserved Th1 responses against *S. pneumoniae* (Peno et al. 2018).

Whereas the cytokine milieu in Spn colonised individuals, along with the $CD4^+$ Th1 profile, suggest an AM polarisation towards the M1 phenotype, human AMs have been

shown to co-express M1/M2 markers and therefore they do not fit neatly in the current macrophage classification (Mitsi et al. 2018, Hussell and Bell 2014). A recent study highlighted that human AMs possess considerable phenotypic diversity (Morrell et al. 2018). By assessing AM gene expression levels, we found that AM population derived from Spn colonised individuals was characterized by increased antigen-presentation and decreased monocytes surface markers signature. Our flow-based data corroborated this result by showing greater AM levels and increased AM to monocyte ratio in the Spn colonised individuals. The positive correlation of AM OPA with genes such as *NT5E* (or CD73) (Eichin et al. 2015) and *TBX21* (a master regulator of Th1 responses) indicate at some degree that monocyte-to-macrophage differentiation and AM polarisation to a more active functional state occur in the human lung, after interacting with the pneumococcus. Studies on human monocytes/macrophages have reported detectable expression of *CD73* in only M(LPS-TNF) polarised cells and increased of *T-bet* mRNA displayed by M1 polarized macrophages (Martinez et al. 2006, Bachmann et al. 2012). Also, our finding on increased expression of TLR8 in the Spn colonised group might be the readout of IFN-γ signalling, as TLR8 is responsive to interferons. Increase in TLR8 levels might lead to enhanced viral sensing and thus have a beneficial effect upon viral infection, such as influenza. Furthermore, it has been reported that activation of TLR8 signalling (TLR8-MyD88-IRAK4 signalling pathway) can reverse the suppression function of $CD4^+$ Treg cells (Peng et al. 2005).

In conclusion, this study emphasises the effect that nasopharyngeal pneumococcal colonisation has upon pulmonary immune system, describing the potential pathways for the development of a robust immune response. It is well accepted that colonisation is a prerequisite for disease. However, disease is more likely caused immediately after acquisition of colonisation (Ghaffar, Friedland and McCracken 1999, Robinson 2004). During longer colonisation episodes, AMs display the increased functionality we described, which in turn could lead to reduced risk of bacterial respiratory infections. It is also important to note that our findings derive from young healthy adults, which are not at high risk of pneumococcal disease. The elderly are at an increased risk of infection and these are colonised less frequently. The lack of repeated colonisation and associated AM (and other immunological) boosting could contribute to their increased susceptibility and explain the paradox of low colonisation and high disease in the elderly.

The seeding of human lung with activated AM that exert prolonged and enhanced opsonophagocytic properties has potential implications for vaccine development. Pneumococcal vaccines that focus solely on inducing a robust Th17 response may not be the best strategy for vaccine targeting serotype-independent protection against pneumonia. On the other hand, such a non-specific boosting of innate lung immunity, may be an alternative attractive strategy to successful pneumonia prevention, especially for the new-borns, whose immune system is still developing, or for the elderly, whose acquired immunity is beginning to wear off. In particular the elderly, who have been described as the age group with the lowest pneumococcal colonisation rates and high incidence of community-acquired pneumonia cases, would benefit from the boosting effect that mucosal stimulation with whole cell pneumococcus confers to the pulmonary immune cell populations. These results, in combination with our previous finding on increased presence of pneumococcal-specific IL-17A secreting T cell in human lung post nasal colonisation (Wright et al. 2013), suggest that a nasally administered live-attenuated pneumococcal vaccine could augment the pulmonary immune responses and confer serotype-independent protection against development of pneumococcal pneumonia.

This study provides insights into the immune responses elicited at the pulmonary mucosa post a colonisation episode of the nasopharynx. We demonstrated that prior nasopharyngeal pneumococcal colonisation results in bacterial aspiration to the alveolar space. Moreover, the duration of this enhanced activity persisted for over three months post the clearance of colonisation. Aspirated pneumococci may act as the stimulus that leads to enhanced AM responsiveness against bacterial pathogens mediated by Th1 cytokines.

In addition, it would be of interest to investigate whether nasal colonisation leads to recruitment of monocytes to the alveolar spaces, which was not possible in the present study due to the single time point sampling. The one-time point sampling also disabled comparisons of immune responses pre- and post- colonisation on an individual level. Therefore, future studies including baseline sampling of the lung mucosa will attribute clarity to the direct effect of pneumococcal colonisation on the pulmonary immune responses.

Chapter 5

Adaptive lung immune responses post nasopharyngeal pneumococcal colonisation.

5.1 Introduction

Despite its pathogenicity, S. *pneumoniae* can colonise the human nasopharynx asymptomatically, a state known as colonisation or carriage (Bogaert et al. 2004). Pneumococcal carriage is critical in transmission and disease but paradoxically it is also essential for the development of adaptive immunity. Through this asymptomatic state, a carriage episode acts as an immunising event, inducing both humoral and cellular responses systemically (Ferreira et al. 2013, Pennington et al. 2016). Regarding the mucosal sites, we previously demonstrated that experimental human pneumococcal colonisation leads to induction of IgG to pneumococcal antigens, such as capsular polysaccharide and pneumococcal proteins (Mitsi et al. 2017, Ferreira et al. 2013) in the nasal lumen. In the lung, we have shown that pneumococcal colonisation increases the frequency of pneumococcal specific IL-17A secreting $CD4^+$ T cells (Wright et al. 2013).

In murine models, the $CD4^+$ Th17 subset has been associated with control of pneumococcal colonisation and protection against pneumonia (Lu et al. 2008, Wilson et al. 2015). Moreover, a recent murine study also emphasised the importance of $CD4^+$ T cells in lung mucosa, when resolution of previous pneumococcal infections seeded the lungs with $CD4^+$ resident memory T cells. These tissue resident memory (TRM) $CD4^+$ T cells found in mice lungs responded to heterotypic pneumococcus stimulation by producing multiple effector cytokines, particularly IL-17A (Smith et al. 2018). The adult human lung contains a large number of $CD4^+$ TRM cells, based on surface staining with

CD69, and at least some of these cells respond to influenza, which suggests that they resulted from prior respiratory infection(Purwar et al. 2011, Sathaliyawala et al. 2013). Upon stimulation, lung $CD4^+$ TRM cells express a variety of cytokines, perhaps reflecting diverse specificities and functions (Purwar et al. 2011, Sathaliyawala et al. 2013). Whether and how the bacterial causes of pneumonia elicit or are influenced by lung $CD4^+$ TRM cells is, to our knowledge, largely unexplored.

In this study, we aimed to further investigate the effect of a recent pneumococcal episode in the adaptive lung immune responses. The immunising effects of colonisation observed by us and others both systemically and in the nasal mucosa, combined with our findings on cognate lung Th17 cells, postulate that other arms of adaptive immunity could be affected in a similar manner. Therefore, we hypothesised that a successful pneumococcal colonisation could induce both cellular and antibody responses. Herein, we performed research bronchoscopies in the EHPC model to expand our observations on Th17 cells to other lymphocyte subsets, including the non-conventional TCR-γδ T cells, as well as resident T cells of the lung mucosa. In parallel with cellular responses, mucosal antibodies against pneumococcus is an important defence mechanism, accelerating bacterial clearance through agglutination (Mitsi et al. 2017) and facilitating bacterial uptake by phagocytes, through opsonisation. We assessed the antibody responses to the challenge strain in the lung mucosa post pneumococcal colonisation by measuring IgG to 6B capsular polysaccharide (6B-CPS) in the BAL fluid.

5.2 Material and Methods

Lung adaptive cellular responses to pneumococcus were measured in volunteers who underwent research bronchoscopy between Day21 to Day50 post inoculation. This time frame was deliberately selected to match with the previous EHPC study that had identified the presence of cognate IL-17A producing $CD4^+$ T cells in the human lung post experimental pneumococcal challenge. On the other hand, pneumococcal-specific antibody levels were quantified in BAL fluid from samples acquired at any day between 1 to 6 months post challenge.

Lymphocytes derived from the BAL samples were separated from alveolar macrophages via an adherence step, following stimulation with HI-spn6B and staining

according to our published protocol (Wright et al. 2013). Full details of the methods used to produce the data presented within this chapter are given in Chapter 2.

5.2.1 BAL Lymphocytes isolation

Lymphocytes from BAL samples were separated from alveolar macrophages after adherence of the latter on a 24-well plate. Full details can be found in section 2.5.9.

5.2.2 Stimulation and Flow cytometry

Isolated BAL lymphocytes were stimulated with heat-inactivated pneumococcus (Spn6B) or left unstimulated, following immunophenotyping and intracellular staining for INF-γ, TNF-α and IL-17A production. Full details of this method can be found in section 2.6.4.4.

5.2.3 IgG ELISA to Spn6B capsular polysaccharide

Levels of IgG to Spn6B- CPS were measured in the BAL fluid using our published ELISA protocol (Mitsi et al. 2017). Full details of the method can be found in section 2.6.7.

5.2.4 Statistical analysis

Statistical analyses were performed using GraphPad Prism (Version 6, GraphPad Software, La Jolla, CA) and R software (version 3.5.1). Two-tailed statistical tests were used throughout the study. If two parametric groups were compared, a two-tailed t test was used for unpaired and paired groups. If two non-parametric groups were compared, a Mann-Whitney or Wilcoxon test was used for unpaired and paired groups respectively. To quantify association between groups, Pearson or Spearman correlation test was used for parametric or non-parametric groups, respectively. Differences were considered significant at $p \leq 0.05$ (*$p< 0.05$, **$p< 0.01$, ***$p< 0.001$, ****$p< 0.0001$).

5.3 Results

5.3.1 Nasal pneumococcal colonisation reduces the frequency of TCR-γδ T cells in the human lung

We assessed carriage-mediated alterations of the frequency of T cell subsets in BAL and compared cellular responses to pneumococcus between Spn colonised and non-colonised groups. Therefore, lymphocytes were immunophenotyped and stained intracellularly for IL17A, IFN-γ and TNF-α production after an 18-hour stimulation. The gating strategy used to identify $CD4^+$, $CD8^+$ and TCR-γδ cells is presented on Figure 5.1A, whereas identification of tissue resident memory $CD4^+$ and $CD8^+$ T cells is illustrated on Figure 5.1B. As over 1/3 of $CD4^+$ $CD69^+$ cells, commonly defined as TRM (Turner et al. 2014), did not express the additional resident memory markers CD103 and CD49a, we defined TRM only as $CD69^+$. In contrast, nearly all $CD8^+CD69^+$ cells also expressed CD103 and CD49a (Figure 5.1B).

The Spn colonised group had similar levels of total $CD4^+$ or $CD8^+$ among total T cells with those measured in the non-colonised (Figure 5.2). On the other hand, Spn colonised group had a 50% reduction in TCR-$\gamma\delta^+$ T cell frequency in the BAL compared to non-colonised group (median=2.39, IQR: 1.62-3.58 in carriage+ vs median= 4.67, IQR: 3.42-6.73 in the carriage- group) (Figure 5.2). A similar pattern was observed in the frequency of the different TRM T cell subsets. Proportions of both $CD4^+$ and $CD8^+$ TRM T cells remained stable post colonisation, whereas frequency of TCR-$\gamma\delta^+$ TRM T cells had a moderate 12% reduction in the Spn colonised group ($p=0.011$) (Figure 5.3).

We also assessed whether there were differences in the frequency of regulatory $CD4^+$ T cells between the two experimental groups. T regs were identified as $CD3^+CD4^+CD25^+Foxp3^+$ (Figure 5.1A) in BAL samples. Percentages of T regs amongst $CD4^+$ T cells did not differ between Spn colonised and non-colonised group (Figure 5.4).

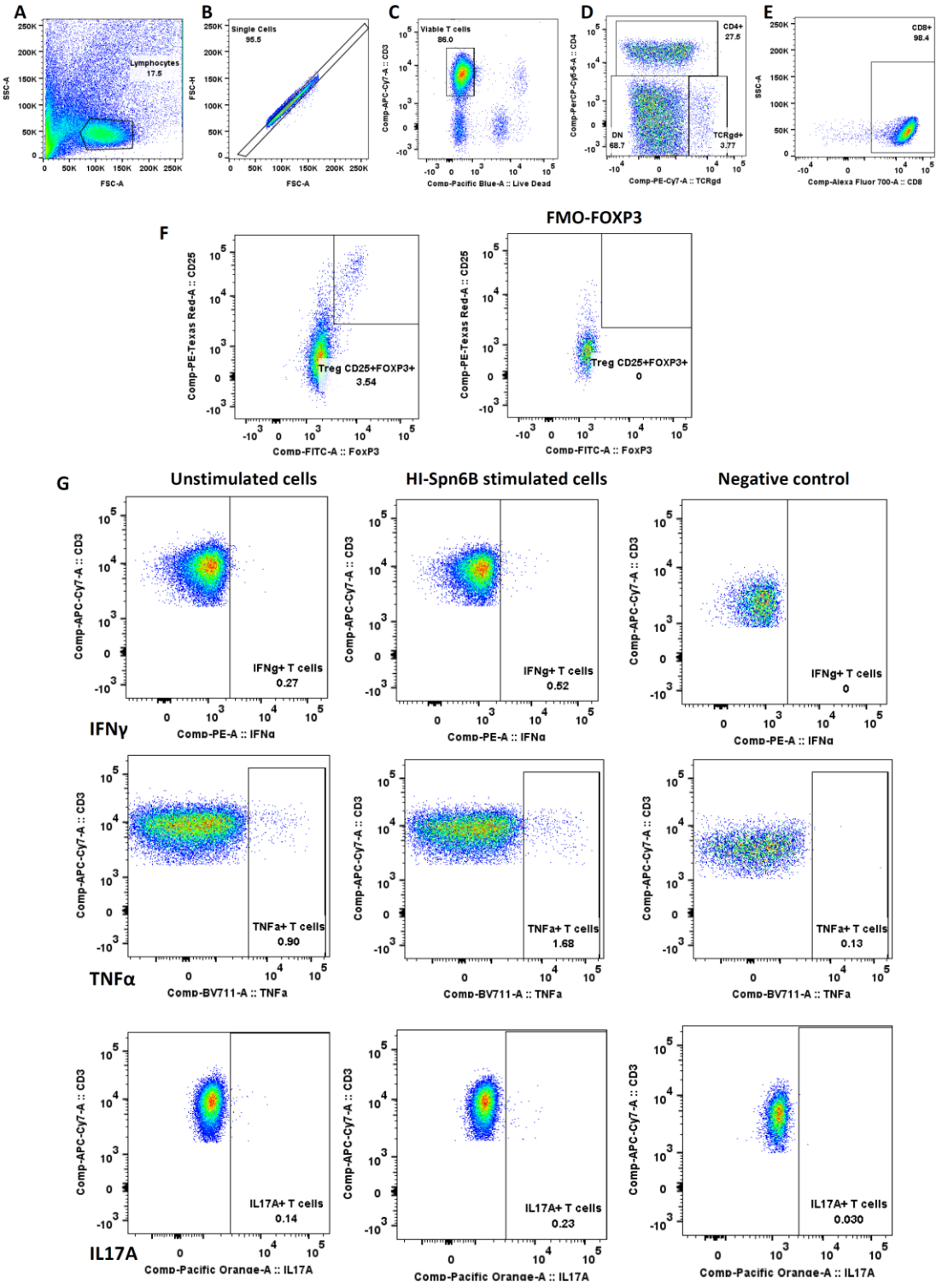


Figure 5.1A. Representative gating strategy to identify T cells and cytokine production by flow cytometry using intracellular staining. The following gating strategy was used: (A) FSC-A/SSC-A to (B) FSC-A/ FSC-H (in order to exclude doublets) to (C) CD3/Viability to (D) CD4/TCR-γδ^{+} to (E) SSC-A/CD8. (F) The markers CD25/FOXP3 (CD25^{hi} and FOXP3^{+}) were used to assess the frequency of regulatory T cells. To assess cellular production of (G) IFN-γ, (H) TNF-α and (I) IL-17A cells were stained intracellularly after overnight stimulation with Spn6B or non-stimulation. Fluorescence Minus One (FMO) controls were used to verify flow cytometric data.

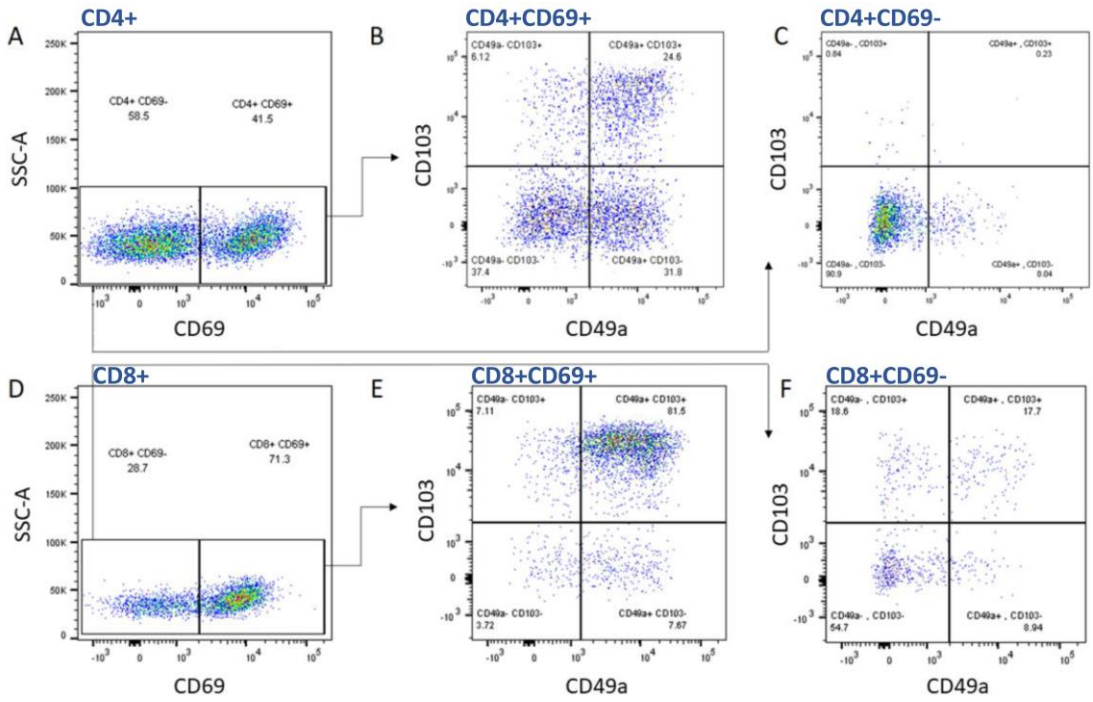


Figure 5.1B. Representative plots of tissue resident memory T cells identified by flow cytometry. The markers CD69, CD103 and CD49a were used to assess the frequency of tissue resident memory cells in human BAL. (A) $CD4^+$ T-cells gated into CD69 negative and positive cells. CD103 and CD49 marker expression are shown for (B) $CD4^+$ $CD69^+$ T cells and (C) $CD4^+$ $CD69^-$ T cells. (D) $CD8^+$ T cells gated into CD69 negative and positive cells. CD103 and CD49 marker expression are shown for (E) $CD8^+$ $CD69^+$ T cells and (F) $CD8^+$ $CD69^-$ T cells.

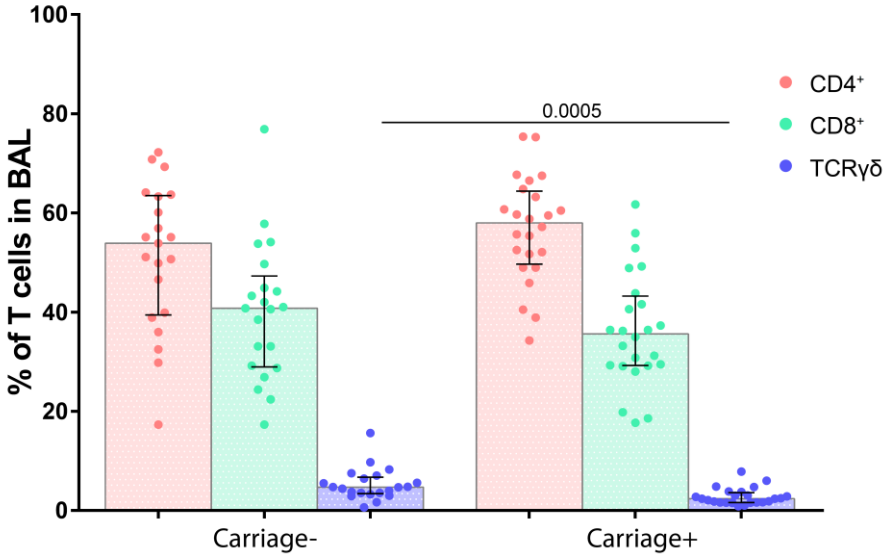


Figure 5.2. Frequency of T cells subsets in the human lung post EHPC. Subset frequencies among viable T cells were measured after an overnight incubation of isolated BAL cells. Error bars depict medians and IQR of T cell subsets percentage among total T cells in carriage- (n=21) and carriage+ volunteers (n=24). Comparisons were made using Mann-Witney tests. P values are shown.

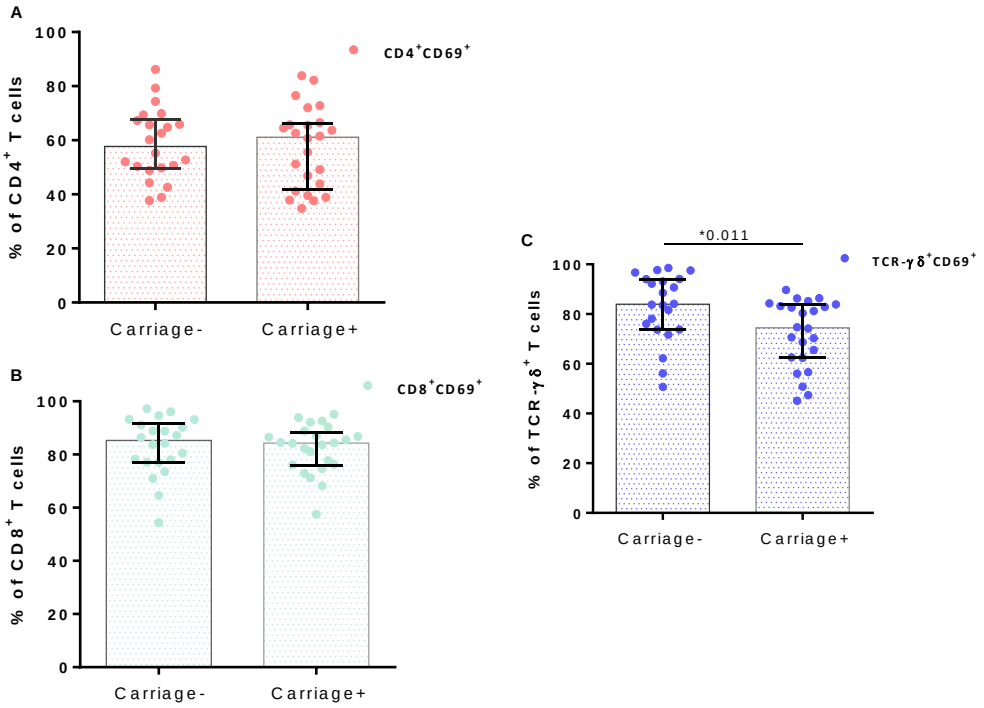


Figure 5.3. Frequency of TRM T cells in the human lung post EHPC. % frequencies of **A)** $CD4^+$ $CD69^+$ of total $CD4^+$ T cells, **B)** $CD8^+$ $CD69^+$ of total $CD8^+$ T cells and **C)** TCR-$\gamma\delta^+CD69^+$ of total TCR-γδ T cells were measured after an overnight incubation of isolated BAL cells. Error bars depict medians and IQR of TRM T cell in carriage- (n=21) and carriage+ volunteers (n=24). Comparisons were made using Mann-Witney tests. P values are shown.

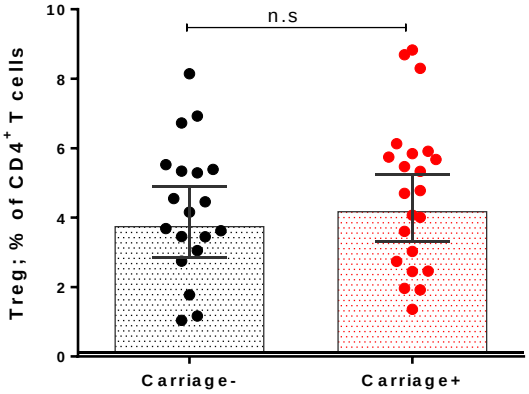


Figure 5.4. Frequency of $CD4^+$ regulatory T cells in the human lung post EHPC. Frequency of unstimulated $CD4^+$ Tregs ($CD3^+$ $CD4^+$ $CD25^+$ $FOXP3^+$) was measured by flow cytometry in human BAL samples of carriage- (n=19) and carriage+ volunteers (n=22). Error bars depict geometric means with 95% CI are shown. Comparisons between the two groups were made by unpaired t test. Abbreviation: ns, not significant.

5.3.2 Significant increase of pneumococcal-specific IL-17A secreting CD4$^+$ T cells in the human lung post Spn colonisation

Here, we used a larger cohort to validate our previous finding on increased lung CD4$^+$ Th17 cells in colonised (Wright et al. 2013). In line with the previous observation, pneumococcal colonised individuals had increased frequency (median 7.5-fold increase, p=0.016) of cognate IL-17A producing CD4$^+$ T cells in the BAL (Figure 5.5A). In contrast, stimulation of lung lymphocytes with the HI-Spn6B elicited production of IFN-γ by CD4$^+$ T cells in both group at a similar level (median 2.5x increase, p=0.026 in carriage- vs 1.8x, p=0.0005 in carriage+ when compared to non-stimulated condition). There was thus not significant induction of Spn specific IFN-γ responses post colonisation (Figure 5.5B). Similarly, stimulation with HI-Spn6B elicited high TNF-α responses by CD4$^+$ T cells irrespectively carriage status, and thus specificity to Spn cannot be attributed to such immune response (Figure 5.5C).

5.3.3 Non-significant increase of pneumococcal specific CD4$^+$ TRM T cells in the human lung post Spn colonisation

We next sought to address whether nasal Spn colonisation contributes to lung immunity by increasing the frequency of cognate CD4$^+$ TRM T cells. Frequency of pneumococcal specific CD4$^+$ TRM T cells did not differ significantly between Spn colonised and non-colonised individuals for either Th17 or Th1 (IFN-γ and TNF-α) responses (Figure 5.6A-C). However, the frequency of spontaneous (non-stimulated) IL-17 producing CD4$^+$ TRM T cells was proportionally more in the Spn colonised group (median= 0.33 with IQR: 0.19-0.50 in the carriage+ vs median= 0.19 with IQR:0.06-0.31 in the carriage- group). Post stimulation, IL-17A producing CD4$^+$ TRM cells was increased by 1.7-fold in both experimental groups. Similarly, IFN-γ and TNF-α producing CD4$^+$ TRM increased in frequency in both groups, when stimulated with HI-Spn6B. Spn colonised exhibited a 2.1-fold increase in frequency of IFN-γ producing CD4$^+$ T cells and a 2.5-fold increase in the TNF-α producing ones. In the non-colonised group, a 1.8-fold increase was observed in the frequency of IFN-γ producing CD4$^+$ T cells and a 2.4-fold increase in the CD4$^+$ T cells that were producing TNF-α upon overnight stimulation with HI-Spn6B. Comparisons of fold changes (Spn-stimulated/ non-stimulated condition) between the two groups did not show statistically significant differences.

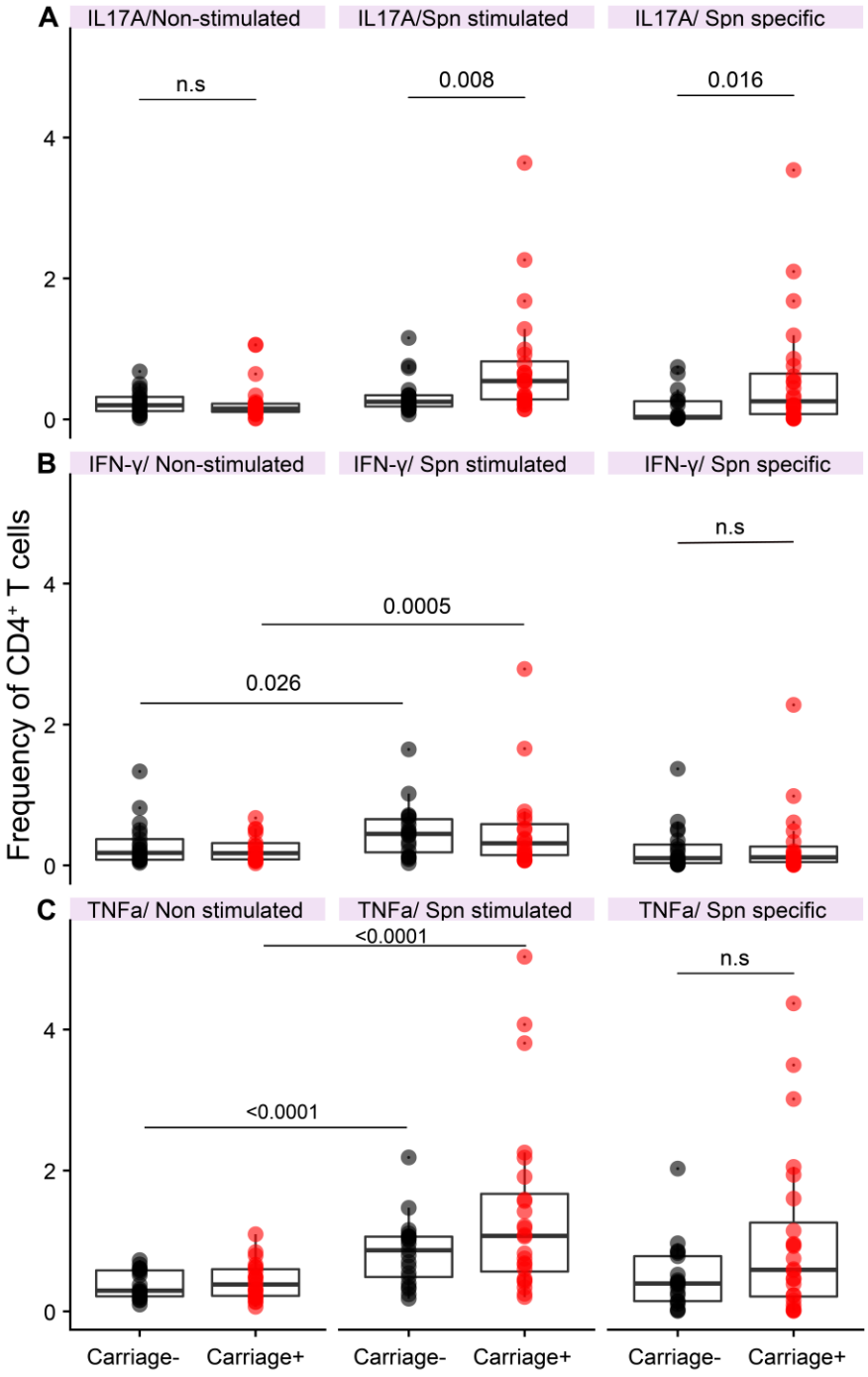


Figure 5.5. Increased frequency of pneumococcal specific CD4⁺ Th17 T cells in the Spn colonised group. % Frequency of cytokine producing CD4⁺ T cells non-stimulated or stimulated with challenge HI-Spn6B in carriage- (black dots, n= 21) and carriage+ (red dots, n= 24). **A)** Frequency of IL-17A, **B)** IFN-γ and **C)** TNF-α producing BAL isolated CD4⁺ T cells per condition. Spn-specific CD4⁺ T cell responses were calculated by subtracting baseline from Spn stimulated values. Medians with IQR are depicted. p values shown on the graph. Mann Whitney and Wilcoxon tests were used.

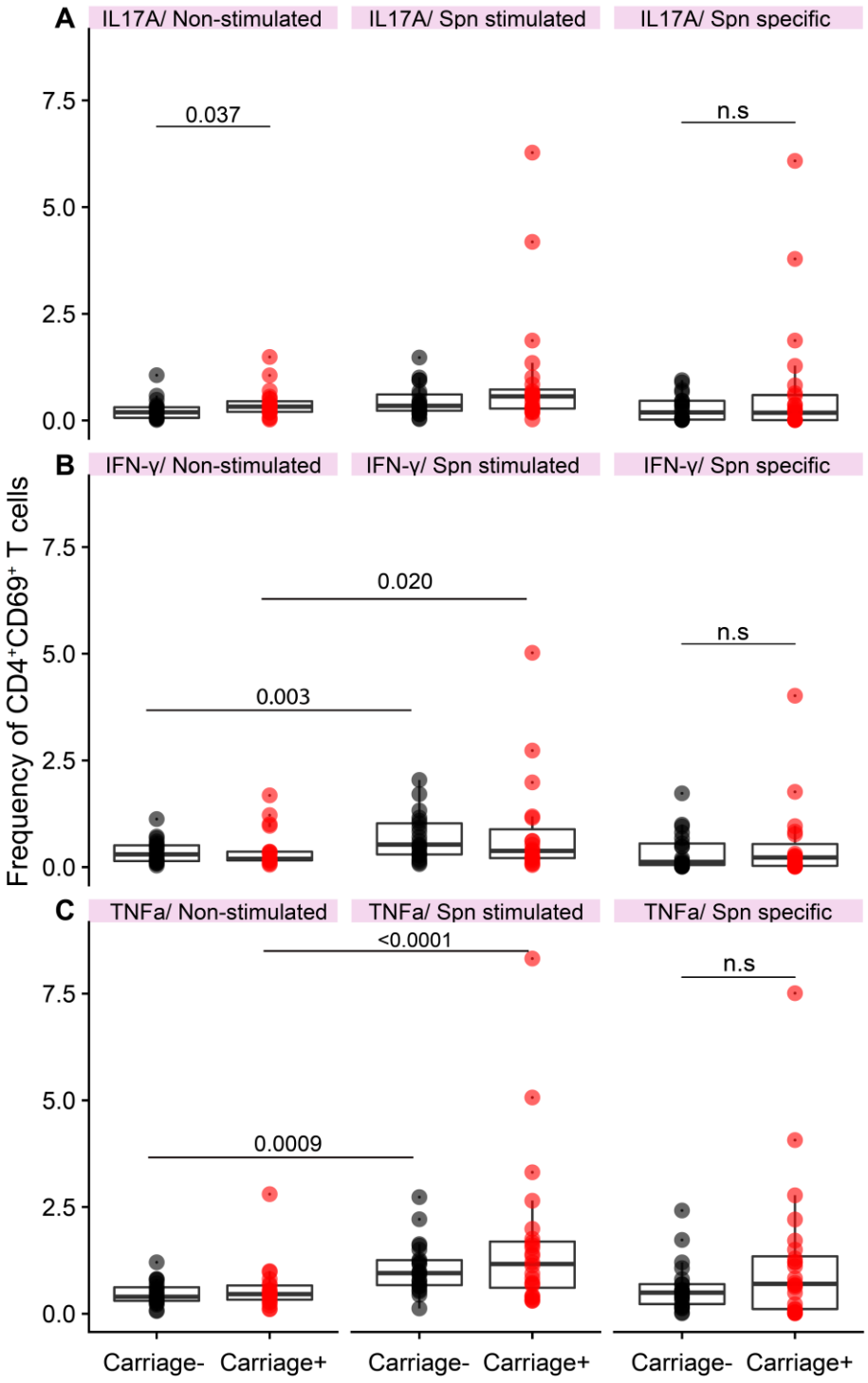
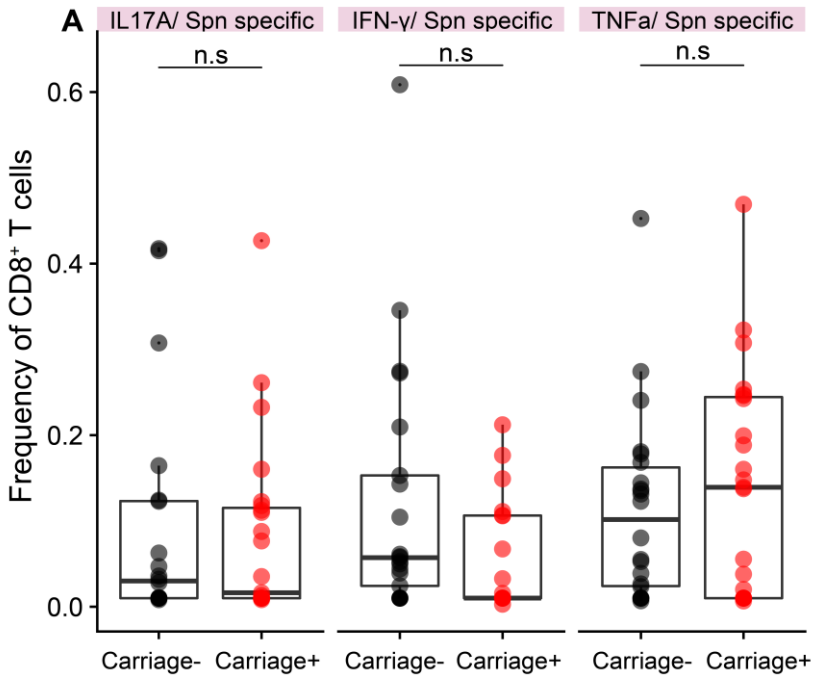


Figure 5.6. Non-significant increase of pneumococcal specific CD4⁺ TRM T cells in the Spn colonised group. % Frequency of cytokine producing CD4⁺ TRM T cells non-stimulated or stimulated with challenge HI-Spn6B in carriage- (black dots, n= 21) and carriage+ (red dots, n= 24). **A)** Frequency of IL-17A, **B)** IFN-γ and **C)** TNF-α producing BAL isolated CD4⁺ TRM T cells per condition. Spn-specific CD4⁺ TRM T cell responses were calculated by subtracting baseline from Spn stimulated values. Medians with IQR are depicted. p values shown on the graph. Mann Whitney and Wilcoxon tests were used.

5.3.4 No responses to pneumococcus by CD8⁺ and CD8⁺ TRM T cells

Next, we studied BAL-derived CD8⁺ T cells responses upon Spn stimulation. As known, CD8⁺ T cells (often called cytotoxic T lymphocytes, or CTLs) are very important for immune defense against intracellular pathogens, including viruses and bacteria. When they recognise their antigen, they become activated and can secrete TNF-α and IFN-γ**.** *In vitro* stimulation of lung CD8⁺ T cells did not elicit any response in either group. In fact, the frequency of such cytokines producing CD8⁺ T cells amongst total lung CD8⁺ population was extremely low. As expected, there was no difference in frequency of IL17A, IFN-γ or TNF-α producing CD8⁺ T cells between the two experimental groups (Figure 5.7A). In line with the total CD8⁺ T cells behaviour, CD8⁺ TRM T cells did not respond to stimulation with the HI-Spn6B (Figure 5.7B).



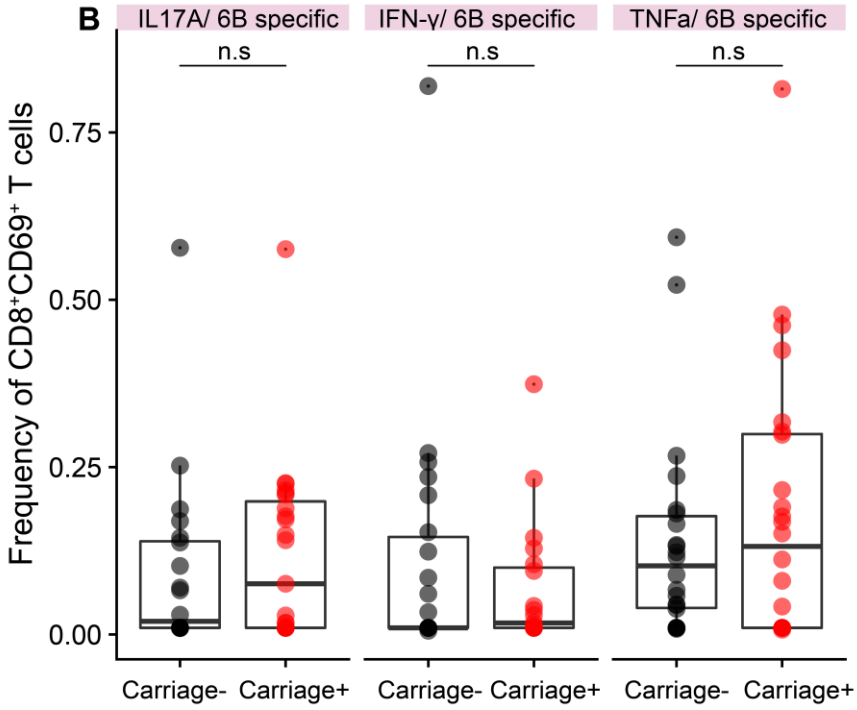


Figure 5.7. Neither $CD8^+$ nor resident $CD8^+$ T cells respond to pneumococcus. % Frequency of pneumococcal specific IL-17A, IFN-γ and TNF-α producing BAL isolated **A)** $CD8^+$ and **B)** $CD8^+CD69^+$ T cells in carriage- (n= 21) and carriage+ (n= 24) upon stimulation with HI-Spn6B. Spn-specific responses were calculated by subtracting baseline from Spn stimulated values. Medians with IQR are depicted. Mann Whitney test was used. Abbreviation: ns, not significant.

5.3.5 IFN-γ and TNF-α but not IL17 responses by TCR-γδ$^+$ T cells to *S. pneumoniae in vitro* irrespectively carriage status

In addition, we studied the direct production of cytokines in lung isolated TCR-γδ$^+$ T cells *ex vivo*, as well as post *in vitro* stimulation with pneumococcus in both experimental groups. TCR-γδ$^+$ cells, a subset of specialized innate-like T cells that can exert effector functions immediately upon activation, play an important role in pulmonary infection (Ivanov et al. 2014, Steele, Oppenheim and Hayday 2000).

In the unstimulated condition, Spn colonised group exhibited a greater proportion of both IL-17A (median 2.3-fold increase) (p=0.038) and TNF-α producing TCR-γδ$^+$ (median 1.9-fold increase) when compared with the non-colonised group (Figure 5.8A and 5.8C). In both cases, less than 2.5% (median % frequency) of the total TCR-γδ$^+$ population was producing these cytokines.

Stimulation with HI-Spn6B did not confer any increase in the frequency of IL-17A producing TCR-γδ$^+$ in either group (Figure 5.8A). However, it elicited robust IFN-γ and TNF-α responses in both groups. In respect with IFN-γ responses, stimulation with pneumococcus increased 6.4x the levels of IFN-γ producing TCR-γδ in the Spn colonised group and 4.5x in the non-colonised group. Frequency of TNF-α producing cells were proportionally increased in a similar manner in both groups (4.3x and 3.6x median increase in carriage- and carriage+, respectively) (Figure 5.8B and 5.8C).

Despite the differential IL-17A and TNF-α responses by spontaneous responding TCR-γδ, observed in the unstimulated lymphocytes fraction between the two groups, there was no statistically significant differences in pneumococcal specific responses upon stimulation (Figure 5.8A and 5.8C).

Tissue resident memory TCR-γδ$^+$ T cells were also defined by CD69 marker, as nearly no such cells were expressing the additional CD49a and CD103 markers. The cytokine expression pattern of lung TCRγδ$^+$ TRM post stimulation resembled the one obverted in total TCR-γδ$^+$ T cells; no IL-17A responses but robust IFN-γ and TNF-α. The identical cytokine responses to spn6B and the fact that TCR-γδ$^+$ was found to constitute only the 0.1-8% of the total T cell population in lung, prohibited any further description of TCR-γδ$^+$ TRM in the lung. We thus focused our interest on describing the total lung TCR-γδ$^+$ population in association with nasal pneumococcal colonisation and its contribution to the lung immunity.

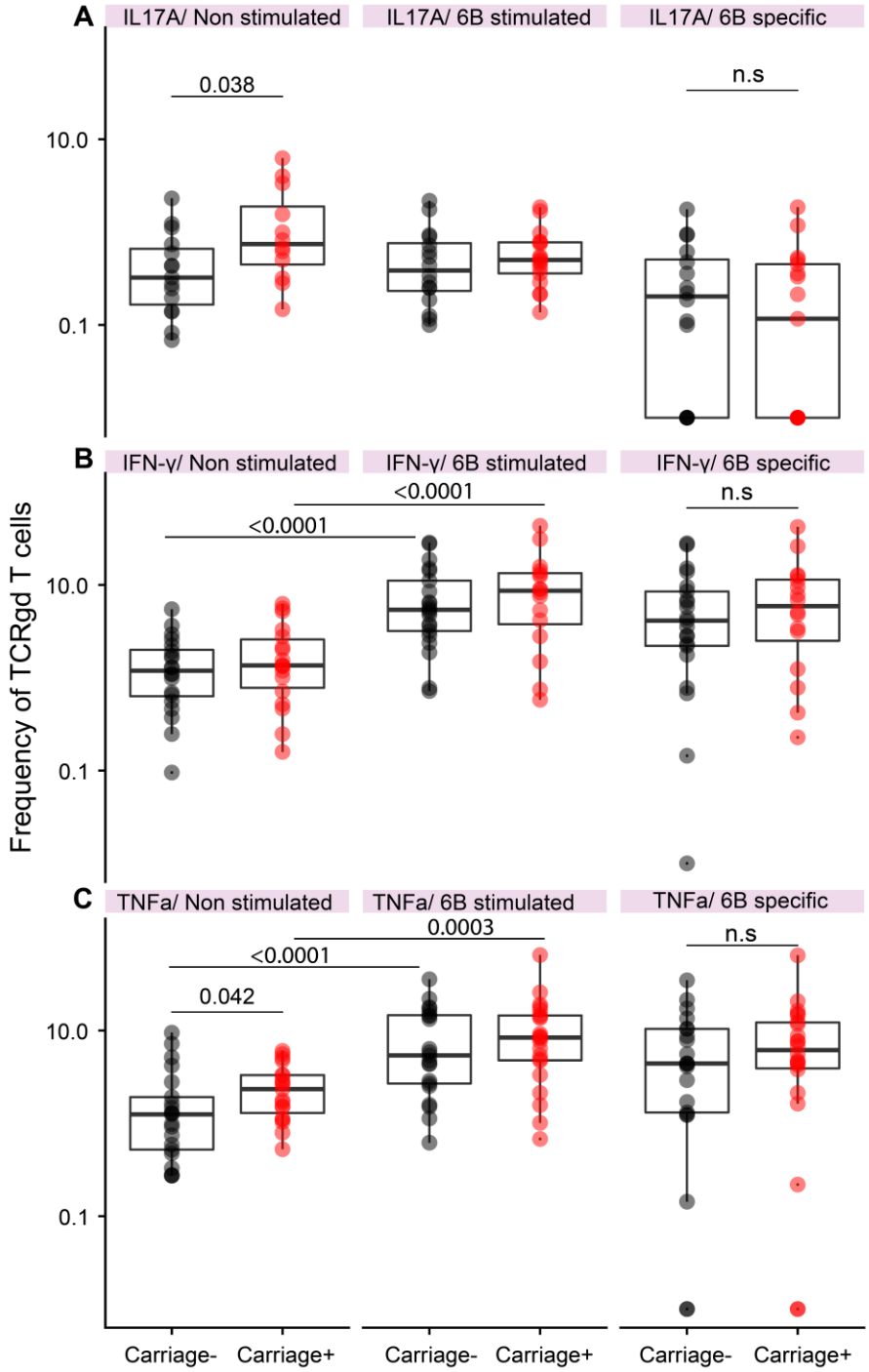


Figure 5.8. Non-significant increase in the frequency of pneumococcal specific TCR-γδ^{+} T cells in the Spn colonised group. % Frequency of cytokine producing TCR-γδ cells non-stimulated or stimulated with challenge HI-Spn6B in carriage- (black dots, n= 21) and carriage+ (red dots, n= 24). **A)** Frequency of IL-17A, **B)** IFN-γ and **C)** TNF-α producing BAL isolated TCR-γδ cells per condition. Spn-specific cell responses were calculated by subtracting baseline from Spn stimulated values. Medians with IQR are depicted. p values shown on the graph. Mann Whitney and Wilcoxon tests were used.

5.3.6 High frequency of pneumococcal responding IFN-γ and TNF-α producing TCR-γδ$^+$ T cells in BAL

Opposed to IL-17A responses, which were confined only to CD4$^+$ T cells, IFN-γ and TNF-α responses were observed in both CD4$^+$ and TCD-γδ$^+$ T cells subsets post stimulation with Spn6B independent of colonisation status. Given that a) the frequencies of such cytokine producing cells differ between the T cell subsets and b) the proportion of CD4$^+$ cells is a log higher from TCR-γδ frequency in the healthy human lung, a direct comparison between cells subsets would be more conclusive.

In terms of IFN-γ responses, spontaneous producing cells were found more frequently detected in CD4$^+$ population. After in vitro stimulation with Spn6B, IFN-γ producing TCR-γδ$^+$ were 8-fold higher ($p < 0.0001$), whereas IFN-γ producing CD4$^+$ and CD4$^+$ TRM T cells were only 2- and 2.5-fold greater, respectively, compared to the unstimulated condition (Figure 5.9A).

A similar pattern was observed in TNF-α responses across the different cell populations. TNF-α producing cells were proportionally more amongst CD4$^+$ T cell population measured *ex vivo*, whereas after the *in vitro* Spn6B stimulation there was no statistical difference between frequencies of TNF-α producing CD4$^+$, CD4$^+$ TRM and TCR-γδ$^+$ amongst the total viable T cells (Figure 5.9B).

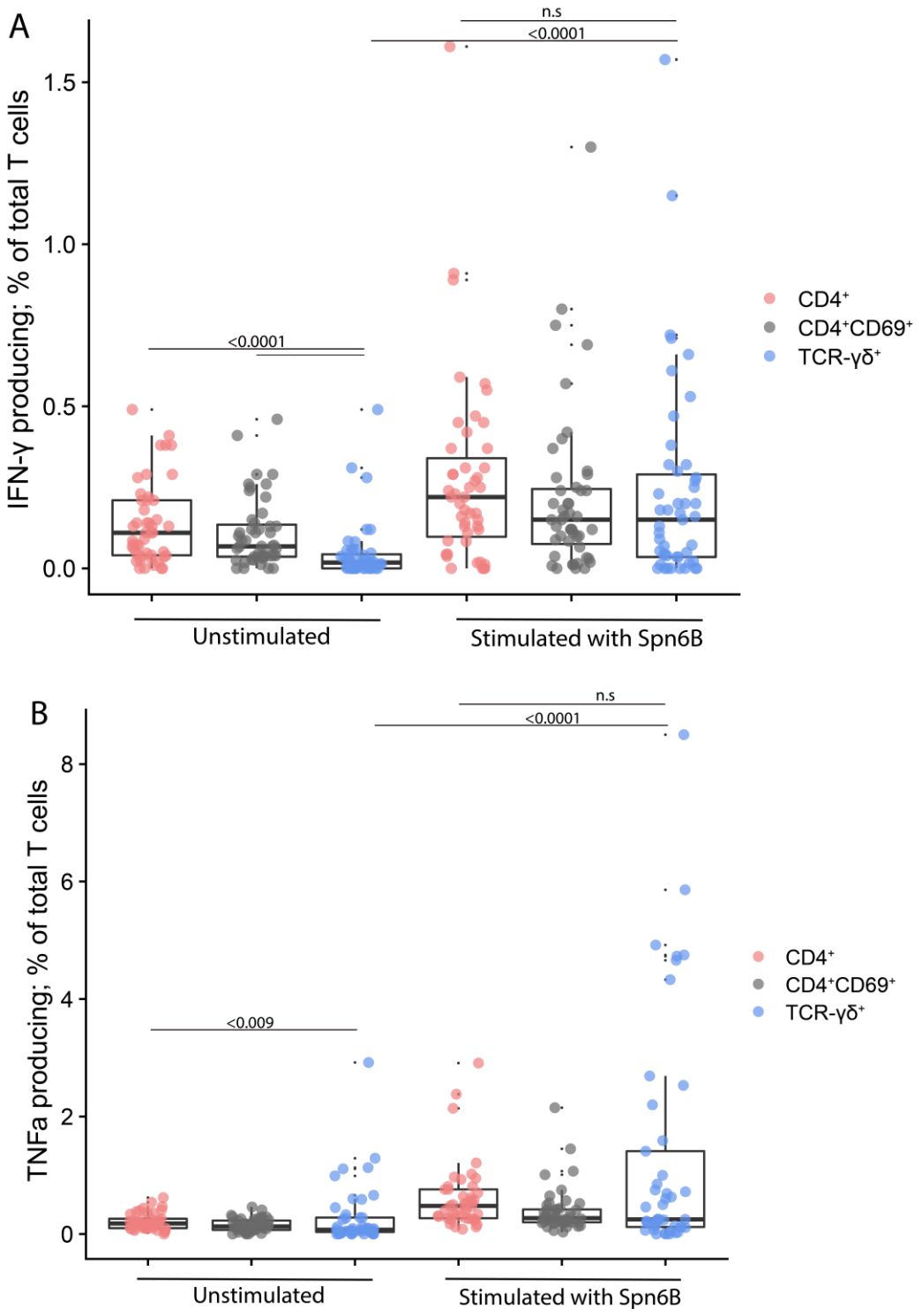


Figure 5.9. Robust IFN-γ and TNF-a responses by TCR-γδ⁺ T cells post stimulation with Spn6B *in vitro.* Frequencies of **A**) INF-γ and **B)** producing T cells subsets (CD4⁺, CD4⁺CD69⁺ and TCR-γδ⁺) expressed as % of total viable T cells in the BAL measured in two condition: unstimulated and post stimulation with HI-Spn6B. Medians with IQR are depicted. p values shown on the graph. Mann Whitney and Wilcoxon tests were used.

5.3.7 Increased levels of IgG to capsular polysaccharide 6B in the human lung post pneumococcal colonisation

We also sought to investigate whether humoral responses to pneumococcus in the lung mucosa are affected by nasopharyngeal pneumococcal colonisation, as previously had been shown in nasal wash samples and blood (ref). Therefore, we measured levels of total IgG against the capsular polysaccharide of the challenge strain (CPS-6B) in BAL samples collected from EHPC participants 1 to 6 months post inoculation. Spn-colonised individuals had 2.6x higher IgG to spn6B-CPS compared to the non-colonised (median= 2.1, IQR: 1.13- 4.64 in Spn colonised vs median= 0.81, IQR: 0.62-1.10 in non-colonised group) (Figure 5.10A) and were sustained at relatively high levels throughout the period of six months (Figure 5.10B). Although, they had started waning off, no significantly decrease was observed at either 4 (Figure 5.11A) or 6 months post (Figure 5.11B) the bacterial challenge.

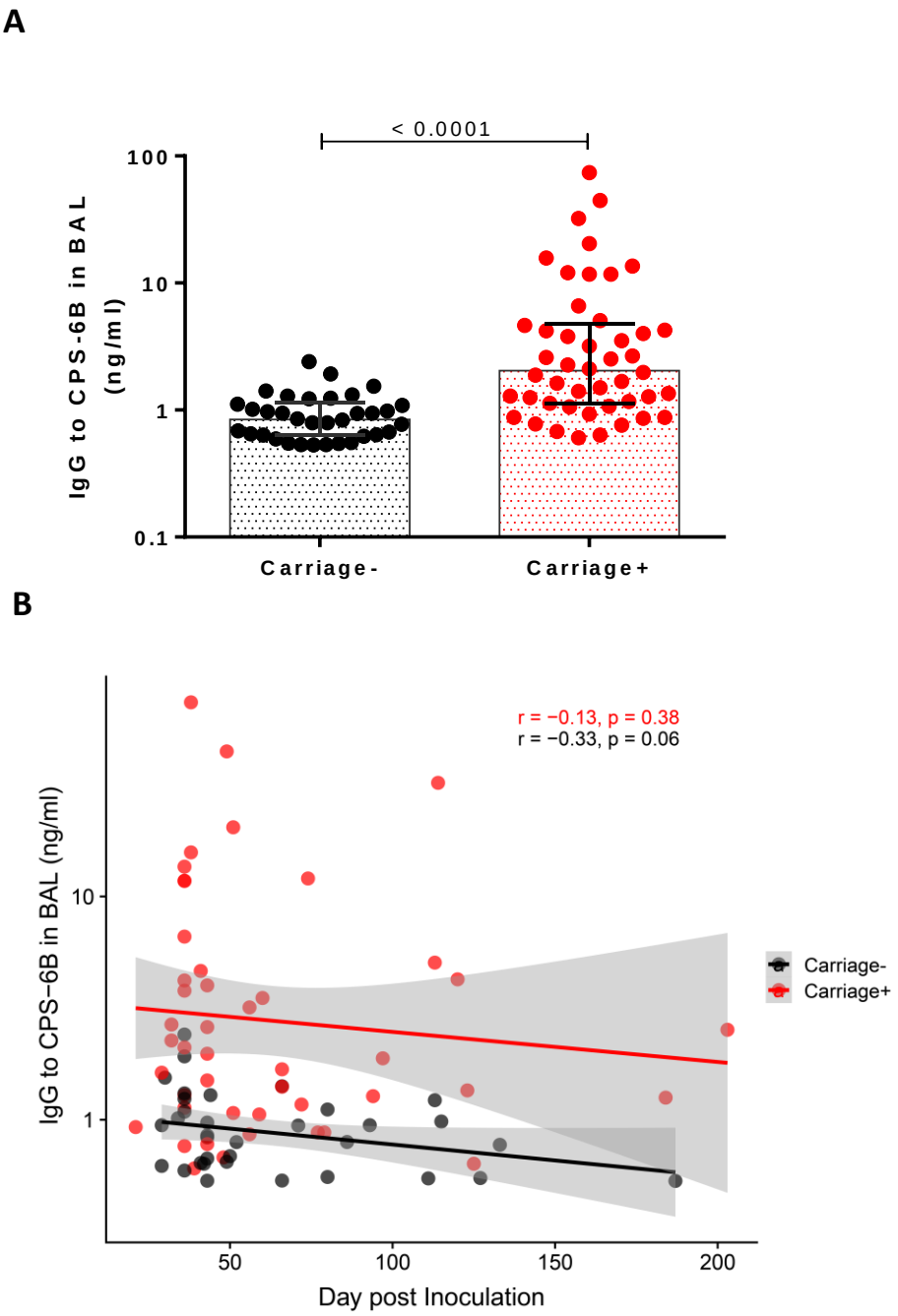


Figure 5.10. Increased IgG to capsular polysaccharide of the challenge strain in Spn colonised group. A) Levels of IgG to capsular polysaccharide of Spn6B measured in the BAL fluid of carriage- (n= 34) and carriage+ (n= 46). Each dot represents an individual. Error bars depict medians with IQR. p value is shown on the graph. Mann Whitney test was used. **B)** Linear model (lines) and observed titers (dots) of IgG to CPS-6B on the day on the bronscoscopy, expressed as day post inoculation for both carriage- and carriage+ group. Association between the two ranked variables per group was tested using Spearman correlation test. R and p values are shown.

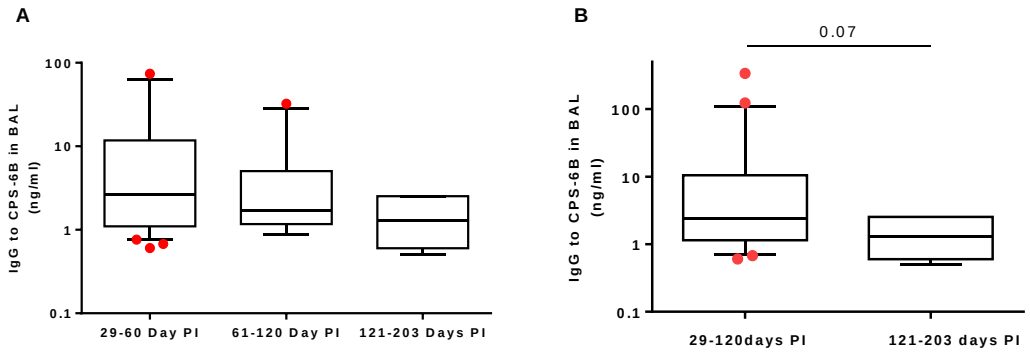


Figure 5.11. Levels of IgG to CPS-Spn6B in BAL per defined time period in the Spn-colonised group. Chronological representation of BAL IgG titers in Spn-colonised (n=46) collected from one to six months post intranasal pneumococcal inoculation divided into **A)** three or **B)** two consecutive time periods. Medians with IQR are depicted. p values shown on the graph. Mann Whitney and Wilcoxon tests were used.

To examine correlative supporting evidence that BAL CPS-Spn6B IgG might be derived from serum leakage, levels of IgG to CPS-Spn6B in BAL of Spn colonised individuals, who had bronchoscopy up to 2 months post the challenge, were correlated with the equivalent IgG levels in serum. Capsular-specific IgG titres measured in serum and BAL fluid per participant were positively correlated (r=0.46, p=0.03) (Figure 5.12).

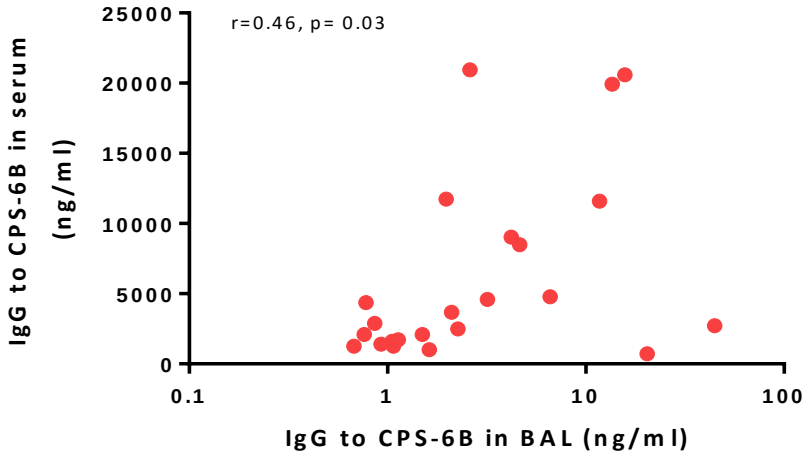


Figure 5.12. Correlation between IgG to CPS-Spn6B in BAL and serum. Levels of IgG to CPS-Spn6B in BAL samples collected from D29 to D60 were correlated with those measured in sera at D29 per individual (n=23, volunteers with paired values). Spearman correlation test result are shown.

5.4 Discussion

Here we investigated the antibody and adaptive cellular responses of lung immunity to pneumococcus. We observed that carriage was not associated with altered frequency of neither $CD4^+$ nor $CD8^+$ T cell populations, but was associated with a 50% reduction in proportion of TCR-$\gamma\delta^+$ T cells in BAL. In regards with tissue resident memory T cells, the frequency of both $CD4^+$ and $CD8^+$ TRM remained unaltered post colonisation, whereas TCR-$\gamma\delta^+$ TRM had a moderate 12% reduction. In consistence with our previous findings (Wright et al. 2013), pneumococcal colonisation resulted in IL-17A responses, seeding the human lung with pneumococcal-specific IL-17A secreting $CD4^+$ memory T cells. However, there was no increase in the frequency of pneumococcal-specific $CD4^+$ TRM, as identified upon *in vitro* stimulation with the challenge strain. Although, we observed greater proportion of both IL-17A producing $CD4^+$ TRM and TCR-γδ T cells in the direct BAL sample of Spn colonised volunteers, stimulation with pneumococcus did not significantly increase their frequency when compared to the non-colonised group. In contrast to IL-17A, frequency of IFN-γ and TNF-α producing cells (both $CD4^+$ T cells and TCR-γδ) were robustly elevated in response to pneumococcus irrespective of carriage status. In addition to cellular responses, pneumococcal colonisation was associated with increased titres of IgG to capsular polysaccharide of Spn6B, which remained high for at least four months post the intranasal challenge.

Human pneumococcal-specific IL-17A responses have been demonstrated previously in peripheral blood (Lu et al. 2008, Lundgren et al. 2012, Mureithi et al. 2009) but in our studies (past and present) we focused on the mucosal compartment where pneumonia occurs – the lung. Using a similar experimental approach, such as challenge with 6B serotype and the backbone of the previous used flow cytometry panel, we replicated the main findings described by our group 5 years ago; pneumococcal colonisation seeds the human lung with cognate $CD4^+$ Th17 T cells. IL-17A responses were a) confined to $CD4^+$ T cell population, b) specific to pneumococcus and c) minimum or non-detectable in the non-colonised group. In fact, pneumococcal-responding IL-17A $CD4^+$ memory T-cells were present at decreased frequency in the healthy adult lung in the absence of colonisation. Many functions have been attributed to IL-17A secreted from Th-17 cells (Kolls and Khader, 2010). It can enhance neutrophil recruitment and phagocytosis (Lu et al. 2008) and increase inducible bronchoalveolar lymphoid tissue

(iBALT) formation (Rangel-Moreno et al. 2011). Given these antimicrobial-related functions attributed to IL-17A, such a cell deposit in lung post colonisation could potentially reduce susceptibility to pneumococcal pneumonia. In addition, it has been described that human Th17 cells persist for longer and are more resistant to apoptosis compared to Th1 cells (Kryczek et al. 2011). This ability highlights the importance of our reproducible finding and makes pneumococcal-specific $CD4^+$ Th17 T cells increase of frequency an attractive goal for vaccinations relying on cellular immunity.

Surprisingly, pneumococcal colonisation was not associated with an increase of cognate $CD4^+$ TRM T cells. A moderate 1.7-fold increase in levels of IL-17A producing $CD4^+$ TRM cells was observed in both experimental groups upon stimulation, which could be the result of heterotypic response by $CD4^+$ TRM generated in the lung due to life-time exposure to other pneumococcal serotypes. The increased frequency, although of low numbers, of IL-17A producing $CD4^+$ TRM T cells in the unstimulated lymphocyte fraction of Spn colonised group, is an observation that seeks further explanation. Theoretically, *in vivo* exposure of cognate $CD4^+$ TRM to aspirated pneumococci could result to their activation. However, the lack of other supportive data weakens this hypothesis. Although, our findings did not showcase the importance of pneumococcal specific $CD4^+$ TRM in lung immunity, they should be interpreted with caution. Unlike $CD8^+$ TRM, which express other tissue resident markers, such as CD49a and CD103, only 1/3 of the total CD4 population express either CD49a or CD103. Therefore, in case of $CD4^+$ T cell population is difficult to define what is a real $CD4^+$ TRM cell.

Conversely to $CD4^+$, total $CD8^+$ T cells did not respond to pneumococcus after *in vitro* stimulation. Although $CD8^+$ T cell functions have been mainly described in the context of viral infections, there is an unconventional $CD8^+$ subpopulation- the mucosal associated invariant T (MAIT) cells. We recently studied MAIT cells in both blood and nasal biopsies collected from challenged volunteers and we found that they may play a role in protection against acquisition of pneumococcal colonisation, through increased production of TNF-α and IFN-γ (Jochems et al, 2019). Unlike the CyTOF panel, the flow cytometry panel used in immunophenotyping lymphocytes in BAL had not included markers that would enable us to study CD8+ MAIT cells in the lung (CD161++TCRvα7.2+).

The last lymphoid population that we studied in the human lung in association with nasal pneumococcal colonisation is TCR $γδ^+$ T cells. γδ T cells are T cells that have a

distinctive T-cell receptor (TCR) on their surface. They are considered unconventional T cells and a bridge between innate and acquired immunity (Nishimura et al. 2004). γδ cells is the least frequent cell population amongst T cells in the BAL, constituting only 1-10% of the total T cells in the healthy adult human lung (Wu et al. 2009, Mwale et al. 2017). Using the EHPC model, we found that pneumococcal colonisation decreased substantially their frequency 3 to 7 weeks post the challenge, with Spn colonised individuals to have 50% less TCR-γδ compared to non-colonised. We can speculate that this carriage-mediated reduction may be associated with either TCR-$\gamma\delta^+$ exhaustion or return to local lymph nodes. A previous study had demonstrated that human TCR-$\gamma\delta^+$ are capable of phagocytosis via Ab opsonisation and CD16 (FcγRIII) and present Ag on MHC class II (Wu et al. 2009). However, the lung is equipped with an enormous number of professional and highly differentiated phagocytes- the alveolar macrophages. As TCR-γδ are highly outnumbered by AM in alveoli, it seems more pragmatic that their presence in the lung is necessitated due to cytokines production, antigen-presentation and to bridge innate and adaptive immunity (Hayday and Tigelaar 2003). Their encounter with pneumococcal cells or particles in the airspaces of Spn colonised volunteers could provide an explanation for the increased frequency of spontaneously producing IL-17A and TNF-α TCR-γδ compared to the non-colonised group. Activated TCR-γδ produce a range of cytokines, including IFN-γ and TNF-α. They also up-regulate CCR7 and home to local lymph nodes where they interact with αβ T cells and other APCs (Devilder et al. 2006, Wu et al. 2009). It has been shown that the activation profile and behaviour of γδ T cells bears resemblances to that of the innate myeloid lineage dendritic cells (Moser and Eberl 2007). It is also important to emphasize that γδ T cells had the greatest IFN-γ response to pneumococcus post *in vitro* stimulation with the challenge strain. The frequency of IFN-γ producing T cells increased 10x post stimulation, reaching levels similar to IFN-g producing $CD4^+$ T cells amongst total T cells. This finding suggests that, although rarely found in alveoli, TCR γδ can be an important source of IFN-γ in the lung mucosa.

The fact that there was no alteration in the frequency of regulatory $CD4^+$ T cells - known for their regulatory function to preventing inflammation-related local tissue damage (Zhang et al. 2011)- post colonisation indicates that nasopharyngeal colonisation is primarily associated with cell stimulation and immunological “boosting” rather acute inflammation or disruption of homeostasis of the lung immune system.

Antecedent pneumococcal colonisation also affected the antibody responses in the lung. Serum immunoglobulins are critical in defence against invasive disease and lung-lining fluid Ig are critical in primary mucosal defence, particularly to opsonophagocytosis of capsulate pneumococci by alveolar macrophages (Gordon et al. 2000). In this study we found that Spn colonised volunteers had 2.6-fold increased IgG against CPS of the challenged strain. Our focus was on IgG because IgA is likely to be insufficient in bacterial opsonisation due to the activity of pneumococcal IgA1 protease to cleave IgA1- the dominant IgA (Janoff et al. 2014). The positive correlation found between serum and BAL pneumococcal specific IgG levels suggests that transudation of antibody from serum into the alveoli may occur and can contribute to the increased pneumococcal-specific IgG in BAL, in support to local antibody synthesis. Pneumococcal fragments (PAMPs) presented by antigen-presenting cells could potentially trigger the maturation of local memory B cells in the lung (Condon et al. 2011). Future studies need to assess whether presence of colonisation elevates antibody levels to conserved pneumococcal proteins amongst the different serotypes in the lung, as we previously reported in nasal mucosal antibodies of Spn colonised individuals (Ferreira et al. 2013). Most importantly, the high-throughput CyTOF platform must be applied on BAL samples to allow us to immunophenotype tissue resident memory B cells to pneumococcus amongst the other rare lung cellular populations.

Collectively, these findings have important implications for vaccine design against pneumococcal pneumonia. Ideally, an effective, non-serotype independent vaccine against pneumonia should induce both cellular and humoral responses. Our data suggest that nasal pneumococcal colonisation triggers both arms of adaptive immunity, without leading to acute inflammation or homeostasis disruption. Enhanced, long-lasting immune memory to pneumococcus might render the host less susceptible to pneumococcal infection, conferring increased protection against development of pneumonia.

Chapter 6

General discussion

6.1 Introduction

Nasopharyngeal pneumococcal colonisation is a natural source of immunisation for the human host (Ferreira et al. 2013, Pennington et al. 2016, Wright et al. 2013). The study of host-pathogen interactions, using a controlled human pneumococcal infection model, can provide insights on immunologic mechanisms that control colonisation and accelerate the development of better or novel vaccines against *S. pneumoniae*. The safe and reproducible experimental human pneumococcal challenge model allows us to describe the dynamics between carriage state and host immune responses in blood, nose and lung, during a known initiation and duration colonisation episode. Amongst these three compartments, lung immune responses induced by nasal colonisation are incompletely described.

This thesis provides new evidence on the relationship between nasopharyngeal pneumococcal colonisation and lung immune responses, focusing on innate immunity and alveolar macrophage behaviour in healthy young adults. The overarching research aims of the present study were:

- To investigate alveolar macrophage polarisation, shift and other phenotypic changes post pneumococcal colonisation
- To test the hypothesis of pneumococcal aspiration in the lungs during nasopharyngeal colonisation
- To investigate the effect of colonisation on lung innate immune responses
- To investigate the effect of colonisation on lung adaptive immune responses

6.2 Discussion of key findings

6.2.1 Human alveolar macrophages phenotypic and gene expression analysis

Assessment of human alveolar macrophage phenotype in adults living in two different geographical locations using flow cytometry, underlined the complexity of this lung resident cell population, revealing a unique phenotype with combined M1/M2 features. We hypothesise that this hybrid phenotype may confer to alveolar macrophages an ability to quickly switch between M1 or M2 associated function allowing for appropriate responses to stimuli and tissue environment. However, it raises the question of whether changes associated with alveolar macrophage polarisation can be detected with the exclusive use of surface markers that have derived from murine models and *in vitro* polarisation of human macrophages using a combination of cytokines. Our transcriptome data showed that AMs from pneumococcal colonised individuals have increased antigen-presentation and their opsonophagocytic capacity was positively correlated with genes that either have been linked with Th1 responses or drive monocyte differentiation towards an "M1-like phenotype". The controversy of our findings emphasises the necessity for the development of a standardised way of assessing alveolar macrophage polarisation in humans. The lack of consensus on how to define macrophage activation/polarisation both in *in vitro* and *in vivo* experiments have been recently highlighted by researchers (Murray et al. 2014). The source of macrophages, the activators, enforcement of feedback and feedforward signalling loops including autocrine production of cytokines and epigenetic and/or developmental effects built into the life history of a macrophage (Ivashkiv 2013, Lawrence and Natoli 2011, Porta et al. 2009) are parameters that can alter macrophage activation state and add into their complexity. Therefore, assessment of AM activation and polarisation mode should rely on combined transcriptome, immunophenotyping and functional data. Additionally, epigenetic and metabolic data may provide a means to dissect intrinsic tissue-resident macrophage activation states.

6.2.2 Micro-aspiration as a proposed mechanism that introduces pneumococci in the human lung during colonisation

The relationship between aspiration of pneumococci from the nasopharynx to the lung has been only studied in presence of respiratory infection. The current paradigm is that high pneumococcal densities, especially in periods of viral co-infection, lead to increased risk of pneumonia development (Howard et al. 2019). Here we demonstrated for the first time that pneumococcal aspiration occurs during a nasal colonisation episode without leading to disease in the healthy human lung. Contrary to the paradigm, it acts as a natural source of lung immunisation against *S. pneumoniae*, with higher pneumococcal densities positively associated with greater alveolar macrophage antimicrobial properties. Our evidences on pneumococcal presence in the lung of colonised individuals, in the form of live pneumococci or intact pneumococcal cells within alveolar macrophages and/or as detected pneumococcal DNA in the bronchoalveolar samples, support the hypothesis that trafficking of bacteria from the nasopharynx to the lung airway is the mechanism that links these two compartments in the context of pneumococcal colonisation. Low levels of micro-aspiration have been reported to occur in patients with obstructive sleep apnea (Beal et al. 2004), as well as in healthy adults during sleep (Gleeson, Eggli and Maxwell 1997). Additionally, a study that combined oral/nasopharyngeal and lung sampling showed that bacteria are present in the lungs of healthy people at low levels compared to the upper respiratory tract. These bacterial populations were indistinguishable in community composition from upper airway microbiota, suggesting that respiratory tract harbours a homogenous microbiota that decreases in biomass from upper to lower tract (Charlson et al. 2011).

6.2.3 Nasal pneumococcal colonisation is an immunising event for the lung, affected both innate and adaptive immune responses

The immunising effects of nasal pneumococcal colonisation on human lung immune responses are presented in this thesis in a comprehensive way. We found that colonisation enhances lung immunity triggering both non-specific and pneumococcal specific responses. Most importantly this immunological “boosting” was not accompanied by acute inflammation, homeostatic disruption or neutrophils infiltration. Although, levels of inflammatory cytokines, regulatory T cells and neutrophils were not

elevated on the day of lung sampling, we cannot rule out that a transient increase of their respective levels could had been occurred during high-density nasopharyngeal colonisation.

6.2.3.1 Augmented alveolar macrophage responsiveness to pneumococcus and unrelated bacterial pathogens

Data presented here are the first to describe *in vivo* priming of alveolar macrophages after colonisation has been successfully established in the nose and provide insights on the mechanism whereby their functional enhancement is achieved. Assessment of alveolar macrophage function post experimental human pneumococcal colonisation demonstrated that they are more able to phagocytose or kill extracellularly both *S. pneumoniae* and other respiratory bacterial pathogens. This enhanced opsonophagocytic capacity persisted for approximately three months after the bacterial challenge. Similar enhanced function, with same duration, and also phenotypic alterations have been previously described in peripheral blood monocytes in the context of vaccination with attenuated bacterial strains (Kleinnijenhuis et al. 2012, Pennington et al. 2019). In particularly, BCG vaccination induces monocytes genetic reprogramming, through the NOD2 receptor, and confers protection against reinfection and heterologous infection (Kleinnijenhuis et al. 2012, Saeed et al. 2014). This relatively long-term, increased responsiveness exhibited by cell populations of innate immunity have been termed as “trained immunity” or innate immune memory (Netea et al. 2016). Only recently, this type of memory was reported in tissue resident macrophages- the alveolar macrophages- in a murine model of respiratory adenoviral infection (Yao et al. 2018). In this study, AM priming required the help of effector CD8 T cells in an IFN-γ- and contact-dependent manner. In our case, AM priming was induced by $CD4^+$ T cells, most likely via an IFN-γ driven axis. The type of infection could explain why AM priming was mediated by different T cell type in each model. During viral infection, CD8 T cells are the major population induced by the virus and it was seen to entry the mouse airway (Yao et al. 2018). In our controlled human pneumococcal infection model, $CD4^+$, but not CD8, responded to *in vitro* stimulation with pneumococcus. Yao et al, also demonstrated that these memory AMs, with sustained changes in surface markers, gene expression, metabolism, and anti-microbial responsiveness, are autonomous and involve a low rate

of *in situ* proliferation independently of circulating monocytes or bone marrow progenitors. In our study, we found that AMs have altered gene expression and heightened responsiveness to respiratory bacterial pathogens post in vivo exposure to pneumococcus. However, lack of epigenetic and metabolic data disables us on concluding whether pneumococcal colonisation mediates innate immune memory in the human lung. Future studies are warranted to explore the potential of pneumococcal colonisation to generate memory macrophages and a long-term protective effect.

Additionally, in our study the decreased monocyte-surface signature found in AMs, in combination with the increased ratio AM to monocytes observed in the lung samples of those who had been colonised, implies to some degree that monocyte-to-macrophage differentiation may be promoted by colonisation. This observation merits further investigation, as this process has mainly been investigated in the mouse system, whereas no data of this and its long-lasting consequences exist in humans (Morales-Nebreda et al. 2015).

Despite hypotheses, such as reprogramming of resident AMs or generation and settlement of a monocyte-derived AM macrophage population in the lung that confers increased lung protection against subsequent infection, as recently shown in a mouse model of influenza infection(Aegerter et al. 2020), we have to consider other plausible alternatives. For instance, the elevated levels of IFN-γ detected in the BAL supernatant up to 50 days post the initial challenge may promote an going immune cell stimulation, sustain AM activated and hence more sufficient against the elimination of bacterial infection.

6.2.3.2 Prompt non-specific Th1 responses and long-term pneumococcal-specific Th17 responses

The role of T helper responses to pneumococcal immunity has been extensively studied in mouse models but little is known about these responses in humans. The immunophenotypic analysis of $CD4^+$ T cells directly after lung sampling allowed us to assess their activation state in the lung mucosa. We found that Th1 (mainly IFN-γ) responses were elicited against the inhaled pneumococci, which were either alive or fragmented when entered the alveolar spaces. Our finding is in line with previous *in vitro* work on human cells, demonstrating that live pneumococci trigger a Th1-biased response (Olliver et al. 2011). *In vitro* stimulation of T cells with heat-killed pneumococci

revealed the presence of cognate, IL-17A secreting memory $CD4^+$ T cells in the human lung post colonisation. These pneumococcal-specific Th17 CD4+ T cells seem to be the acquired memory result of the lung cellular interactions with *S. pneumoniae*. Pneumococcal fragments released after bacterial lysis are presented by APCs, such as dendritic cells, and may trigger Th17 responses, seeding the human lung mucosa with cognate Th17 $CD4^+$ T cells. Schlitzer et colleagues showed that in both humans and mice a subset of IRF4 expressing CD11b+ dendritic cells, secrete IL-23 and control Th17 cell responses in the lung mucosa (Schlitzer et al. 2013).

Whereas previously we have shown that treatment with exogenous IL-17A augmented AM opsonophagocytic capacity to pneumococcus (Wright et al. 2013), in the current study AM pneumococcal uptake was not correlated with the levels of IL-17A producing $CD4^+$ T cells post stimulation with heat-killed pneumococci. The different source and/or higher concentrations of IL-17A may offer a plausible explanation of such a discrepancy. Moreover, the importance of Th1 response and AM-$CD4^+$Th1 synergy against pneumococcal infection had been overlooked in the previous study, as immunity to pneumococcus post EHPC was only investigated under the prism of pneumococcal-specific T cell memory.

6.2.3.3 Alveolar macrophage and T cells synergy in the host defence to *S. pneumoniae*

The alveolar macrophage capacity to kill pneumococci *in vitro* was enhanced during co-culture with autologous $CD4^+$ T cells irrespective of nasal carriage status. AM treatment with exogenous IFN-γ conferred a similar phenotype at low doses, including a dose that mimicked the relative levels of IFN-γ in the BAL of colonised volunteers, whereas neutralisation of IFN-γ receptor abrogated this effect. We hypothesise that during pneumococcal aspiration bacteria are internalised by alveolar macrophages and subsequently skew the responses to Th1. Production of IFN-γ by activated $CD4^+$ Th1 cells in turn enhances AM capacity to take up pneumococci, leading to further IFN-γ secretion and a positive feedback loop between AM and Th1 cells. γδ-T cells, despite their low numbers in the lung mucosa, produce large amounts of IFN-γ upon stimulation and therefore might act as an additional source of this cytokine. Data derived from *In vitro* human monocytes-$CD4^+$ co-culture suggest that the Th1 response to live pneumococci is

induced post pneumoccal internalisation and is dependent on the presence of monocyte-derived IL-12p40 (Olliver et al. 2011). In the same study, it was also shown that the IFN-γ response to live pneumococci was 100-fold higher than to heat-killed pneumococci, while the IL-17 response was not inhibited by heat killing (Olliver et al. 2011). We speculate that IL-17A production is restrained as long as AM can promptly remove pneumococci from the alveolar spaces. This is supported by our data, as IL-17A was not elevated, neither neutrophil infiltration occurs in the lung mucosa post colonisation. It is also possible that the amount of pneumococci present in the alveolar spaces was not enough to induce detectable IL-17A in the BAL sample.

In the scenario that AM are unable to control infection, neutrophils are recruited to the lung via IL-17A production (Lu et al. 2008). Recruited neutrophils reinforce the lung immune responses but also cause tissue damage (Pechous 2017). In the setting of intracellular pathogens, IL-17 can induce the production of IL-12 in dendritic cells and drive Th1 immunity and intracellular bacterial control (Kolls and Khader 2010). Recently, Ercoli and collegues demonstrated replication of *S. pneumoniae* inside splenic macrophages in mice, questioning the previous dogma of pneumococcus as a strictly extacellular pathogen (Ercoli et al. 2018). Our preliminary data on pneumococcal presence inside AM isolated from prior colonised volunteers, days after natural or antibiotic-mediated pneumococcal clearance in the nasopharyngeal, suggest a fundamental shift on the way that we understand the pathogenesis of community acquired pneumonia. Therefore, it is yet to be investigated whether and under what circumstances alveolar macrophages can act as a pneumococcal reservoir.

6.3 Implications

6.3.1 Implications for pneumococcal vaccine development

Current licenced pneumococcal vaccines have been successful in reducing the incidence of invasive pneumococcal disease by mainly conferring B cell-mediated protection. However, this type of immune response is less effective against mucosal infections. Additionally, vaccine escape serotypes, serotype replacement, compartmentalisation of the human immune responses are reasons that advocate the development of mucosally effective serotype-independent vaccination strategies against pneumococcal pneumonia.

Quick clearance of bacteria from the lung by alveolar macrophages is the key aspect for the prevention of disease development at the very early stages of infection. Also, it prevents neutrophil recruitment that can trigger excessive lung inflammation. Our data highlight the importance of alveolar macrophages and Th1 responses in achieving this ideal immunological state in lung mucosa and suggest that the research interest for pneumonia prevention has to be shifted towards innate immunity and Th1/Th17 responses, rather than focuses on new vaccine strategies that exclusively elicit Th17 responses. Colonisation of nasopharynx with pneumococcus confers such ideal immunological "boosting" to the lung mucosa and lack of it could explain the paradox of low colonisation and high disease in the elderly. Adaptive Immunological memory has reached plateau in this age group (Patin et al. 2018), and thus current licensed pneumococcal vaccine may have minor benefits in this age group. Additionally, there are theoretical concerns that PPV may cause immunological harm resulting from exhaustion of present memory B-cells (Clutterbuck et al. 2012). Therefore, an inhaled live-attenuated pneumococcal vaccine that enhances innate and adaptive immunity could confer increased and broader protection to the elderly.

In other risk groups (smokers, COPD, HIV-infected individuals, infancy) increased susceptibly to pneumonia is mainly associated with impaired AM function or perturbed communication between AMs and lung epithelium cells or abnormal/underdeveloped $CD4^+$ Th1 responses, which support the importance of the AM-Th1 cells cross talk described here. These groups most likely require targeted therapeutic strategies that would aim to correct the defective immunological factor and restore the cellular communication.

6.3.2 Implications for pneumococcal biology and pneumonia treatment

Alveolar macrophages are central players of the lung defence against extracellular pathogens, but they can provide a niche for intracellular pathogens, such as *Mycobacterium tuberculosis*. Presence of intact pneumococcal cells within human alveolar macrophages, derived from volunteers that cleared colonisation days before bronchoscopy or after treatment with amoxicillin, is an unexpected but important finding, which suggests a potential role of AMs as pneumococcal reservoir. Influenced by the findings of sepsis mouse model on replication of pneumococci within splenic

macrophages, our results of prolonged pneumococcal survival in the human lung imply an intracellular phase of pneumococcus within alveolar macrophages. If this is the case, macrolides, classically known as *protein synthesis inhibitors,* might be more effective than amoxicillin for pneumonia treatment, due to their ability to penetrate macrophages and act intracellularly. Although known for their immunoregulatory effects on excessive inflammation, a mice colonisation model study showed that macrolides promote macrophage recruitment, through CCL2, and subsequently clearance of pneumococci from the nasopharynx (Iwanaga et al. 2015).

6.4 Future work

The findings presented within this thesis open up new avenues of research. Further characterisation of lung cellular populations using high throughput techniques, such as single cell RNA sequencing (sc-RNAseq) and CyTOF, will i) yield important insights into the mechanisms responsible for generation of protective lung immune responses, ii) enable the identification and study of rare lung cell population and iii) allow us to study alveolar macrophage population at the single cell level. The latter is of major importance, as can shed light on the developmental origin of AMs during the adult life and identify molecular signatures that either enhance AM responsiveness to pathogens or promote pneumococcal survival within them. Avenues that seek further exploration as the following:

6.4.1 Assess the potential of colonisation to generate innate immune memory in the lungs

Analysis and comparison of alveolar macrophage transcriptome pre and post pneumococcal colonisation will further inform us on the direct effect of nasopharyngeal colonisation upon AM in the level of individual, reducing the variation introduced to the model due to differential immunological profile and respiratory tract microbiome. Applying this approach to the single cell level will allow us to define AM subpopulations with changed functionality, their underlying transcriptomic networks and subsequently their functional differences. Additionally, assessment of changes in the availability of DNA for transcription (epigenetic changes) and alterations on metabolic pathways will provide evidences on whether pneumococcal colonisation seeds the lung with memory AMs, conferring a long-term increased lung defence to respiratory pathogens.

6.4.2 Pneumococcal survival assessment in the healthy and susceptible lung

In vitro investigation of pneumococcal survival within AM from both healthy adults and risk populations (etc COPDs, HIV-infected, elderly), as well as people with ongoing influenza infection will inform us on how apiece lung conditions facilitate the development of pneumococcal pneumonia. High-resolution phenotypic (CyTOF) and transcriptomic analysis of AM from these different groups will yield important insights on the mechanisms that drive such a phenomenon. Additionally, combination of EHPC with optical molecular imaging techniques of the distal lung will enable us to visualise inhaled pneumococci *in vivo* and define potential lung deposits.

6.4.3 Controlled human pneumococcal infection combined with pneumococcal protein lung delivery.

Data presented here bring to the table the topic of immune compartmentalisation and highlight the importance of lung immunisation. Mucosally delivered pneumococcal vaccines may be a more suitable vaccine strategy for pneumonia prevention, as they could activate local cell population and confer increased lung immunity. Recent advances on vaccine-antigens delivery systems have made a step forward to the administration of such vaccines to the human lung (Goncalves et al. 2019). The EHPC model could serve as a platform for testing lung delivered pneumococcal protein-based vaccines by assessing their ability to immunise the human lung and comparing it with the systemic and mucosal immunising effects of pneumococcal colonisation. A randomised control clinical trial that would include the following experimental arms: a) intranasal inoculation of live wild type *S. pneumoniae,* b) inoculation with saline (control group), c) lung administration of pneumococcal protein-based vaccine (protein loaded to nanoparticle or nanogels) and d) combined intranasal inoculation of live pneumococcus and lung delivery of the pneumococcal protein-vaccine would be required to test the superiority of each single-immunisation approach versus the other, as well as over the combined immunisation.

6.6 Conclusion

In conclusion, we have demonstrated that nasopharyngeal pneumococcal colonisation acts an immunising event for the lung mucosa. We found evidences of the natural process that links the two mucosal sites during colonisation and provided insights into the mechanism that promotes increased lung immune responses (Figure 6.1). The benefits of nasopharyngeal colonisation were beyond those specific to pneumococcus, as it influences immune responses to non-related bacterial pathogens. Additionally, the presented work provides a significance advance in understanding the development of pneumococcal pneumonia.

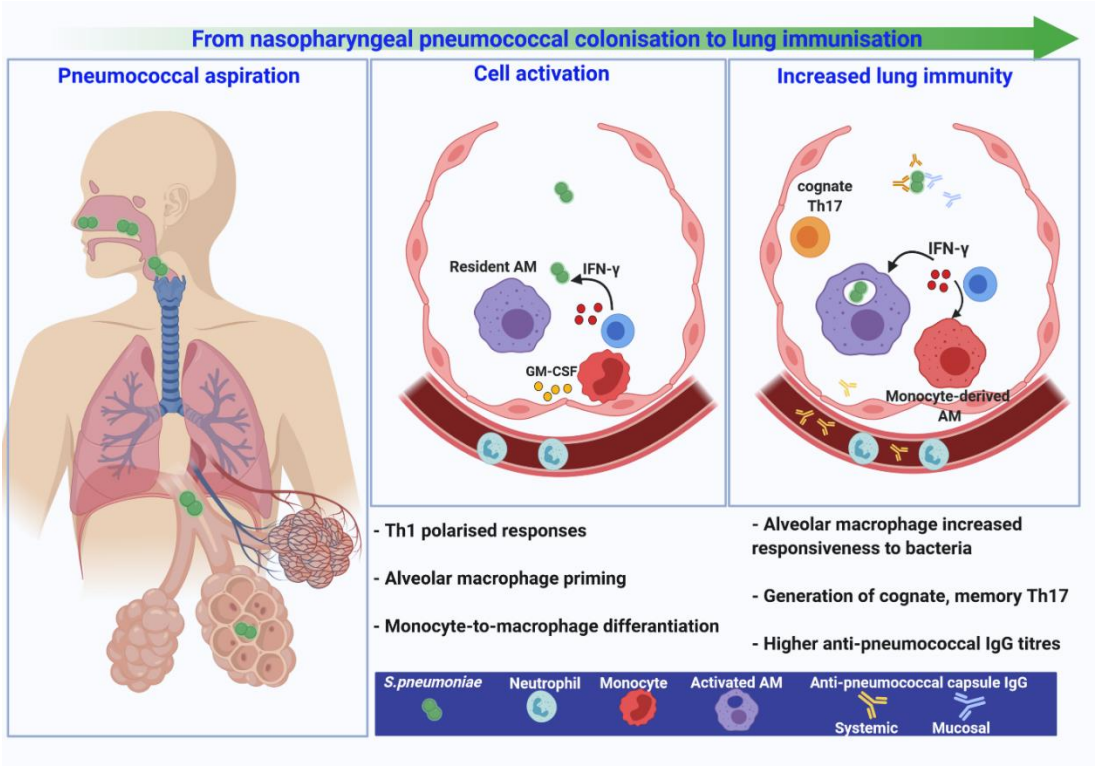


Figure 6.1. Schematic overview of the events that result in augmented lung immunity during a nasal pneumococcal colonisation episode.

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Appendix: Additional tables from Chapter 4

Cytokine	Median concentration Carriage neg.	Median concentration Carriage pos.	p-value	adjusted p-value
IL2	0.17	0.17	0.936	0.936
IL17	0.3	0.3	0.142	0.634
TNFα	0.42	0.42	0.741	0.931
FGF Basic	0.65	0.65	0.183	0.634
GM-CSF	0.49	0.96	0.034	0.484
EGF	1.01	1.01	0.619	0.912
IL 10	0.92	1.11	0.443	0.831
IL 1β	1.09	1.09	0.290	0.725
IL 4	1.25	1.24	0.617	0.912
Eotaxin	1.19	1.33	0.914	0.936
RANTES	1.79	1.79	0.903	0.936
IL 5	2.3	2.78	0.361	0.831
IFN-γ	1.89	3.82	0.048	0.484
IFNα	2.96	3.61	0.044	0.484
MIG	2.07	7.37	0.111	0.634
IL 13	4.87	6.03	0.100	0.634
IL 12	8.13	7.07	0.884	0.936
MIP 1α	9.35	9.57	0.429	0.831
MIP 1β	11	11.42	0.503	0.887
IL 15	16.56	11.29	0.232	0.634
IL 6	13.49	16.22	0.203	0.634
IL 2R	17.62	22.65	0.406	0.831
IL 7	17.58	25.34	0.177	0.634
IP 10	23.07	29.58	0.212	0.634
G-CSF	33.03	43.81	0.733	0.931
MCP 1	51.34	48.13	0.895	0.936
VEGF	56.4	61.4	0.638	0.912
HGF	79.2	73.13	0.800	0.936
IL 8	61.92	90.55	0.538	0.897
IL 1RA	959.97	775.27	0.745	0.931

Table I. Levels of 30 cytokines and chemokines measured the in the BAL fluid of carriage negative (n=20) and carriage positive (n=22) volunteers, who underwent research bronchoscopy up to 50 days post the pneumococcal inoculation. Levels are expressed as pg/ml and are ordered from low to high values. Median per group, p-values by Mann-Whitney test and p-values corrected by multiple-comparison testing (Benjamini-Hochberg) are displayed.

pathway	LeadingEdge
chemokine cluster (I) (M27.0)	*("CXCL2", "CXCL11", "CXCL10", "IL8", "CCL13", "CCL18", "PPBP", "CCL20")*
regulation of antigen presentation and immune response (M5.0)	*("HLA-DQA1", "CLEC7A", "LY96", "HLA-DPB1", "CD247", "FYN", "TLR1", "LILRB1", "IRAK3", "TLR7", "PTPN22", "CD19")*
TLR and inflammatory signalling (M16)	*("LY96", "TLR8", "CSF3R", "TLR1", "LILRB1", "IRAK3", "TLR7", "LILRA6", "CXCR2")*
immune activation - generic cluster (M37.0)	*("LY96", "CEACAM6", "PAX5", "IL1R2", "TLR1", "PTGS2", "TLR7", "ENTPD1", "CD19", "CTSS", "CXCR2", "ITGAM", "CXCL1", "IL18RAP", "PTK2")*
cell cycle and transcription (M4.0)	*("CD36", "PRKCD", "TGFBI", "CD163", "IL1RN", "PLAUR", "FCGRT", "ITGAX", "CSF1R", "CD14", "PTAFR")*
enriched in monocytes (II) (M11.0)	*("FCGRT", "S100A8", "ITGAX", "CSF1R", "CD14", "PTAFR")*
Monocyte surface signature (S4)	*("FCGR2A", "TLR4", "TNFRSF1B", "LILRB2", "LTBR", "CD36", "TNFSF12", "CD163", "MARCO", "PYCARD", "CD4", "FCGRT", "PTAFR")*

Table II: List of differentially expressed genes presented in the pathways identified by gene set enrichment analysis

Gene	log2FoldChange	p-value	adjusted p-value
C1QA	-1.73	0.001	0.425
CD14	-1.86	0.004	0.500
CSF1R	-1.84	0.008	0.500
IRF4	1.77	0.008	0.500
C1QB	-1.66	0.009	0.500
CXCL11	1.96	0.011	0.500
GPI	-1.27	0.011	0.500
NT5E	1.38	0.018	0.500
CCND3	-1.45	0.018	0.500
CLEC7A	2.37	0.021	0.500
CEACAM6	1.29	0.022	0.500
LY96	2.19	0.022	0.500
TAPBP	-1.34	0.024	0.500
TNFSF4	1.32	0.026	0.500
HLA-DRB3	-3.36	0.028	0.500
ITGAX	-1.83	0.029	0.500
IL13	1.18	0.030	0.500
FCGRT	-1.32	0.032	0.500
CMKLR1	-1.55	0.033	0.500
TNFSF13B	1.78	0.034	0.500
CD164	2.01	0.035	0.500
S100A8	-1.46	0.035	0.500
CXCL2	2.08	0.036	0.500
PYCARD	-1.02	0.036	0.500
TBX21	1.42	0.037	0.500
TAGAP	1.08	0.037	0.500
KLRC4	1.24	0.038	0.500
CCRL1	1.27	0.039	0.500
GAPDH	-1.42	0.041	0.508
IL10RA	-1.14	0.045	0.543
TLR8	1.56	0.047	0.545
KIR3DL2	1.25	0.048	0.545
ITGB2	-1.41	0.049	0.545

Table III. List of differentially expressed genes (DEG with $p < 0.05$) in sorted AMs on the day of the bronchoscopy (36 to 115 days post intranasal inoculation), compared *Spn* colonised (n=5) to non-colonised (n=5) individuals. Log2fold change (carriage positive over carriage negative), p-values by Mann-Whitney test and corrected p-values by using Benjamini-Hochberg procedure are displayed.

Gene	p value	Rho
KLRD1	0.007	0.818
SLAMF1	0.008	0.806
IL13RA1	0.011	0.760
CCL15	0.016	0.758
KIR3DL1	0.018	0.745
KLRAP1	0.018	0.745
IL16	0.021	0.733
PRDM1	0.024	0.721
CCR10	0.028	0.709
LAG3	0.028	0.709
TRAF4	0.028	0.709
IRF8	0.030	0.681
EDNRB	0.035	0.669
KLRK1	0.035	0.669
IL6R	0.035	0.685
NT5E	0.035	0.685
ZAP70	0.035	0.685
DPP4	0.039	0.657
CD7	0.039	0.673
CEACAM6	0.039	0.673
FCER1A	0.039	0.673
LILRA4	0.039	0.673
IL12A	0.042	0.650
BCL2	0.044	0.661
MASP2	0.044	0.661
TBX21	0.044	0.661
TNFRSF9	0.044	0.661
HLA.DOB	0.049	0.648
IRF5	0.049	0.648
LILRA3	0.049	0.648
LILRA5	0.049	0.648
SELL	0.049	0.648
TLR8	0.049	0.648
TNFRSF14	0.049	0.648

Table IV. List of genes for which expression significantly positively correlates with AM opsonophagocytic activity.