Cellular immune responses against *Streptococcus pneumoniae* in the human lung

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

Doctor of Philosophy

By

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DECLARATION

This thesis is the result of my own work except were indicated. The study presented in this thesis was done in conjunction with other studies and in some instances the work was shared with colleagues. This research was carried at Malawi-Liverpool Wellcome Trust Clinical Research Programme and University of Leicester. My contributions for the reported work were as follows:

Activity	Responsibility
Sample collection	Anstead Kankwatira, Rose Malamba and Miriam Manduwa
Sample processing	Shared
Pneumococcus binding assay	Shared
Invasion assay	Shared
Outgrowth assay	Shared
Confocal microscopy analysis	Shared
Microbiological analysis	Shared
Flow cytometry data analysis	Sole
Statistical data analysis and presentation	Sole
Thesis preparation and writing	Sole

The material contained in the thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification elsewhere.

Tinashe Kenny Nyazika

LIST OF ABBREVIATIONS

Abbrevation	**Full name**
AMs	alveolar macrophages
APC	allophycocyanin
APCs	antigen presenting cells
ART	antiretroviral therapy
ATP	adenosine triphosphate
AUC	area under the curve
BAL	bronchoalveolar lavage
BV	brilliant violet
C3	convertase 3
$CaCl_2$	calcium chloride
CAP	community acquired pneumonia
CbpA	choline-binding protein A
CD	cluster of differentiation
CDC	cholesterol-dependent cytolysin
CFU	colony forming units
CHIM	Controlled Human Infection model
ChoP	phosphorylcholine
CL2	containment level 2
CO_2	carbon dioxide
COMREC	College of Medicine Research Ethics Committee
COPD	chronic obstructive pulmonary disease
CPS	capsular polysaccharides
CR1	complement receptor 1
CR2	complement receptor 2
CR3	complement receptor 3
DAMPs	damage-associated molecular patterns
DAPI	4′,6-diamidino-2-phenylindole
DC	dendritic cells
DNA	deoxyribose nuclease
DNases 1	deoxyribose nucleases 1
EAD	Extracellular adenosine
E. coli	*Escherichia coli*
EHPC	Experimental human pneumococcal challenge
EI	Extracellular inhibition
ETEC	enterotoxigenic *E.coli*
FBS	foetal bovine serum
Fc	fragment crystallizable
FITC	fluorescein isothiocyanate
FMO	flourescent minus one
FSC-A	forward scatter area

FSC-H	forward scatter height
G-CSF	granulocyte colony stimulating factor
GAVI	Global Alliance for Vaccines and Immunization
γδ T-cells	gamma delta T-cells
GFP	green fluorescent protein
GM-CSF	granulocyte monocyte colony stimulating factor
GPA	gentamicin protection assay
HBSS	Hanks balanced salt solution
HIV	human immunodeficiency virus
hpIgR	human polymeric immunoglobulin receptor
IFN	interferon
Ig	immunoglobulin
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interluekin
IPD	invasive pneumococcal disease
IQR	Interquartile range
IV	Intravenous
IVIG	Intravenous immunoglobulin
LAMP-1	lysosomal associated membrane protein-1
log	logarithmic
LRI	lower respiratory infection
LSTM REC	Liverpool School of Tropical Medicine Research Ethics Committee
LTA	lipoteichoic acid
MAiT	mucosal associated invariant T cells
MASP-2	Mannan-Binding Lectin Associated Serine Protease-2
MBL	mannose-binding lectin
Mcl	myeloid cell leukemia
MDM	monocyte derived macrophages
MFI	median fluorescent intensity
MgCl2	magnesium chloride
MHC	major histocompatibility complex
Mins	minutes
MIP	macrophage inflammatory protein
MLW	Malawi-Liverpool-Wellcome Trust Clinical Research Programme
MoDC	monocytes derived dendritic cells
MOI	multiplicity of infection
MPO	myeloperoxidase
MR1	major histocompatibility complex related protein 1
MRC-1	mannose receptor C-1
MyD88	myeloid differentiation factor-88

NALT	nasopharynx associated lymphoid tissue
NaN3	sodium azide
NanA	neuraminidase A
NanB	neuraminidase B
NanC	neuraminidase C
NETs	neutrophil extracellular traps
NF-kb	nuclear factor-κβ
NIBSC	National Institute for Biological Standards and Control
NKT cells	natural killer T-cells
NLR	nucleotide- binding oligomerization domain-like receptor
NLRs	Nod-like receptors
NO	nitric oxide
OD	optical density
Ops	opsonised
PAD4	peptidylarginine deiminase 4
PAF	platelet-activating factor
PAF-R	platelet-activating factor receptor
PAMPs	pathogen-associated molecular patterns
PavA	pneumococcal adhesion and virulence A
PBS	phosphate buffered saline
PCV	pneumococcal conjugate vaccine
PD-1	Programmed cell death protein-1
PE	phycoerythrin
PFA	parafomaldehyde
PGN	peptidoglycan
PI	pathogenicity island
PiaA	pneumococcal iron acquisition A
PitA	pneumococcal iron transporter
PiuA	pneumococcal iron uptake A
PPD4	peptidyl deaminase 4
PPSV23	pneumococcal polysaccharide vaccine 23
PRR	Pathogen Recognition Receptors
PsaA	pneumococcal surface adhesin A
PspA	pneumococcal surface protein A
PspC	pneumococcal surface protein C
QECH	Queen Elizabeth Central Hospital
RLRs	RIG-1-like receptors
RNA	riboxynuclease
RNS	reactive nitrogen species
RONS	reactive oxygen and nitrogen species
ROS	reactive oxygen species
RPM	revolution per minute

RPMI	Roswell Park Memorial Institute Medium
rSD	robust standard deviation
RSV	respiratory syncytia virus
S. pneumoniae	*Streptococcus pneumoniae*
SBG	Sheep blood agar
SEM	standard error of the mean
SipA	signal peptidase- like protein A
SIV	Simian Immunodeficiency virus
Srt	Sortase
SSA	Sub-Saharan Africa
ST	serotype
STGG	skimmed milk tryptone glucose and glycerine media
TGF-b	Transforming growth factor beta
Th17	T helper 17
THY	Todd Hewitt broth supplemented with 0.5% yeast
TLR	toll-like receptor
TNF	tumor necrosis factor
Tregs	T-regulatory cells
UnOps	unopsonised
URT	upper respiratory tract
WGA	wheat germ agglutinin

Publication and Grants

Publications arising during course of thesis

1. **Tinashe K. Nyazika**, Alice Law, Todd D. Swarthout et al. Influenza-like illness is associated with high pneumococcal carriage density in Malawian children. *Journal of Infection* 2020 doi: 10.1016/j.jinf.2020.06.079 Author #Joint 1st
2. **Tinashe K. Nyazika**, Rabelani Kaela, Mathias Mugoni et al. Implementation of Antibody Rapid Diagnostic Testing versus Real-Time Reverse Transcription-PCR Sample Pooling in the Screening of COVID-19: Case of Different Testing Strategies in Africa. *mSphere* 2020 doi: 10.1128/mSphere.00524-20. Author#1st
3. Frank Chinowaita, Wendy Chaka, **Tinashe K. Nyazika** et al. Sepsis in cancer patients residing in Zimbabwe: Spectrum of bacterial and fungal aetiologies and their antimicrobial susceptibility patterns. *BMC infectious diseases* 2020; 20:161.
4. Newton Kalata, **Tinashe K. Nyazika** et al. Pneumococcal pneumonia and carriage in Africa before and after introduction of pneumococcal conjugate vaccines, 2000–2019: protocol for systematic review. *BMJOpen* 2019 doi: 10.1136/bmjopen-2019-030981. Author #Joint 1st
5. Brenda Nherera, Kudakwashe Mhandire, **Tinashe K. Nyazika** et al. Comparison of non-invasive methods of assessing liver fibrosis in combination ART-experienced Zimbabweans. *SAJHIVMED* 2019; 20: a844.
6. **Tinashe K. Nyazika**, Joseph K. Tatuene et al. Epidemiology and aetiologies of cryptococcal meningitis in Africa, 1950–2017: protocol for a systematic review. *BMJ Ope*n 2018; 8: e020654. Author#1st
7. Ferry Hagen, …, Tinashe **K. Nyazika**, et al. Importance of Resolving Fungal Nomenclature: The Case of Multiple Pathogenic Species in the Cryptococcus Genus. *mSphere* 2017; 2: e00238-17.

Manuscripts under preparations

8. Tinashe K. Nyazika, Mathias Mugoni, Simbarashe Chiwanda, Kondwani C. Jambo, Cuthbert Musarurwa. The potential impact of COVID-19 pandemic on healthcare supply chains associated with HIV, tuberculosis and malaria in Africa. *BMC Medicine*. Under review. Author#1
9. Tinashe K. Nyazika, Lusako Sebale, Joseph Phiri et al. Airway neutrophils exhibit increased binding to pneumococcus compared to alveolar macrophages *ex vivo*. *Manuscript under preparation*. Author#1
10. Tinashe K. Nyazika, Lusako Sebale, Christopher Mkandawire et al. The presence of extracellular *S*. *pneumoniae* in the airway is propagated by intracellular bacterial persistence within AMs *ex vivo*. *Manuscript under preparation*. Author#1

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2. 2019: Federation of Africa Immunology Society (FAIS) Legacy award. *Investigation of the persistence of pneumococcus within alveolar macrophages*. Principle investigator

Abstract

Background

Streptococcus pneumoniae is the leading cause of community-acquired pneumonia in all ages, with the greatest incidence occurring in children, the elderly and HIV-infected individuals. HIV-infected adults are at least 25-fold at risk of bacterial pneumonia compared to age-matched HIV-uninfected individuals, but the immunological mechanisms for this risk are still unclear. Alveolar macrophages (AMs) are the major sentinel phagocytic population found in the airway responsible for the early clearance and control of respiratory pathogens. I hypothesise that HIV infection is associated with impaired airway alveolar phagocyte killing function, leading to survival and propagation of pneumococci.

Methods

Three groups of participants aged between 18 to 60 years were recruited, consisting of asymptomatic HIV-uninfected adults, HIV-infected adults on short-term or long-term antiretroviral therapy (ART) residing in Blantyre, Malawi. Lower airway samples (bronchoalveolar lavage fluid) were collected from the participants and used to investigate airway mediated defence immunity against *S. pneumoniae* in an *ex vivo* model. The airway cells were infected with pneumococci (serotype 3 strain) and the number of alveolar phagocytes associated with bacteria were measured and phenotyped using flow cytometry. Intracellular and extracellular bacterial killing was measured using quantitative culture in a gentamicin protection assay (GPA) and bacterial outgrowth assay, respectively. Confocal microscopy was used to visualise pneumococci within AMs following infection.

Results

Alveolar macrophages were the principal phagocytic cells associated with IgG-opsonised pneumococcal-ST3 following *ex vivo* infection, irrespective of HIV status. AM from HIV-infected adults did not exhibit impaired binding and internalisation of IgG-opsonised pneumococcal-ST3, irrespective of ART duration. Furthermore, airway cell-mediated control of extracellular *S. pneumoniae* outgrowth during early infection was also not impaired in HIV-infected adults on ART. However, HIV-infected adults on ART demonstrated reduced AMs-mediated intracellular killing capacity of *S. pneumoniae* during the late killing phase (24-hours post infection) with pneumococci persisting in $CD206^{+}$AMs. The presence of extracellular *S. pneumoniae* 24-hours post infection in a bacterial outgrowth assay was propagated by intracellular bacterial persistence.

Conclusion

In summary, the data from this thesis demonstrates that AMs from HIV-infected adults on ART have impaired late bacterial killing capacity, leading to intracellular bacterial persistence and propagation of extracellular pneumococcal outgrowth. Persistence of the pneumococci within $CD206^{+}$AMs could serve as a reservoir for pneumococcal infection in the lung, potentially contributing to increased susceptibility to pneumococcal pneumonia in HIV-infected individuals.

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May your awesome deeds be revealed to your servants,

as well as your splendour to their children.

May your favour be on us, Lord our God;

make our endeavours successful;

yes, make our endeavours secure!

Psalms 90:16-7

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CHAPTER 1

1.0. Introduction

1.1. History of *Streptococcus pneumoniae*

Streptococcus pneumoniae (the pneumococcus) was first isolated and described independently by two scientists; Louis Pasteur (France) and George Miller Sternberg (America) in 1880-1881 before being confirmed by Albert Fraenkel as one of the common causes of pneumonia, and later confirmed to be the major cause of mortality during the 1918 influenza pandemic (Brundage and Shanks, 2008; Fraenkel, 1884; Pasteur, 1881a, 1881b; Sternberg, 1881; Watson et al., 1993; Wever and van Bergen, 2014). This organism has gone through several name changes over the years; initially being named *Microbe septicemique du salive* by Pasteur, then referred to as *Micrococcus pasteuri* by Sternberg, the name *Pneumococcus* was suggested by Fraenkel in 1886 but it was later renamed *Diplococcus pneumoniae* in 1920 because of its propensity to cause pulmonary disease (Fraenkel, 1886; Pasteur, 1881b; Sternberg, 1885; Winslow CEA et al., 1920). It was only until 1974 that a consensus was reached, and pneumococcus was given its present name *S. pneumoniae* based on its growth characteristic in liquid media and its proclivity to cause pneumonia (Deibel and Seeley Jr, 1974).

1.2. *S. pneumoniae* the organism

S. pneumoniae is an invasive Gram-positive, diplococcus (but may also be observed as single organism in chains), optochin positive, catalase negative, aerotolerant anaerobic bacterium (Brooks and Mias, 2018; Facklam, 2002; Henriques-Normark and Tuomanen, 2013). When *S. pneumoniae* is cultured on sheep blood agar (7%) supplemented with gentamicin (5µg/L) (SBG), it forms smooth whitish colonies approximately 2 - 5µm in diameter (Figure 1.1).

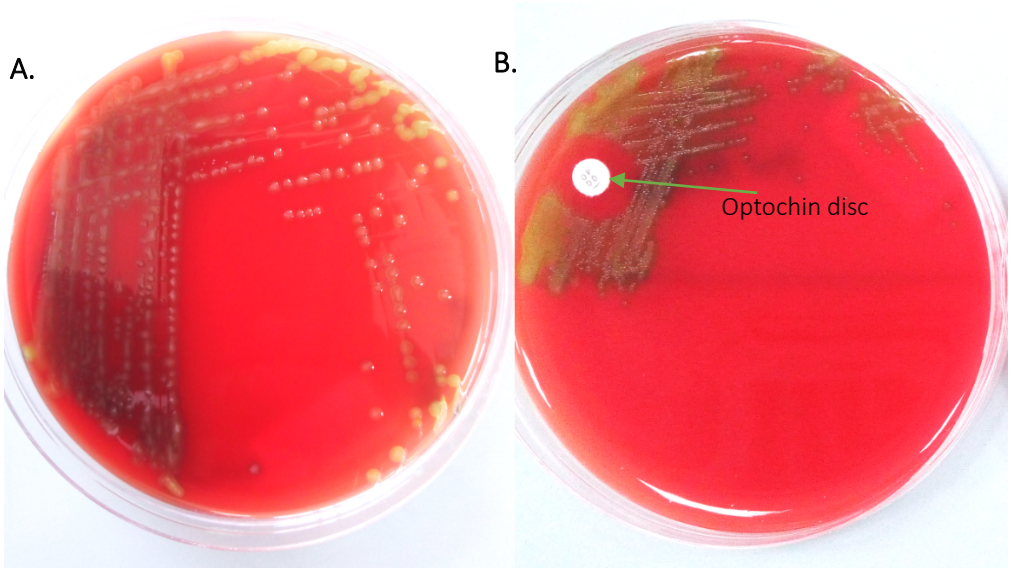


Figure 1.1. **Growing *S. pneumoniae* on sheep blood agar supplemented with gentamicin. A.** colonial morphology of *S. pneumoniae* serotype 3 on SBG agar plate **B.** Optochin sensitive *S. pneumoniae* on SBG agar plate.

S. pneumoniae is a commensal and an extracellular bacterium that frequently colonises the human nasopharynx without causing disease, a state known as carriage, but has potential to cause mild and severe diseases such as otitis media, sinusitis, pneumococcal pneumonia, meningitis and bacteraemia (Austrian, 1999; Brooks and Mias, 2018). *S. pneumoniae* can be divided into serotypes based on capsule chemical composition and a factor (typing) sera that reacts with the capsule giving serogroups which contain antigenically related serotypes (Bentley et al., 2006). At least 98 serotypes (in 46 serogroups) of *S. pneumoniae* are known to exist and vary in the frequency at which they are carried within the nasopharynx with only a few implicated in disease causation (Bentley et al., 2006; Chaguza et al., 2017; Dube et al., 2018; Geno et al., 2015; Kalata et al., 2019; Ziane et al., 2016). The pathogenic potential of pneumococcal serotypes varies and similarly, their frequencies or prevalence also vary by geographic location (Gámez et al., 2018; Jedrzejas, 2001; Mitchell and Mitchell, 2010). Virulence factors are key determinants in the establishment of pneumococcal disease.

1.3. Virulence factors involved in pneumococcal pathogenesis

Several virulence factors have been identified and these can be grouped based on their function namely adhesion, colonisation, breaching tissue barriers, and immune evasion (Gámez et al., 2018; Voss et al., 2012).

1.3.1. The Bacterial capsule

The capsule is one of the major virulence factors expressed by *S. pneumoniae* and has been studied for more than a century. The capsule thickness, steric hindrance and negative charge has been shown to determine the ability of a capsular type to inhibit surface deposition of complement, C-Reactive Protein, mannose- binding proteins, and antibodies that can bind to receptors found on phagocytes (Henriques-Normark and Tuomanen, 2013). The *S. pneumoniae* capsule inhibits the activation of classical and alternative complement pathways, through binding of IgM, IgG to the pneumococci, as well as inhibiting the degradation of C3b to iC3b which reduces the opsonophagocytosis process (Hyams et al., 2010a, 2013a). The ability to regulate the production of the capsule at the transcriptional, translational or post-translational level is important for the survival of *S. pneumoniae* in different host environments (Kadioglu et al., 2008).

The capsule is not the only virulence factor required to induce pneumococcal infection. Other virulence factors (see Figure 1.2) are also found to be glycoconjugated to various proteins on the pneumococcus surface and have been associated with high pathogenic potential (Gámez et al., 2018).

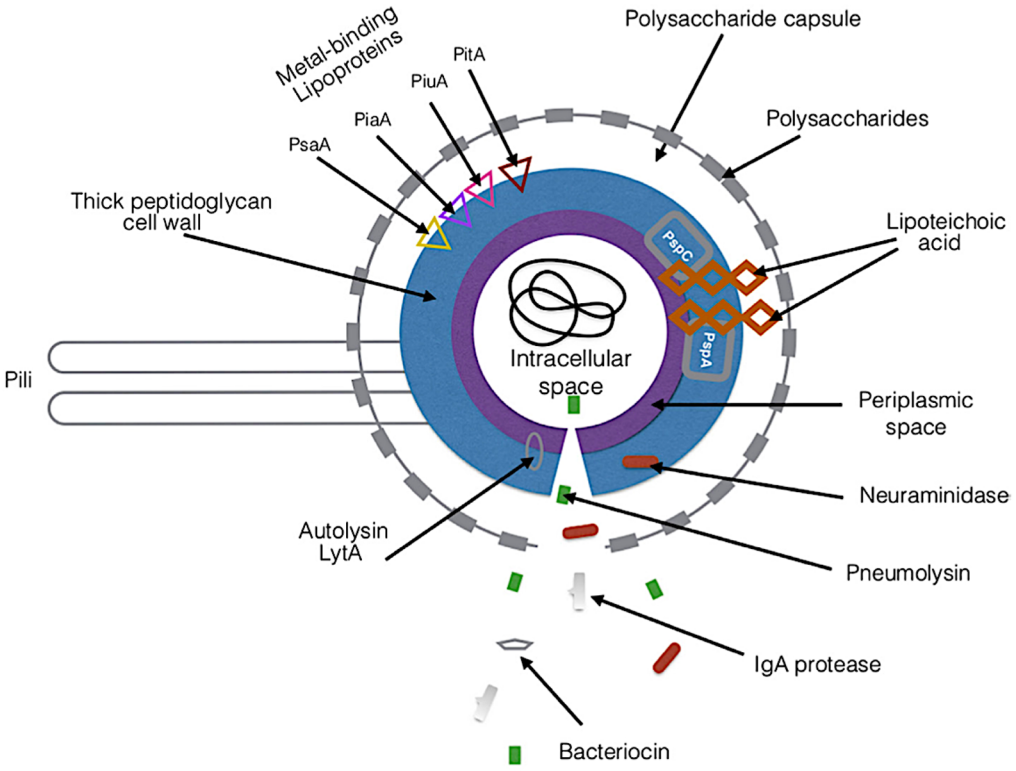


Figure 1.2. Schematic drawing of *S. pneumoniae* structure showing virulence factors involved in host pathogenesis. A number of proteins and toxins are expressed by *S. pneumoniae* and thesedrive its pathogenesis within the host. The major virulence factors are highlighted in the figure. Abbreviations: PsaA, pneumococcal surface adhesin A; PspA, pneumococcal surface protein A; PspC, pneumococcal surface protein C; PiaA, pneumococcal iron acquisition A; PiuA, pneumococcal iron uptake A; PitA, pneumococcal iron transporter. *Adopted from* (Brooks and Mias, 2018).

1.3.2. Neuraminidases

S. pneumoniae is known to express neuraminidases, NanA, NanB, and NanC, with NanA and NanB genes present in almost all clinical *S. pneumoniae* isolates while NanC gene is present in ~50% of isolates (Pettigrew et al., 2006). Neuraminidases are critical in cleaving sialic acid-containing substrates from epithelial cell surfaces thereby promoting pneumococcal colonisation and exposing host cell receptors for bacterial adherence (Tong et al., 2001). Sialic acid is a glycoconjugate and a major source of carbon required for the propagation and growth of pneumococcus in the upper airway (Burnaugh et al., 2008; Siegel et al., 2014). In a mouse co-infection model, neuraminidase from influenza virus and pneumococci were shown to promote pneumococcal growth and load during colonisation but this was dependent on acquisition of the host sialic acid residues from airway mucins (Siegel et al., 2014). In another study, NanA was reported to enhance complement activity in whole blood through cleaving sialic acids from cell surfaces and predisposing sensitised red blood cells to complement and pneumolysin mediated lysis (Syed et al., 2019).

1.3.3. Pili

Pneumococci also uses its multimeric filamentous pili encoded by the pathogenicity islet (PI) 1 and 2 to attach to the respiratory mucosa (Bagnoli et al., 2008; Barocchi et al., 2006; Henriques-Normark and Tuomanen, 2013; Novick et al., 2017; Shaik et al., 2015). At least 20 – 30% of clinical isolates possess the PI-1 and its presence has been shown to be critical in pneumococci interaction with different types of host cells, including both epithelial and endothelial cells (Moschioni et al., 2008). It has been shown that clinical isolates with PI-1 islet present are more virulent, readily adhere to lung epithelia and induce severe disease in murine model infections compared to pili deficient *S. pneumoniae* isolates (Barocchi et al., 2006). PI-1 is also known as the *rlrA* and encodes three pilins (RrgA, the adhesin; RrgB, the backbone; and RrgC, the bridging element) and three class C sortases (Barocchi et al., 2006; Izoré et al., 2010; LeMieux et al., 2006; Shaik et al., 2015). The RrgA has been shown to be the major adhesin

protein on the pilus-1 that binds to the nasopharyngeal epithelium (Iovino et al., 2020; Nelson et al., 2007). Also, the PI-1 type pilus binds to toll like receptor-2 (TLR2) using the adhesin. The PI-2 islet encodes two pilins; pneumococcal iron transporter (PitA and PitB), two sortases (SrtG1 and SrtG2) and one signal peptidase- like protein (SipA) important in bacterial adhesion to host cells (Shaik et al., 2015; Shenoy and Orihuela, 2016; Zähner et al., 2010).

1.3.4. Pneumolysin

Another important virulence factor implicated in pneumococcal disease is the pneumolysin, a 53kDa pore forming toxin released during bacterial autolysis and also localises within the pneumococcal cell wall, thereby becoming accessible to Pathogen Recognition Receptors (PRR) (Price and Camilli, 2009). It belongs to the cholesterol-dependent cytolysin (CDC) family of toxins and oligomerises with membrane cholesterol to form macromolecular pores that perforate the host cell membranes thus mediating cell lysis (Rai et al., 2016). Pneumolysin has also been shown to dampen the pro-inflammatory cytokine secreting potential of alveolar macrophages and dendritic cells thus, facilitating intracellular residency of pneumococci (Subramanian et al., 2019). Pneumolysin has also been shown to induce inflammation during colonisation which in an infant mouse model, was shown to promote shedding of the bacteria from the inhospitable host (Zafar et al., 2017). Pneumolysin has also been shown induce dysregulation of the production of reactive oxygen species (ROS) which in turn causes damage to host double-stranded DNA (Rai et al., 2015, 2016). Other key virulence factors and their role in the pneumococcal pathogenesis have been summarised in Table 1.1.

Table 1.1. Summary of selected virulence factors important for pneumococcal evasion

Virulence factor	Known function
Capsule	• Inhibits complement and antibody mediated opsonophagocytosis. • Prevents mucus entrapment. • Allows the pneumococci to escape neutrophil extracellular traps.
Pneumolysin	• Induce lysis of neutrophils leading to expulsion of neutrophil elastase which impairs AMs phagocytosis. • Binds to host membranes cholesterol. • Binds to the mannose receptor (MRC-1/CD206) enabling it to evade the lysosomal killing pathway. • Inhibits DCs pro-inflammatory cytokine responses and TLR signalling • Inhibits DCs maturation. • Activates complement.
PspC/CbpA	• Allows pneumococci to traverse from the apical to basal epithelia through binding of the human polymeric immunoglobulin receptor (hpIgR) or secretory IgA. • Invades the host epithelia through binding to platelet-activating factor receptor (PAF-R) via ChoP. • Improves complement resistance by binding to factor H.
IgA protease	• Cleaves human IgA1
RrgA	• Adhesin protein on the pilus-1 that binds to the nasopharyngeal epithelia. • Interacts with CR3 on AMs and DCs thereby promoting internalisation of pneumococcus
Neuraminidase	• Cleaves sialic acid from epithelia cells. • Aids bacterial colonisation by revealing receptors for adherence. • Promotes pneumococcal growth and survival
Autolysin	• Digests the pneumococcus peptidoglycan, releasing pneumolysin and causing lysis of incompetent cells.
PavA	• Inhibits pneumococcal internalisation by DCs and cytokine induction.
PspA	• Prevents binding of C3 onto pneumococcal surface. • Also binds lactoferrin and inhibits *S. pneumoniae* killing by lactoferrin

Table is taken from (Brooks and Mias, 2018) and adapted for this thesis.

Abbreviations: AMs – alveolar macrophages; C3 – convertase 3; CbpA – choline-binding protein A; ChoP – phosphorylcholine CR3 – complement receptor 3; DCs – Dendritic cells; IgA – immunoglobulin A; PAF-R – platelet-activating factor receptor; PavA – pneumococcal adhesion and virulence A; PspA – pneumococcal surface protein A PspC – pneumococcal surface protein C; TLR – toll like receptor; hpIgR – human polymeric immunoglobulin receptor

The list of pneumococcal virulence factors in the table, is not exhaustive but only few critical ones have been selected and are shown.

1.4. From Pneumococcal colonisation to disease

1.4.1. Pneumococcal Carriage

S. pneumoniae is a common coloniser of the mucosal surfaces of the human upper respiratory tract (URT) occurring in 5 – 90% of individuals (Abdullahi et al., 2012; Conklin et al., 2016; Esposito et al., 2016; Heinsbroek et al., 2015; Petraitiene et al., 2015; Weiser et al., 2018; Zhou et al., 2015). Pneumococcal colonisation in the absence of disease is higher amongst children, ranging from 40 – 90%, whereas in adults it ranges between 5 – 50% (Adetifa et al., 2018; Heinsbroek et al., 2015; Hill et al., 2006, 2008; Neal et al., 2019; Regev-Yochay et al., 2004; Sutcliffe et al., 2019; Swarthout et al., 2020; Usuf et al., 2015). Nasopharyngeal colonisation is the first step in the progression to pneumococcal pneumonia and yet the carriage dynamics remain incompletely understood (Dube et al., 2018). Once colonisation has been established, an individual strain can be carried for weeks to months before eventual clearance (Hill et al., 2008; Kadioglu et al., 2008; Murad et al., 2019). The nasopharynx has been classified as the main reservoir for pneumococcus in humans (Brooks and Mias, 2018). Successful colonisation with pneumococcus depends on its ability to outcompete for its niche with other microbes, evade mucociliary clearance, withstand environmental factors, availability of nutrients and host immunity (Jochems et al., 2017, 2019; Siegel et al., 2014). Long-term carriage is thought to be a proxy for immunity, in the sense that exposure to pneumococcal antigens via repeated episodes of nasopharyngeal colonisation is key to acquiring sustained protective anti-pneumococcal adaptive immunity (Adler et al., 2017; Weinberger et al., 2008). The strength for this protective effect is thought to vary according to serotype, age and the individual's immune status (Weinberger et al., 2008).

Nasopharyngeal carriage occurs naturally and frequently in children under the age of 5-years but can also be promoted by the disturbance of the nasopharyngeal environment by an upper respiratory viral infection. Acquisition is therefore more frequent in winter compared to summer (Gray et al., 1980; Jochems et al., 2018).

Similarly, surveillance studies conducted in Malawi have demonstrated that pneumococcal disease peaks during the colder and drier months and association with seasonality has also been reported to be higher in HIV-infected adults and in children (Everett et al., 2011; Wall et al., 2014). Separate studies conducted in Kenya, Malawi and Mozambique showed that HIV-infected adults are more frequently colonised with pneumococcus (estimated point prevalence of 24.8% - 52%) compared to asymptomatic healthy individuals (estimated point prevalence 9.7% - 26.8%), with increased carriage reported in HIV-infected individuals on antiretroviral therapy (ART) compared to the ART naïve (Conklin et al., 2016; Glennie et al., 2013; Heinsbroek et al., 2015; Nzenze et al., 2015; Sepako et al., 2014). Moreover, the duration of carriage has been observed to be longer (39.5 vs 28.5 days) in children under 5-years compared to their mothers (Heinsbroek et al., 2015). Furthermore, some serotypes frequently found to cause invasive disease are not often isolated from the nasopharynx of healthy children, suggesting these serotypes colonise for a short duration (Brueggemann et al., 2003; Sutcliffe et al., 2019; Sandgren et al., 2004).

The transition from colonisation to disease is a watershed in the relationship between *S. pneumoniae* and its host. The link between pneumococcal colonisation and subsequent development of various forms of pneumococcal disease (see Figure 1.3) has demonstrated in experimental murine models of meningitis, pneumonia and bacteraemia; as well as inferred from studies on otitis media, bacteraemia, meningitis and pneumonia in humans (Adler et al., 2017; Albrich et al., 2012; Blacklock and Guthrie, 1933; Chiavolini et al., 2008; van Ginkel et al., 2003; Gray et al., 1980; Kelly and Gussin, 1924; Neill et al., 2014). The transition involves a major switch in the expression of virulence determinants as the pathogen adapts to the altered microenvironment (Kadioglu et al., 2008).

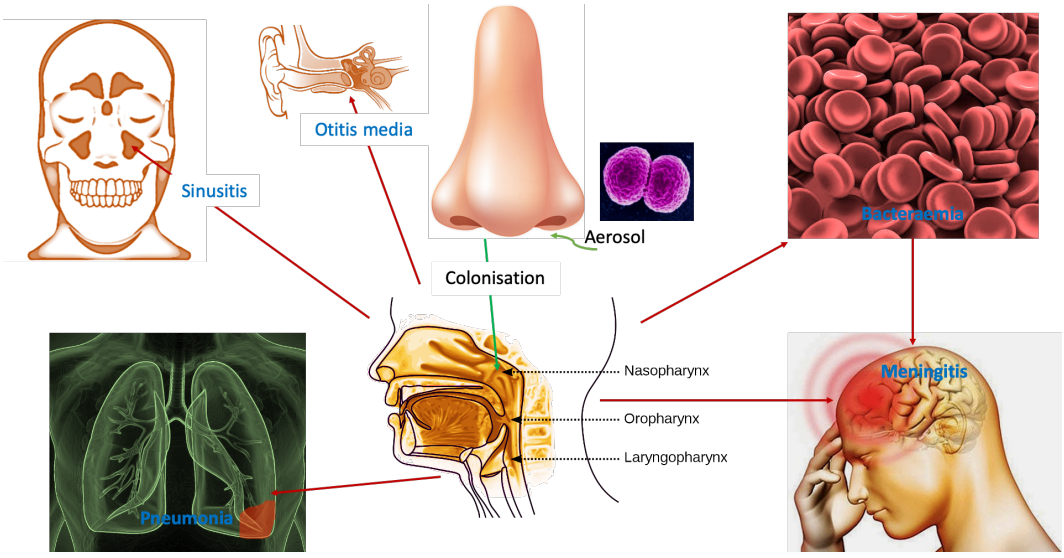


Figure 1.3. **Pathogenesis and spread of *S. pneumoniae*.** *S. pneumoniae* is thought to be transmitted from person to person via aerosols and contaminated hands resulting in nasopharyngeal colonisation. Colonisation is tightly regulated by the host upper airway immunity. During a nasopharyngeal airway disturbance, pneumococcus can evade the host immunity resulting in its spread to different parts of the body and causing diseases such as sinusitis, otitis media, pneumonia, bacteraemia and meningitis.

1.4.2. Otitis Media and Sinusitis

Otitis media and sinusitis are known to be facilitated through local bacterial spread and are non-invasive or fatal in nature. Otitis media is a common paediatric manifestation, affecting at least 80% of children by the age of 3-years (Bergenfelz and Hakansson, 2017). *S. pneumoniae* is the leading bacterial cause of otitis media (between 30 – 50%) ahead of *Haemophilus influenzae* and *Moraxella catarrhalis* (Bergenfelz and Hakansson, 2017; Revai et al., 2008). A newly acquired carriage serotype has often been implicated as a cause of otitis media amongst children with established carriage (Revai et al., 2008).

S. pneumoniae, *M. catarrhalis*, *H. influenzae*, *S. pyogenes* and *Staphylococcus aureus* are common bacterial pathogens associated with community acquired sinusitis (Brook, 2011; Olarte et al., 2014). *S. pneumoniae* has been reported to represent an important pathogen among the aerobic isolates in chronic sinusitis, particularly in acute exacerbations of chronic sinusitis and in younger children (Olarte et al., 2014). Respiratory viral infections are known to predispose

individuals to sinusitis through microbial synergy, induction of local inflammation leading to blockage of the sinus ostia, increased bacterial attachment to the epithelial cells, and disruption of the local immune defence (Brook, 2011).

1.4.3. Pneumococcal pneumonia

Bacteremic pneumococcal infection is associated with a high morbidity and mortality and typically occurs as a complication of pneumonia (bacteremic pneumococcal pneumonia) or, less often, the organism will spread directly from its niche to the bloodstream (Kadioglu et al., 2008). Previously, in a study conducted in Vietnamese hospitalised children, increased pneumococcal load in nasopharynx were associated with radiologically confirmed pneumonia and viral co-infection (Vu et al., 2011). In another study conducted in South Africa, invasive/bacteremic pneumococcal pneumonia was associated with HIV infection, influenza and high *S. pneumoniae* colonisation density and these were also independent predictors of disease severity (Wolter et al., 2014). However, in humans, it is still not clear whether higher pneumococcal colonisation density in the upper airway precedes pneumonia or is actually an effect of lower airway disease (Siegel et al., 2014).

1.5. Burden of pneumococcal infections

Pneumococcal disease has been present throughout history and it is postulated that the pneumococcus bacterium evolved in parallel with the human immune system (Adler et al., 2017; Kilian et al., 2008). The nasopharynx is the reservoir as well as a source of transmission and disease. Once the pneumococcus invades the respiratory epithelia, it can potentially cause life-threatening diseases such as bacteraemia, pneumonia and meningitis (Kadioglu and Andrew, 2004; Klugman and Feldman, 2001). Pneumococcus is the leading cause of pneumonia and invasive bacterial infections in all ages, with the greatest incidence being in children, the elderly and HIV-infected individuals (Azzari et al., 2016; Troeger et al., 2017) see Table 1.2. Between these ends of the age spectrum, healthy young adults have a much lower incidence and severity of pneumonia (Guillon et al.,

2020). Moreover, the incidence of pneumococcal infections varies between age groups, populations and countries, with differences being attributed to several factors such as overcrowding, poverty, underlying immune status, co-infection with influenza and malnutrition (Cohen and Levy, 2017; Nightingale et al., 2019). Identifying mechanisms protecting healthy young adult lungs against pneumonia is a pressing research priority (Dela Cruz et al., 2018; Guillon et al., 2020).

With the introduction of pneumococcal vaccines in children, the burden of pneumococcal disease in both adults and children has dropped, although a gap in studies estimating the true burden still exists (Troeger et al., 2017; Wahl et al., 2018a). Current pneumococcal vaccines are efficacious in reducing invasive pneumococcal disease (IPD) but less effective against pneumonia (Klugman et al., 2003; Silaba et al., 2019). It is estimated that pneumococcus causes IPD in approximately 1.5-million individuals every year and is the leading cause of community acquired pneumonia ahead of influenza, *H. influenzae* B and respiratory syncytial virus (RSV) among children under the age of 5, the elderly and HIV infected individuals (Aston et al., 2019; GBD 2013 Mortality and Causes of Death Collaborators, 2015; O'Brien et al., 2009; Troeger et al., 2017). In 2015, pneumococcal pneumonia was estimated to have caused 55.4% of deaths from lower respiratory infections (LRI) in all ages, totalling 1,517 388 deaths (95% uncertainty interval (UI), 857 940–2.183 791) (Troeger et al., 2017; Wahl et al., 2018a). Lower respiratory infections (LRI) are among the leading top five cause of mortality in people of all ages (GBD 2013 Mortality and Causes of Death Collaborators, 2015). Sub-Saharan Africa (SSA) and southeast Asia are estimated to have the highest disease burden and mortality of pneumococcal pneumonia (Troeger et al., 2017). Moreover, the burden of the disease is greater in SSA due to the high HIV prevalence, malnutrition, poverty, persistent high pneumococcal carriage and transmission rates and this is despite the availability of pneumococcal conjugate vaccines (PCVs) (Chisti et al., 2009; Cohen et al., 2017a; Heinsbroek et al., 2015; Iroh Tam et al., 2017; Troeger et al., 2017). In Malawi, pneumococcus is known to be the commonest cause of meningitis and second

commonest cause of bacteraemia and community acquired pneumonia (CAP)(Aston et al., 2019; Everett et al., 2011; Wall et al., 2013; Wall E.C. et al., 2014). Furthermore, the incidence of invasive pneumococcal disease in adults is estimated at 58.1/100,000 person years with higher incidences (108.8/100,000 person years) occurring in the HIV-infected individuals aged between 35-40 years (Bar-Zeev et al., 2015; Everett et al., 2011).

Table 1.2. Pneumococcal infections

Comparator	Community acquired Pneumonia	Bacteraemia	Meningitis	Otitis media
Common age group	Children<5-years, the elderly, and HIV-infected	Children<5-years, the elderly, and HIV-infected	Children<5-years, elderly	Children <5-years
Contribution of pneumococcus to aetiology	20 – 30%	–	20 – 25%	30 – 50%
Cases/year*	8 910 000 in children<5-years	326 000 in children<5-years	83 900 in children<5-years	~300 million
Risk factors	Viral respiratory infection (e.g. influenza), HIV, malnutrition	Viral respiratory infection (e.g. influenza), HIV, malnutrition	Viral respiratory infection (e.g. influenza), HIV, malnutrition	Viral respiratory infection
Vaccine	PCV-10/13, although it is less efficacious against preventing vaccine type CAP.	PCV-10/13 are efficacious against IPD. PPSV in the elderly.	PCV-10/13 are efficacious against IPD	PCV-10/13, although it is less efficacious against preventing otitis media
Invasiveness	Invasive	Invasive	Invasive	Non-invasive
Common models for studying disease	Mice	Mice	Mice	Mice

References (Borsa et al., 2019; Loughran et al., 2019; Troeger et al., 2017; Wahl et al., 2018a)

*The number of cases/year is limited to children as data for adults is not available

Abbreviations: HIV - IPD – invasive pneumococcal disease, PCV-10/13 – 10/13 valent pneumococcal conjugate vaccine.

1.6. Mucosal innate immunity against *S. pneumoniae*

1.6.1. Mucosal airway innate immunity

The human airways (50 – 100m^2) are exposed to large number of airborne pathogens as a result of inhalation of 10,000 litres of air every single day (Hartl et al., 2018; Joseph et al., 2013; Martin, 2005). The integrity of the airway depends on a highly regulated host defence machinery with both innate and adaptive immunity playing pivotal roles (Bals and Hiemstra, 2004). Mucosal innate immune responses are critical in the control and clearance of pneumococcus infection in the airway. The host's innate responses have to be robust and efficient. Compromised immune responses result in pneumococcus colonisation, dysbiosis, followed by infection of the airway and lung parenchyma (non-bacteremic pneumonia), together with systemic spread to other anatomical sites (Herta et al., 2018; Shenoy and Orihuela, 2016). The innate mucosal immunity involved in pneumococcal defences include such cells as epithelial cells, AMs, dendritic cells, monocytes and neutrophils. The innate immune response uses pattern recognition receptors (PRR) including transmembrane Toll-like receptors (TLRs), cytosolic Nod-like receptors (NLRs), RIG-I-like receptors (RLRs) and various cytosolic DNA sensors to bind to conserved molecular patterns termed pathogen-associated molecular patterns (PAMPs) found on the pneumococcus cell surface (Bals and Hiemstra, 2004; Byrne et al., 2015; Janssens and Beyaert, 2003; Kohler et al., 2016b; Martin, 2005; Medzhitov and Janeway, 1997). Epithelial cells, dendritic cells and AMs are the initial checkpoints that encounter micro-aspirated pneumococcus and can either trigger proinflammatory or tolerogenic/anti-inflammatory downstream immune responses (Hartl et al., 2018; Weight et al., 2019). The control of *S. pneumoniae* invasion in the lower airway in humans is yet to be fully understood, hence defining the mechanistic basis for host cellular control of *S. pneumoniae* is critical for the development of therapeutics (Mitsi et al., 2019; Weight et al., 2019).

1.6.2. How *S. pneumoniae* hijacks the airway epithelia cells for its survival

The airway epithelium is a fundamental part of the lung innate immunity, forming an interface between internal milieu and the external environment, being responsible for the binding and transportation of antibodies, bacterial sensing via their PRR and rapidly transducing signals to recruit innate and inflammatory immune cells to the site of inflammation (Bals and Hiemstra, 2004; Weight et al., 2019). These epithelial cells can be subdivided into bronchial and alveolar cells, which are primarily responsible for ion exchange, regulating gas exchange, mucin production, inflammation, and providing physical barrier though tight junctions and desmosomes (Eisele and Anderson, 2011; Rose et al., 2001).

Alveolar epithelial cells are divided further into type I and type II cells. The type I alveolar cells are responsible for gaseous exchange and are also capable of sensing invading pathogens (Eisele and Anderson, 2011). Type II alveolar epithelia (also called type II pneumocytes) function as the lower airway host defence, through their ability to sense pathogens, secretion of antimicrobial products such as surfactants, lysozymes, lactoferrins cytokines and chemokines (Eisele and Anderson, 2011). Airway epithelial cells express low levels of $CD14^{+}$ and toll-like receptor (TLR) 1-6 and -9, and sense pneumococcus in the mucociliary fluid by the same TLR-dependent mechanisms (TLR2, TLR4, TLR9) used by alveolar phagocytes (Dessing et al., 2009; Dudek et al., 2016a; Gordon et al., 2013; Janssens and Beyaert, 2003; Tomlinson et al., 2014). TLR2 is known to be activated by lipoteichoic acid (LTA), peptidoglycan (PGN) and bacterial lipoprotein; TLR4 is activated by pneumolysin whilst TLR9 is activated by the pneumococcal unmethylated CpG-DNA (Albiger et al., 2007; Domon et al., 2018; Kohler et al., 2016a; Moffitt et al., 2014; Thorburn et al., 2016; Tomlinson et al., 2014).

Upon ligand recognition, the nuclear factor-κβ (NF-κβ) pathway is activated within airway epithelia cells, resulting in the production of proinflammatory cytokines and chemokines including IL-1β, IL-6, TNF, and IL-8 (Dudek et al., 2016a; Jochems et al., 2018; Weight et al., 2019). The NF-κβ (a transcriptional factor) and the myeloid differentiation factor-88 (MyD88; a central adaptor molecule) are crucial to most TLR signalling and their absence has been shown to result in uncontrollable pneumococcal outgrowth in the airways (Albiger et al., 2005, 2007; Kohler et al., 2016a).

Most of the data on the interaction of *S. pneumoniae* and airway cells is derived from murine models. *S. pneumoniae* also exploits the airway epithelia machinery using its virulence factors such as the pili to attach to the respiratory epithelia. Once *S. pneumoniae* initiates attachment to mucosal cells, the bacteria partially sheds its polysaccharide capsule at the site of adhesion as to provide access to the respiratory mucosa and facilitate exposure of adhesive molecules embedded within the bacterial cell wall or cytoplasmic membrane (Novick et al., 2017).

Following attachment of *S. pneumoniae* to the airway epithelia, the bacteria may invade the cells through the binding of phosphorylcholine (ChoP), to the platelet-activating factor receptor (PAF-R) found on epithelial surfaces lining the nasopharynx of the host thus, triggering host cell signalling (Balachandran, 2002; Kadioglu et al., 2008; Maestro and Sanz, 2016; Novick et al., 2017; Rosenow et al., 1997). The Chop found in pneumococcus mimics the platelet-activating factor (PAF) and is used as a decoy to evade host response (Kadioglu et al., 2008). Also the choline-binding protein A (CbpA also referred to as PspC or SpsA) in the *S. pneumoniae* non-covalently anchors to ChoP (Kadioglu et al., 2008). Additionally, the binding of CbpA to PAF-R is capable of initiating the PAF-R recycling pathway, leading to the entry of *S. pneumoniae* into the host cells, and bacterial transportation to the basal membrane of the epithelial cells (Hergott et al., 2015; Kadioglu et al., 2008; Maestro and Sanz, 2016; Rosenow et al., 1997). Notably, pneumococcus may use ChoP esterase (also known as CbpE) to hydrolyse ChoP from host-derived PAF in the lumen of the airway resulting in the PAF-mediated suppression of neutrophil activation and effectively limiting bacterial clearance (Hergott et al., 2015).

S. pneumoniae is also able to bind to the human polymeric immunoglobulin receptor (hpIgR) or to secretory IgA through the CbpA, thus exploiting the recycling pathway and traversing from the apical to the basement of the alveoli epithelia membrane (Hammerschmidt et al., 1997; Li et al., 2020; Maestro and

Sanz, 2016; Moreno et al., 2012). The hpIgR is an integral membrane protein required for transcytosis of IgA and IgM across the mucosal epithelial cells and its interaction has been proposed to promote the translocation of *S. pneumoniae* across the mucosal barrier and cause disease (Balachandran, 2002; Li et al., 2020; Novick et al., 2017). Thus, by exploiting micro-invasion and hijacking the host recycling and trafficking system, the pneumococcus may carefully calibrate the host innate immune/inflammatory response thereby propagating its survival or dissemination (Weight et al., 2019). The schematic Figure 1.4. summarises the mechanism pneumococci utilises to hijack and evade epithelia defences.

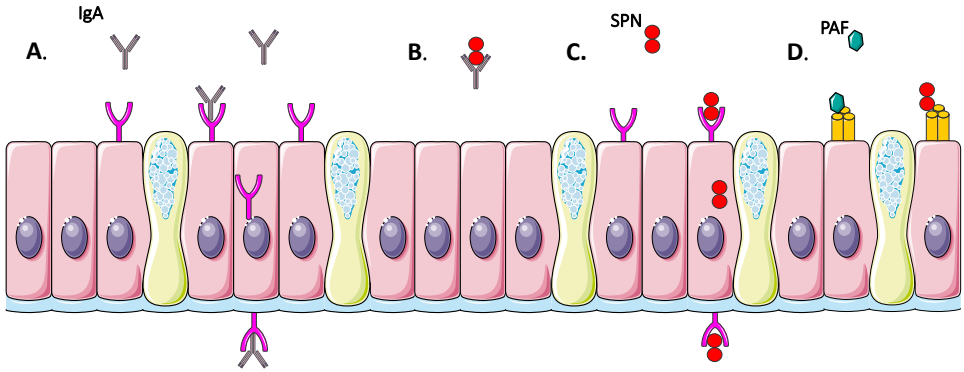


Figure 1.4. **Hijacking of the epithelial human polymeric immunoglobulin and platelet-activating factor receptors by *S. pneumoniae*. A.** Mucosal epithelial cells transport sIgA across their basolateral and apical surfaces through the hpIgR (pink), the receptor is endocytosed and recycled back to the basolateral surface. **B.** *S. pneumoniae* protects itself by secreting IgA1 protease which cleave host sIgA. **C.** *S. pneumoniae* is able to bind to the empty hpIgR (pink) and transported across the epithelia basolateral and apical surfaces. **D.** PAF (green) binds PAF-R (orange) to mediate intercellular signalling. The *S. pneumoniae* Chop mimics PAF and able to bind to the PAF-R thus initiating the PAF-R recycling pathway resulting in the bacteria being transported across the epithelia basolateral and apical surfaces. Abbreviations: HpIgR – human polymeric immunoglobulin receptor, IgA – immunoglobulin A, PAF – platelet factor A, SPN –*Streptococcus pneumoniae*. This figure contains some artwork produced by Servier Medical Art (http://smart.servier.com), under creative commons license 3.0.

1.6.3. Role of neutrophils in control of pneumococcal infection

Neutrophils are critical phagocytes in the airway but are not abundant during homeostatic state (Mwale et al., 2018). It has been reported that the neutrophil lung marginated pool, constitutes the most prominent reservoir of neutrophils in systemic circulation (~40% of total body neutrophils) (Hartl et al., 2018). There is mounting evidence that neutrophils are plastic and display phenotypic and

functional heterogeneity under different disease states ranging from proinflammatory to anti-inflammatory subsets (Deniset and Kubes, 2018). Neutrophils are rapidly sequestered in the pulmonary microvasculature and migrate into the lung parenchyma and alveolar spaces where they perform numerous effector functions (Gomez et al., 2015). However, their effector functions can be detrimental in acute and chronic inflammation as hyperactivation of these cells leads to lung tissue injury (Tak et al., 2013). Hence, a delicate balance is required between neutrophil recruitment and mucosal maintenance (Hidalgo et al., 2019). The role of these innate immune cells during infection is varied and complex (Bou Ghanem et al., 2015).

Interestingly, peripheral neutrophils from HIV infected individuals (ART-naïve and ART experienced) have been shown to have reduced effector functions including chemotaxis, phagocytosis and oxidative burst (Campillo-Gimenez et al., 2014; Elbim et al., 1994; Gupta-Wright et al., 2017; Hensley-McBain and Klatt, 2018; Lazzarin et al., 1986). The contribution of this dampened effector function in the pathogenesis of pneumococci in lung pneumonia has not been established in HIV infected individuals. Patients who are neutropenic are known to be at increased risk for pneumonia, and in mice studies, depletion of neutrophils prior to *S. pneumoniae* infection or delay in neutrophil recruitment into the lungs resulted in higher pulmonary bacterial loads. On the contrary, enhanced airway recruitment of neutrophils following *S. pneumoniae* infection results in reduced survival of the bacteria.

Neutrophils exhibit the greatest anti-bacterial activity compared to AMs and are capable of killing pneumococcus through phagocytosis, and through use of oxygen dependent and oxygen independent mechanisms (Kobayashi et al., 2018; Silva and Correia-Neves, 2012). Neutrophils can also kill bacteria using neutrophil extracellular traps (NET) mediated killing (Jhelum et al., 2018; Kobayashi et al., 2018). Through the oxygen dependent bactericidal pathway, neutrophils produce superoxide (O_2^-) via NADPH oxidase, which converts the superoxide into reactive oxygen species (ROS), which are released and deposited onto the bacteria thus

degrading DNA, RNA and extracellular proteins (Kobayashi et al., 2018). Neutrophils can use serine granules like neutrophil elastase present in their cytoplasm to kill internalised and extracellular bacteria including pneumococci, in an oxygen independent manner (Domon et al., 2018).

High levels of neutrophil elastase have been reported in cell free bronchoalveolar lavage fluid in severe pneumonia patients compared to that observed in fluid from non-severe pneumonia patients (Wilkinson et al., 2012). Paradoxically, neutrophil elastase has also been shown to contribute to the severity of pneumococcal pneumonia due to its toxic effect on the host extracellular-matrix proteins, as well as epithelial cadherin, a cell-cell adhesion molecule pivotal in epithelial cell behaviour and tissue formation thus causing lung epithelial disruption (Boxio et al., 2016; Domon et al., 2016, 2018; Hagio et al., 2008). In addition, pneumolysin; a cholesterol binding and pore forming toxin found in *S. pneumoniae* has been shown to induce lysis of neutrophils leading to expulsion of neutrophil elastase which is known to impair AMs phagocytosis through the cleavage of TLR2 and TLR4 enabling immune evasion by the pathogen and AMs unresponsiveness (Craig et al., 2009; Domon et al., 2016, 2018).

The extracellular release of proteases and oxidases (e.g., neutrophil elastase and myeloperoxidase) within inflamed airways following granule mobilisation to their cell surfaces is thought to be the hallmark of neutrophil activity (Hartl et al., 2018). NETs comprises of chromatin decorated with various antimicrobials peptides such as neutrophil elastase, myeloperoxidase (MPO) and peptidyl deaminase 4 (PPD4) (Cortjens et al., 2017; Jhelum et al., 2018; Mohanty et al., 2019). NETs are thought to be the option of last resort to kill invading bacteria, but pneumococcus has developed ways to escape NETs including their degradation by endonucleases such as DNases 1 (Beiter et al., 2006; Cortjens et al., 2017; Jhelum et al., 2018).

In mice models it has been demonstrated that the efficacy of neutrophils changes during the course of pneumococcal pneumonia disease from clearing bacteria

early on to promoting infection at later time points (Bou Ghanem et al., 2015). Extracellular adenosine (EAD) production has been shown to be crucial for the ability of both murine and human neutrophils to kill *S. pneumoniae* with CD73 controlling the neutrophil antimicrobial phenotype during infection (Siwapornchai et al., 2020). Upon cellular damage due to a variety of insults including infection, adenosine triphosphate (ATP) is thought to leak from damaged cells into the extracellular space and is converted into EAD by the sequential action of two exonucleosidases, CD39 and CD73 (Siwapornchai et al., 2020; Thompson et al., 2004). In Figure 1.5. I summarise the mechanism neutrophils uses to control pneumococci.

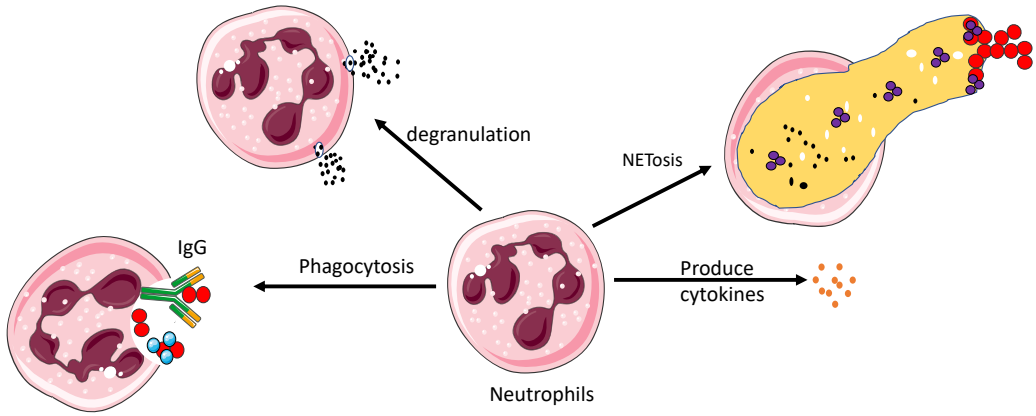


Figure 1.5. **Mechanisms employed by neutrophils in killing *S. pneumoniae*.** Neutrophils can kill pneumococcus through phagocytosis of bacteria which have been opsonised through complement and antibody or unopsonised bacteria. Neutrophils are also able to control the growth of pneumococci through the release of microbiocidal toxic granules (neutrophil elastase and myeloperoxidase) extracellularly to inhibit and kill. Neutrophils are able to release cytokines (TNF, IL-β, IL-6, MIP-2) to mobilise, recruit or activate other cells. Also, neutrophils can form neutrophil extracellular traps (NETs). Neutrophils undergo a process of NETosis, which leads to the formation of NETs under influence of various triggers (e.g. cytokines and bacteria). These NETs consist of extracellular DNA packed with nuclear (e.g. histones) and granule (e.g. MPO, elastase) proteins, can trap and/or neutralise pneumococci. Abbreviations: IgG – immunoglobulin. This figure contains some artwork produced by Servier Medical Art (http://smart.servier.com), under creative commons license 3.0.

1.6.4. Macrophages

Macrophages were first described in 1892 by Metchnikoff, and were first recognised for their ability to phagocytose microorganisms (Byrne et al., 2015; Cavaillon, 2011). Alveolar macrophages constitute the major resident phagocytic and antigen presenting cells in the airway (Guillon et al., 2020; Hartl et al., 2018). Metabolically, AMs exhibit a high basal glucose consumption and respiratory rate but a low respiratory burst activity (Hartl et al., 2018). Alveolar macrophages recognise immunoglobulin (IgM and IgG) opsonised pneumococci through their Ig receptors or complement opsonised pneumococci through the complement receptor 1 (CR1) which is covered in later text (sub sections 1.6.6). For example, the RrgA present on the pili, has been shown to interact with complement receptor CR3 on the surface of macrophages thereby promoting bacterial internalisation (Andre et al., 2017).

Alveolar macrophages also express various PRRs such as scavenger receptors and TLRs that are involved in innate immunity signalling and phagocytosis in response to pneumococcal infections (Albiger et al., 2007). Some TLRs are also located within phagosome membranes, and can be activated by phagocytosed bacteria (Periselneris et al., 2019; Wolf et al., 2011). AMs produce proinflammatory mediators such as IL-1β, IL-6, TNF and NO in response to TLR stimulation and these are critical in the clearance and control of pneumococci. Notably, NOD2 which is part of the family of intracellular PRR found on macrophages and dendritic cells; has been shown to sense pneumococcal peptidoglycan fragments following their digestion by lysozymes and is also thought to be important during bacterial internalisation (Craig et al., 2009; Liu et al., 2017; Wiese et al., 2017). For bacteria with an intracellular phase, NOD1 and NOD2 receptors have direct access to the peptidoglycan and other potential ligands on the cell wall (Wolf et al., 2011). However, shedding of the pneumococcal capsule has been shown to impair its recognition by the NOD2 receptor (Wiese et al., 2017). NOD2 receptors function by activating inflammasome, responsible for mediating the activation of caspase-1 which in turn promotes the cleavage and secretion of IL-1β and IL-18

(Fang et al., 2011; Paterson and Orihuela, 2010). An inflammasome is a cytosolic multiprotein complex composed of nucleotide- binding oligomerisation domain-like receptor (NLR) molecule and procaspase (McNeela et al., 2010; Witzenrath et al., 2011). Pneumolysin has been reported to be critical in NOD2 sensing of pneumococcus (Wiese et al., 2017). Furthermore, pneumolysin has been shown to induce the maturation and secretion of IL-α, IL-1β and IL-18 following NOD2 activation but not their expression on AMs and DCs during *S. pneumoniae* infection (McNeela et al., 2010; Shoma et al., 2008; Wiese et al., 2017; Witzenrath et al., 2011). In Figure 1.6. I summarise the AMs control of pneumococci.

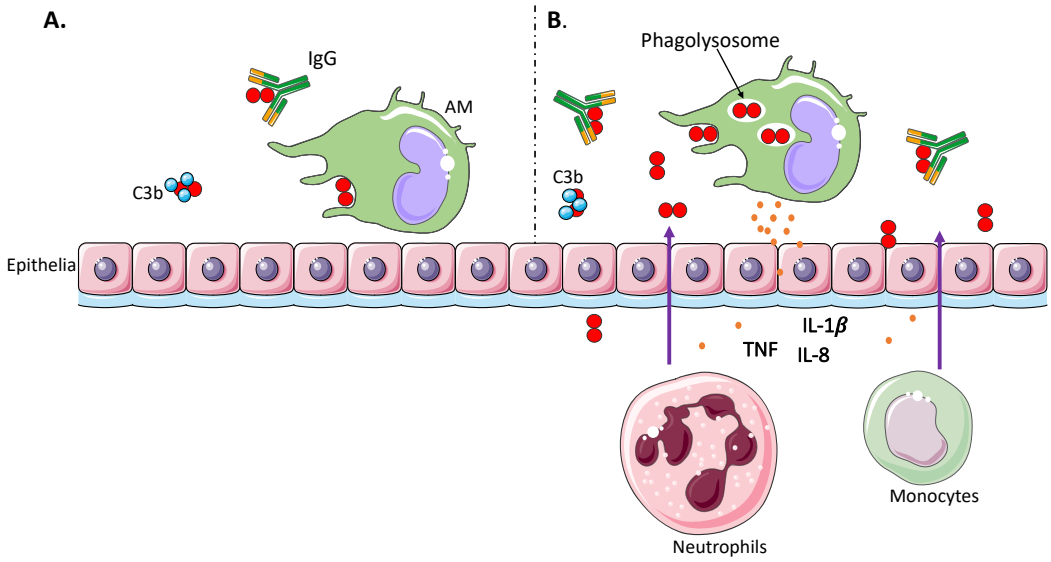


Figure 1.6. **Pneumococcal control by alveolar macrophages. A.** Once the pneumococcus microaspirates into the lower airway, it is recognised by AMs via the pattern recognition receptors or as immunoglobulin/complement opsonised pneumococci through their immunoglobulin/complement receptors and cleared from the airway. **B.** When the AMs fail to clear the pneumococcus, they release cytokines such as TNF, IL-1β and IL-8 resulting in the recruitment and activation of AMs, DCs, neutrophils and monocytes leading to pneumococcal clearance or disease. Arrow in purple represents the chemotaxis of towards site of pneumococcal infection. Abbreviations: AMs – alveolar macrophages, DC – dendritic cells, IgG – immunoglobulin G, TNF – tumour necrosis factor. This figure contains some artwork produced by Servier Medical Art (http://smart.servier.com), under creative commons license 3.0.

1.6.5. Dendritic cells

Dendritic cells (DCs) are professional antigen presenting cells, which can initiate primary immune responses and represent 0.6 – 1.0% of all total leukocytes in the airway (Mwale et al., 2018; Tsegaye and Pöhlmann, 2010). Chronic HIV has been shown to be associated with disruption of the myeloid but not plasmacytoid dendritic cells in the airway (Mwale et al., 2018). Immature DCs have been shown to be particularly adept in antigen capture and processing antigens into cell surface-presentable forms, whilst mature DCs efficiently present antigen to T cells (Noske et al., 2009; Tsegaye and Pöhlmann, 2010). Maturation of DCs is associated with changes including; surface expression of MHC, expression of adhesion and co-stimulatory molecules (CD40, CD80, and CD86), enhanced production cytokine (IL-6, IL-12, TNF) and results in DCs migrating to tissue draining lymphoid organs like the mediastinal lymph node, where they can activate antigen specific T-cells (Noske et al., 2009). Interestingly, *in vitro* studies have shown that pneumococcal pneumolysin inhibits human DCs maturation, induction of proinflammatory cytokines and activation of the inflammasome (Khan et al., 2014; Littmann et al., 2009). Furthermore, it has also been reported that the pneumococcal adherence and virulence factor A (PavA); a virulence factor present on cell outer surface of *S pneumoniae* inhibits pneumococcal internalisation by DCs and cytokine induction (Holmes et al., 2001; Kanwal et al., 2017; Noske et al., 2009; Obert et al., 2006). Recently, it was reported that the binding of pneumolysin to the MRC1 (CD206) inhibited pro-inflammatory cytokine responses and TLR signalling in on human DCs (Subramanian et al., 2019). Figure 1.7. summarises how DCs aid in the control of pneumococci.

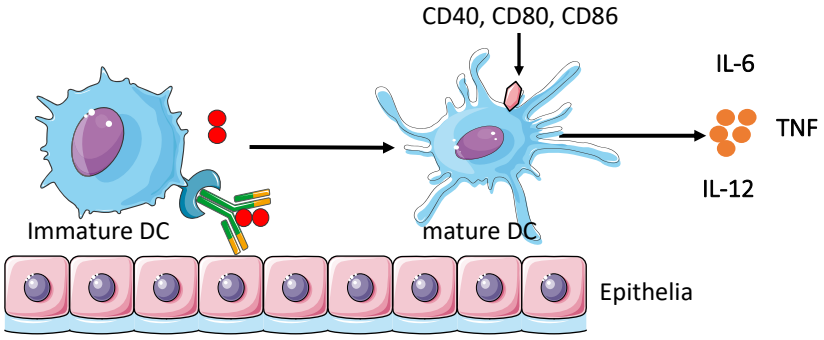


Figure 1.7. **Pneumococcal control by dendritic cells.** Once pneumococcus microaspirates into the lower airway, it is recognised by immature DC via the pattern recognition receptors or as immunoglobulin opsonised pneumococci through their Ig receptors and processed for presentation to B and T-cells. Through this process immature DCs mature and start expressing costimulatory and adhesion molecules (CD40, CD80 and CD86) and cytokine production. Abbreviations: CD- Cluster of differentiation, DC – dendritic cells, TNF – tumour necrosis factor. This figure contains some artwork produced by Servier Medical Art (http://smart.servier.com), under creative commons license 3.0.

1.6.6. Role of complement in control of pneumococcus

Complement is a system composed of a network of fluid-phase and membrane-bound proteins that act as a rapid immune surveillance system, triggering an enzymatic cascade of protein-protein interactions upon stimulation (Huson et al., 2015; Ricklin et al., 2010). It is composed of more than 40 soluble and cell surface anchored proteins with the soluble ones being produced mainly by the liver (Merle et al., 2015). Complement–driven opsonophagocytosis has been demonstrated to be important for opsonisation of the bacteria for neutrophil phagocytosis and in systemic clearance of *S. pneumoniae* infection (Ali et al., 2012; Andre et al., 2017; Brown et al., 2002; Hyams et al., 2010b). Complement is important in innate immunity to *S. pneumoniae* as it provides protection through both antibody-dependent and -independent mechanisms (Ali et al., 2012; Brown et al., 2002) see Figure 1.8. Complement is activated by three enzyme cascades: the classical, the alternative, and the mannose-binding lectin (MBL) pathways (Walport, 2001). Complement receptors (CRs) found on phagocytes important in pneumococcal control include CR1 (CD35), CR3 (CD11b/CD18), and CR4 (CD11c/CD18) (Dustin, 2016; Hajishengallis and Lambris, 2010; Rosales and Uribe-

Querol, 2017). CR1 reportedly has high affinity for C3b and lower affinity for iC3b, but also binds C4b, C1q, and MBL (Ren et al., 2004; Roozendaal and Carroll, 2006; Walport, 2001). CR2 (CD21) is a non-phagocytic receptor reportedly binding to C3dg/C3d thereby influencing B cell activation and development (Tedder et al., 1997). Contrary to CR1, CR3 and CR4; CR2 has higher affinity to iC3b lower affinity for C3b (Roozendaal and Carroll, 2006; Tedder et al., 1997). CR3 is an important adhesion molecule involved in migration of neutrophils and monocytes from the periphery to sites of inflammation (Ren et al., 2004).

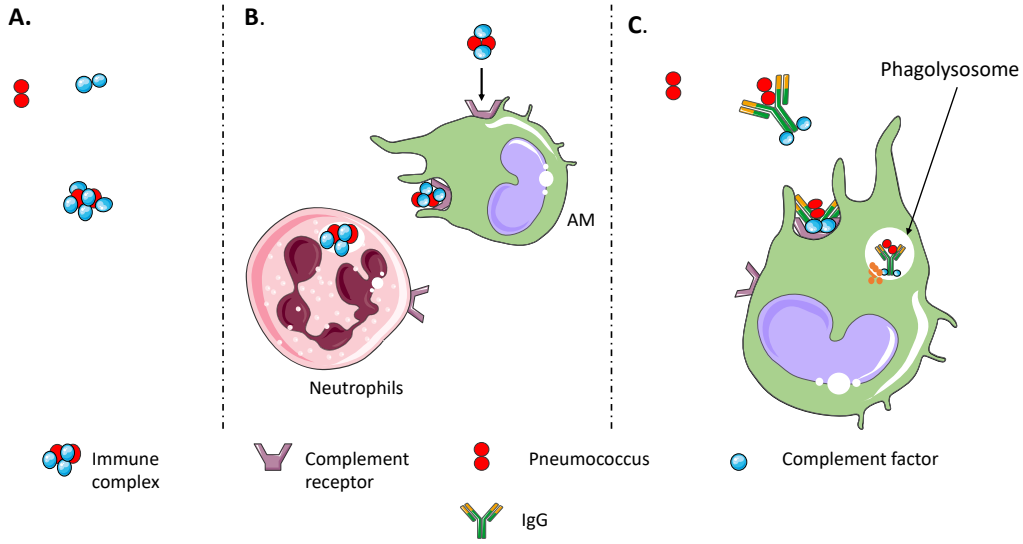


Figure 1.8. Pneumococcal control by complement. **A.** The mannose binding pathway lectin pathway may be activated directly by the bacterial cell surface components resulting in the clearance of pneumococcus through a downstream cascade reaction. **B.** In the alternative pathway, complement may opsonise pneumococcus through an antibody independent mechanism resulting in its internalisation via the complement receptors found on the surface of the phagocytes **C**. The classical pathway may be activated by an antibody (IgM) pneumococcus complex via its Fc portion resulting an immune complex which is then internalised through the complement receptors found on the surface of phagocytes. After internalisation the phagosome formed fuses with lysosomes to form a phagolysosome resulting in the killing and clearance of pneumococcus. This figure contains some artwork produced by Servier Medical Art (http://smart.servier.com), under creative commons license 3.0.

The classical pathway mediated by the binding of C1q to the Fc portion of IgM/IgG–antigen (phosphorylcholine) complexes on the bacterial surface, is the most important portion of the complement system for innate immunity to *S. pneumoniae* (Brown et al., 2002; Kang et al., 2006). The alternative and MBL pathways are activated directly by bacterial cell surface components and are also

considered to be effectors of the innate immune response (Ricklin et al., 2010; Walport, 2001).

Interestingly, HIV infection has been shown not to affect the deposition of C3b/iC3b (major opsonin of complement system) on *S. pneumoniae* and the levels were found to be similar in the HIV-uninfected (Hyams et al., 2010b). Furthermore, the internalisation of complement opsonised *S. pneumoniae* by AMs was also not affected by HIV infection (Gordon et al., 2001). In murine models, it has been reported that mice lacking C3 failed to control growth of pneumococci within the lungs and bloodstream (Kerr et al., 2005). Mice deficient of Mannan-Binding Lectin Associated Serine Protease-2 (MASP-2), were shown to be highly susceptible to pneumococcal infection and failed to opsonise *S. pneumoniae* in the none-immune host (Ali et al., 2012). Moreover, in mice splenic macrophages, it was reported that SIGN- R1, a transmembrane C-type lectin expressed by macrophages, can recognize carbohydrates on the pneumococcal surface and binds to C1q (Kang et al., 2006). All the three complement pathways are important in the control of pneumococci; however, some pneumococcal virulence factors have been reported to inhibit complement activities and these are listed in and Table 1.3.

Table 1.3. Pneumococcal virulence factors: anti-complement activities

Factors	Target	Molecule
Capsule	IgM, IgG, CRP, C3, and iC3b	Prevents complement activation. Inhibits bacterial opsonization by C3b and iC3b.
PspA[a]	CRP and FB	Inhibits CRP deposition and C3 convertase formation
PspC	FH, C4BP, Vitronectin, C3	Inhibits C3 convertase formation and MAC assembly.
Ply	C1q, IgG, and L-Ficolin	Activates complement away from bacterial cells and depletes complement components.
Phts[a]*	FH and C3	Inhibits C3 convertase formation
NanA/BgaA/StrH	–	Deglycosylate complement components.
LytA[b]	FH, C4BP, C3b, and iC3b	Inhibits C3 convertase formation and reduces opsonisation by iC3b.
LytB[a]	–	Lytic activity promotes bacterial dispersion which limits complement deposition
PepO[c]	C1q, C4BP, PLG	Activates and depletes complement. Inhibits C3 convertase formation.
GAPDH[acd]	C1q, PLG	Activates and depletes complement.
Eno[cd]	C4BP, PLG	Inhibits C3 convertase formation and depletes complement.
PGKe	C5, C7, C9, and PLG	Inhibits MAC formation and depletes complement.
Tuf_{sp}[e]	FH, FHL-1, CFHR-1, and PLG	Inhibits C3 convertase formation and depletes complement.
RrgA	CR3	Promote adhesion to and invasion of macrophages.

Taken from (Andre et al., 2017) and adapted for this thesis.

[a]*Possible mechanism; not completely elucidated.*

[b]*Indirect activity. Inhibits binding between C1q and CRP.*

[c]*Explores PLG in order to improve adhesion and colonization.*

[d]*Possibly cleaves/depletes complement components by binding to PLG, but this was not investigated.*

[e]*Binding to PLG possibly improves adherence and invasion, but this was not investigated.*

**Two studies found conflicting results for Pht binding to FH.*

1.6.7. Cytokines

The cytokine milieu in the lung plays an essential role in shaping both the cellular composition and immune response in this compartment (Jambo et al., 2017; Yosef and Regev, 2016). The outcome of pneumococcal infection is strongly influenced by the efficiency of the innate and adaptive immune responses in controlling and clearing *S. pneumoniae* (Madouri et al., 2018; Wilson et al., 2015). In the lung, macrophages and other innate immune cells serve central immune defence functions through phagocytosis, communicating with adaptive immune cells through antigen presentation and secretion of and response to cytokines and chemokines (Amit et al., 2016; Lavin et al., 2015; Wynn et al., 2013; Yosef and Regev, 2016).

During pneumococcal infection the host cells release or respond to cytokines and chemokines induced by the invading pathogen. It has been shown previously, that pneumococcal carriage results in a modest increases in levels of GM-CSF, IFN-γ and IFN-α in airway fluid (Mitsi et al., 2019). In mice, TNF and IL-1β are among the first cytokines that become immediately detectable in the airway and have been demonstrated to be important in the recruitment and activation of AMs, dendritic cells and neutrophils leading to pneumococcal clearance (Hatta et al., 2010; Kafka et al., 2008; Puchta et al., 2016). On the contrary, increased levels of TNF as observed in the elderly, predisposes them to pneumococcal pneumonia with inflammatory monocytes being implicated as the major source cause (Puchta et al., 2016). Reduction or neutralization of TNF is detrimental as patients treated with anti-TNF-α therapies have been shown to be at increased risk of invasive pneumococcal disease (Ali et al., 2013; Takashima et al., 1997). Therefore, both decreased and increased levels of pro-inflammatory cytokines lead to immune alteration and dysregulation of pneumococcal control (Puchta et al., 2016; Yende et al., 2008). In HIV-infected adults, IL-1 and TNF have been shown to be higher in cell culture supernatants following *ex vivo* infections of AMs with pneumococci-ST1 compared to HIV-uninfected cell cultures (Gordon et al., 2013). Furthermore, unstimulated airway supernatants from chronic HIV infected individuals have

been shown to be associated with disruption of key cytokine networks involved in immune defence and maintenance of immune cell homeostasis in the lung (Jambo et al., 2017).

Whilst a robust inflammatory response is generally thought to be protective against infection, high levels of circulating pro-inflammatory and anti-inflammatory cytokines (TNF, IL-6, IL-8, IL-10, IL-18 and G-CSF) during community acquired pneumonia (CAP) have been associated with more severe disease and higher mortality (Bordon et al., 2015; Haugen et al., 2015; Paats et al., 2013; Yende et al., 2008). On the contrary in mice models, pneumolysin has been shown to be associated with dampening of pro-inflammatory cytokine responses (TNF, IL-1β, IL-6 and IL-12) with heightened anti-inflammatory (IL-10 and TGF-β) responses. This is thought to promote intracellular residency of pneumococci within AMs and DCs expressing MRC-1/CD206 (Subramanian et al., 2019). On the other hand, the pneumococcal capsule and extracellular bacteria have been shown to induce enhanced macrophage pro-inflammatory cytokine responses (TNF and IL-β) following *S. pneumoniae* infection compared to unencapsulated strains (Periselneris et al., 2019). It has also been demonstrated previously that early induction of IL-10 during pneumococcal infection resulted in reduced bacteria clearance, reduced TNF and IFN-γ levels and detrimental effects on host resistance (Poll et al., 1996; Siwapornchai et al., 2020).

In mice models, neutrophils have been shown to be the major producers of IFN-γ during pneumonia induced by *S. pneumoniae* and *S. aureus*, but not in pneumonia caused by *Escherichia coli* and *Pseudomonas aeruginosa* (Gomez et al., 2015; Yamada et al., 2011). IFN-γ has been reported to be important in regulating the induction of NETs and in priming macrophages to take up pneumococcus (Gomez et al., 2015; Mitsi et al., 2019; Zhang et al., 2006). Furthermore, pneumolysin has been reported to be involved in the induction of IFN-γ from human adenoid cells following pneumococcal stimulation (Zhang et al., 2006).

1.7. Adaptive immunity against *S. pneumoniae*

1.7.1. T-cell mediated immunity

The establishment and maintenance of immune responses, homeostasis, and memory depends on T-cells (Kumar et al., 2018). T-cells express a receptor which has potential to recognise diverse antigens from pathogens including *S. pneumoniae*, the environment and also to maintain immunological memory and self-tolerance (Kumar et al., 2018). T lymphocytes can be categorised into conventional T-cells ($CD4^+$ and $CD8^+$) and non-conventional T-cells ($\gamma\delta$ T-cells, natural killer T-cells) based on their T-cell receptor.

It has been previously observed in the Experimental Human Pneumococcal Carriage (EHPC) model that pneumococcal carriage increases the proportion of lung IL-17A secreting $CD4^+$ memory T-cells which are thought to be key in the maintenance of mucosal surfaces through recruitment of neutrophils and augmenting AMs killing of opsonised pneumococci (Wright et al., 2013). Furthermore, in another study, it was demonstrated that there's a dynamic relationship between T regulatory (Tregs) and T helper 17 (Th17) cells in nasopharynx associated lymphoid tissue (NALT). The ratio of Th17:Treg appears to be a key determinant in pneumococcal clearance and increases with age and in carriage negative individuals (Mubarak et al., 2016).

In another study, using a mice murine model, it was shown that pneumococci - induced Th17 mediated immunity was critical in sustaining local recruitment of neutrophils, monocytes and macrophages in the upper airway, since this was important in the clearance pneumococci (Zhang et al., 2009). Autologous $CD4^+$ T-cells have been shown to prime and increase AMs uptake of pneumococcus in carriage positive and carriage negative individuals compared to AMs alone in a coculture *ex vivo* experiment (Mitsi et al., 2019). MRC-1 found on macrophages and DCs has also been shown to induce immune tolerance of T-cells, with pneumolysin required for robust regulatory T-cell induction (Subramanian et al., 2019). Interestingly, mice deficient of the major histocompatibility complex class

(MHC) II have been shown to have prolonged carriage, which indicates an important role for $CD4^+$ T cells rather than humoral immunity in pneumococcal clearance (Basset et al., 2007; Kadioglu and Andrew, 2004; Malley et al., 2005). In another study of pneumonia in a mouse model, it was shown that although $CD8^+$ T-cells were required, $CD4^+$ T-cells were dispensable for resistance to serotype (ST)3 pneumonia. However, the authors failed to identify the specific phenotype of the $CD8^+$ T-cells and how these cells mediate the responses against pneumococcus (Weber et al., 2011). Furthermore, the same study also suggested that the Th17 cells could play different roles during colonisation and acute infection in naïve and immune hosts (Weber et al., 2011).

In addition, there is increasing appreciation of the role of non-conventional T-cells in orchestrating early responses to extracellular pathogens such as *S. pneumoniae* and *S. aureus* (Godfrey et al., 2015). These include natural killer T-cells (NKT), gamma delta ($\gamma\delta$) T-cells and mucosal associated invariant T (MAiT) cells with features of both innate and adaptive cells (Ivanov et al., 2014; Kurioka et al., 2018; Nakasone et al., 2007). When these cells emerge from the thymus, they have the capability of undertaking cytolysis and cytokine release (Ivanov et al., 2014). Natural killer T-cells are a subset of non-conventional T-cells capable of recognising and being activated by lipid antigens presented by the non-polymorphic CD1d on antigen presenting cells (Chandra et al., 2018). Whereas $\gamma\delta$ T-cells can be activated by Class I like molecules such as T10/T22 (in mice) and members of CD1 family; they can also be activated by MHC-unrelated molecules such as viral glycoproteins and F1-ATPase complex in humans (Gao and Williams, 2015; Kalyan and Kabelitz, 2013; Russano et al., 2007; Uldrich et al., 2013). $V\alpha14^+$ NKT and $V\gamma4$ $\gamma\delta$ T-cells have been reported to accumulate in the lower airway of inflamed tissue during murine pneumococcal pneumonia (Nakasone et al., 2007; Kawakami et al., 2003). It was further reported that these cells accelerate the infiltration of neutrophils into the lower airways by promoting the synthesis of macrophage inflammatory protein (MIP)-2 and TNF-α (Kawakami et al., 2003; Nakamatsu et al., 2007; Nakasone et al., 2007). MIP-2 is major chemokine

responsible for attracting neutrophils to the site of infection whilst TNF-α enhances the adhesion of neutrophils to endothelial cells (Nakamatsu et al., 2007). γδ T-cells have also been implicated as key regulators of AMs and DCs during the resolution of *S. pneumoniae*-mediated lung inflammation (Kirby et al., 2007). Other unconventional T-cells like MAiT cells are capable of recognising *S. pneumoniae* through small molecules derived from the riboflavin metabolic pathway presented by, a non-polymorphic major histocompatibility complex related protein (MR) 1 (Hartmann et al., 2018; Kurioka et al., 2018).

It has been shown that MAiT cells may be activated in an MR1-dependent manner in the presence of macrophages and through innate cytokines (IL-12 and IL-18) dependent manner in the presence of monocytes (Kurioka et al., 2018). It was also reported in another study, that MAIT cells were capable of recognising and responding to DCs and airway epithelia cells infected with carriage and invasive isolates of *S. pneumoniae* (Hartmann et al., 2018). However, how the precise role of non-conventional T-cells during pneumococcal infection in humans has not been fully established. Figure 1.9. summarises how T-cells aid in the control of pneumococci.

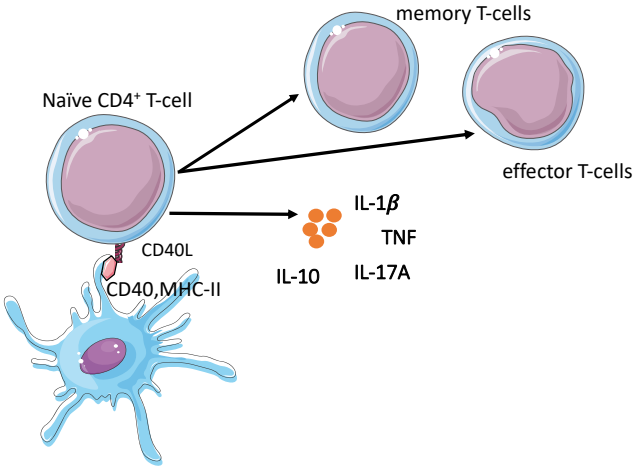


Figure 1.9. **Pneumococcal control by T-cells.** Mature DCs migrate to the regional lymph nodes, where they encounter cognate T-cells and crosslink resulting in T-cell activation**.** The antigen specific T-cell is then capable of undergoing activation and proliferation resulting in the production of cytokines, differentiation of naïve T-cell to memory and effector T-cells. Abbreviations: CD- Cluster of differentiation, TNF – tumour necrosis factor. This figure contains some artwork produced by Servier Medical Art (http://smart.servier.com), under creative commons license 3.0.

1.7.2. B-cell mediated immunity

B cells play a central role in pneumococcal defence as they produce immunoglobulins directed against antigens (capsule, polysaccharide, lipoprotein, pneumolysin) embedded within the bacterial cell wall, thus promoting pathogen clearance (McKay et al., 2015a; Moore et al., 2001). When a naïve B cells encounters pneumococcal antigen, it can differentiate into IgM^+ memory B cells to produce pneumococcal-specific IgM without T-cells help (T-independent activation); later, during somatic hypermutation the activated B-cell undergoes class switching, and some pneumococcal- specific IgM^+ B cells will differentiate to pneumococcal-specific IgG^+ or IgA^+ memory B cells or plasma cells (Richards et al., 2010; Zhang et al., 2015). T-independent antigens are highly repetitious structures present on bacterial cell walls (lipoproteins, pneumococcal polysaccharide, proteoglycan) and form a major protective immune response generated against encapsulated microbes (Moore et al., 2001). On the contrary, T-dependent immune response, requires a complex interplay between antigen presenting cells (APC), antigen, direct B-cell and T-cell interaction (Moore et al., 2001). Following phagocytosis, the APC processes the antigen into small peptide fragments and expresses them together with the MHC class II, resulting in the complex being recognised by the T-cell receptor. The binding of the TCR to the MHC class II and antigen complex on the APC, results in the co-stimulation of the $CD4^+$ T-cell and the expression of CD40 ligand (CD40L) on its surface (Doherty et al., 2018; Moore et al., 2001; Parker, 1993). The CD40L then binds to the CD40 receptor molecule on B-cells resulting in its ligation and activation of B-cells (Doherty et al., 2018; Parker, 1993). This results in the expression of cytokine receptors on the B-cells which can now bind to cytokines secreted by the $CD4^+$ T-cell and activate the production and isotype switching of immunoglobulins (Doherty et al., 2018; Parker, 1993).

It has been shown previously that serum IgM antibodies to pneumococcal serotypes 3, 9 and 22 are undetectable at birth but progressively increase with age, as well as systemic IgM memory B cells (Kruetzmann et al., 2003).

Furthermore, in the same study it was shown that the absence of IgM memory predisposes individuals to encapsulated bacterial infections (such as invasive pneumococcal disease) and leads to an impairment in their response to polysaccharide vaccine (Kruetzmann et al., 2003). In another longitudinal study of infants followed from birth, it was shown that serum anti-capsular IgG titres were highest in the cord sample (reflecting maternally-derived antibodies), with a decline in serum concentration of IgG antibodies within the first 6-months of age which was then followed by a modest increase in the serum IgG concentrations (Turner et al., 2013). It was also observed that the mean serum IgG antibody titres to various pneumococcal proteins declined rapidly after birth to a nadir at 4 months, with a subsequent increase in serum IgG antibody titres thereafter which was largely attributed to post-natal pneumococcal exposure (Turner et al., 2013). In an EHPC model, systemic anti-capsule antibodies were only detected in subjects that developed carriage after challenge with serotype 6B (Ferreira et al., 2013a). Moreover, the authors, also further demonstrated in a murine model that the passive transfer of post carriage sera to mice confers significant protection against invasive disease (Ferreira et al., 2013a). Figure 1.10. summarises the T-cells dependent and independent ways B-cells use in the control of pneumococci.

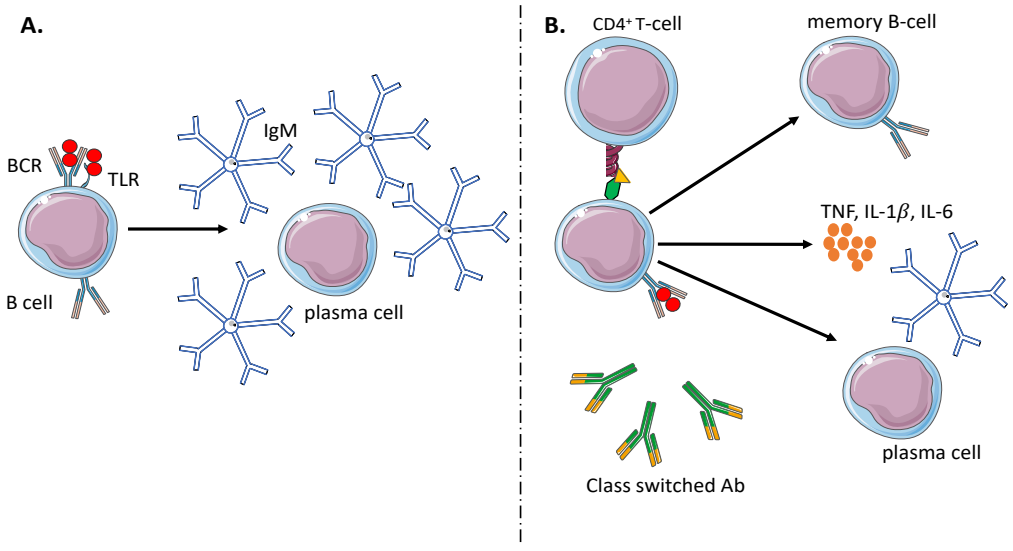


Figure 1.10. **Pneumococcal control by B-cells. A.** During T-independent activation, a naïve B-cell encounter pneumococcal antigen through the B-cell receptor, upon getting a second signal via the toll-like receptors the B-cell differentiate into IgM+ memory B cells or plasma cells capable of producing IgM antibodies**. B.** During T-dependent activation, an antigen presenter such as a B-cell

encounters pneumococcus, internalise it and process it into small peptides (yellow) for presentation together MHC class -II. Upon encounter of a cognate T-cells and crosslink with the MHC-II and peptide, the B cells is activated, and it differentiates into a plasma cell, memory B cell and resulting in the production of class switched antibodies such as IgG2, IgA and cytokines. Abbreviations: Ab- antibody, CD – cluster of differentiation, TLR – toll-like receptor, TNF – tumour necrosis factor. This figure contains some artwork produced by Servier Medical Art (http://smart.servier.com), under creative commons license 3.0.

1.8. Impact of HIV infection on anti-pneumococcal immunity in humans

1.8.1. Systemic/peripheral blood responses

HIV infection is known to preferentially deplete $CD4^+$ T-cells in peripheral blood, lymphoid and mucosal tissues (Brenchley et al., 2004; Sedaghat et al., 2008). HIV infection is associated with an impaired proliferation response and upregulation of CD154 (CD40L) on activated $CD4^+CD69^+$ T-cells to pneumococci in individuals with a CD4+≤ 500 cells/mm^3 but not T-cell activation ($CD4^+CD69^+$) in response to pneumococci (Glennie et al., 2011). Furthermore, it was noted that pneumococci IL-17A $CD8^+$ T-cells together with IL-17A, TNF, IL-2 cytokines produced in response to pneumococci were preserved but not IFN-γ producing $CD8^+$ T-cells (Glennie et al., 2011).

Similarly, in another study, the same authors demonstrated that the proliferative capacity of $CD3^+CD8^-$ T-cells was impaired in HIV-infected untreated, treated and symptomatic individuals compared to the HIV-uninfected in response to pneumococci antigens (Glennie et al., 2013). The authors further showed that IL-17A producing $CD3^+CD8^-$ T-cells were preserved in HIV infected untreated, treated and symptomatic individuals but not IFN-γ producing $CD3^+CD8^-$ T-cells, though the proliferative capacity was restored in HIV symptomatic and treated individuals (Glennie et al., 2013). It has also been further shown that IFN-γ producing and proliferating $CD4^+$ T-cells responding to pneumococci antigens increase in proportion following initiation of antiretroviral therapy (ART), with similar proportions observed at 12-months ART between HIV-uninfected and HIV infected individuals (Sepako et al., 2014).

Untreated HIV infection disrupts B cell populations causing reduction in the pool of memory and naïve resting B cells as a consequence of lymph node destruction or by the direct effect of HIV leading to apoptosis of B cells (Boliar et al., 2012; Kardava et al., 2018; Longwe et al., 2010; Planchais et al., 2018). Phenotypic perturbations include B cell hyperactivation, poor expression of $CD19^{+}CD21^{+}$ (naive mature B cells) and increased frequency of immature/transitional B cells ($CD10^{+}CD21^{low}CD27^{-}$) in the peripheral blood (Iwajomo et al., 2015; Longwe et al., 2010; Moir and Fauci, 2013; Moir et al., 2010). HIV infection leads to functional perturbations of B cells including hypergammaglobulinemia associated with polyclonal and HIV-specific activation of B cells, decreased B-cell responses to specific immunogens and non-HIV pathogens such as pneumococcus, and a hypo responsiveness to vaccines (Buckner et al., 2013; Kardava et al., 2018; Moir et al., 2010; Tsachouridou et al., 2015). However, early initiation of ART has been shown to rescue and preserve some degree of B cell immune capacity but not the amount of circulating isotype-switched memory B cells nor their functional activity (Moir et al., 2010; Planchais et al., 2018).

In a study conducted in children, it was observed that the recovery in proportions of circulating memory B cells specific to pneumococcal antigens such as CbpA, PspA, pneumolysin and PsaA was slow and only became apparent 12-months following commencement of ART (Iwajomo et al., 2015). It is hypothesised that the pneumococcal-specific B cell memory function may lag behind other indices of B cell recovery (Iwajomo et al., 2015). In contrast, another study demonstrated an increased serum anti-pneumolysin IgG and IgA and slightly increased anti-PspA IgA but no increase in anti-PspA IgG amongst HIV infected untreated individuals compared to healthy adults (Collins et al., 2013). It has also been shown that an inverse correlation exists between plasma levels of HIV RNA and peripheral blood $CD4^{+}$ with serum opsonic activity against serotype 3 and type 9 strains of *S. pneumoniae* as detected in asymptomatic HIV-infected persons (Segal et al., 2011; Titanji et al., 2006). This effect may be attributed to polyclonal B-cell activation and hyperglobulinaemia resulting in poorly opsonic immunoglobulins, thus

increasing the risk of invasive pneumococcal disease amongst the HIV infected individuals (Collins et al., 2013). It is generally thought that even effective ART may not possibly fully restore B-cell function (Zhang et al., 2015).

1.8.2. Airway responses

The presence of HIV infection in the lower airway has been shown to disrupt airway cellular homeostasis resulting in $CD8^+$ alveolitis, increase in B cells and γδ T-cells with a reduction in $CD4^+$ T-cells (Collini et al., 2018; Gordon et al., 2013; Mwale et al., 2018). Furthermore, HIV infection is also known to perturb the airway cytokine microenvironment in individuals with untreated chronic HIV-1 infection and long-term ART does not completely restore it (Jambo et al., 2017).

Despite the low proportions of alveolar $CD4^+$ T cells in the bronchoalveolar lavage (BAL) of HIV untreated individuals compared to HIV uninfected healthy controls; no perturbations in the proportions of IL-17A, TNF and IFN-γ-producing alveolar $CD4^+$ T-cells were observed following airway cell stimulation with *S. pneumoniae* in cell culture supernatants of ART- naïve HIV-infected and HIV-uninfected adults (Jambo et al., 2011; Peno et al., 2018). At the same time, it has also been demonstrated that long-term ART results in an increased proportion of IL-17A and TNF but not IFN-γ-producing alveolar $CD4^+$ T-cells against *S. pneumoniae* in HIV-infected adults (Peno et al., 2018). However, it has been demonstrated that an alveolar subset of the CD103 expressing airway $CD8^+$ MAiT cells were selectively depleted in untreated HIV-infected adults compared to healthy controls (Mvaya et al., 2019). This subset has been shown to be critical for mucosal maintenance and recently it has been shown that human MAiT cells respond to a diverse strains of *S. pneumoniae* through MR1 or co-stimulation provided by innate cytokines (Kurioka et al., 2018). So, the depletion of MAiT cells by HIV could potentially affect early pneumococcus control in the lung.

Previous reports have also shown that HIV infection results in the accumulation of B-cells in the lower airway (Mwale et al., 2018). It has been shown previously that airway levels of total IgG and total IgM and pneumococcal-specific IgG and IgM

are increased in HIV-infected subjects compared to their HIV negative counterparts (Gordon et al., 2003). Furthermore, following invasive pneumococcal infection, HIV-infected patients had higher bronchoalveolar lavage levels of pneumococcal specific IgG than HIV-infected patients without recent infection (Gordon et al., 2003). It has also been demonstrated that HIV-infected patients have higher levels of pneumococcal specific IgG but not IgA (anti-pneumolysin and anti-PspA) in their airway than healthy controls (Collins et al., 2013). Yet, another study demonstrated impaired bacterial binding of pneumococcus serotype-1-specific IgG and impaired opsonophagocytosis of pneumococci in asymptomatic HIV-untreated individuals compared to healthy volunteers (Eagan et al., 2007). Taken together, it is possible that HIV infected individuals have limited airway IgG diversity despite the increased local production within the airway (Eagan et al., 2007). The increased IgG responses observed in airway fluid in the HIV-infected adults might be a consequence of their higher rates of carriage or HIV-induced hypergammaglobulinemia (Collins et al., 2013; De Milito et al., 2004; Heinsbroek et al., 2015).

AMs are susceptible to HIV infection through CCR5 and CXCR4 receptors on their surfaces. These are similarly expressed on $CD4^+$ where these receptors mediate the entry of the HIV into cells (Boliar et al., 2019; Clayton et al., 2017; Jambo et al., 2014a; Kruize and Kootstra, 2019). In HIV-infected individuals, approximately 3 – 10% of whole bronchoalveolar fluid AMs are infected with HIV and this reservoir persists even during long-term ART (Costiniuk et al., 2018; Jambo et al., 2014a). Following HIV infection, it has been shown that HIV impairs the functions of AMs such as phagosomal proteolysis (Jambo et al., 2014b). On the contrary, it has also been demonstrated that during early pneumococcal *ex vivo* infection, pathogen binding and internalisation are similar between HIV-uninfected and HIV-infected individuals (Gordon et al., 2000, 2001; Jambo et al., 2014b). Interestingly, despite the HIV associated classical activation observed in AMs, the AMs and pneumococcal interaction were not impaired by HIV in *ex vivo* infection studies (Gordon et al., 2013). Classical activation occurs following activation of AMs by a

cognate $CD4^+$ T-cell and is mediated by gamma interferon (IFN-γ) released by the $CD4^+$ T-cell resulting in increased expression of major histocompatibility complex class II (MHC-II), antigen presentation, increased intracellular killing, cytokine production and microbicidal functions (Gordon et al., 2005, 2013). In a separate study from the United Kingdom, (Collini et al., 2018) demonstrated a selective deficit in delayed, apoptosis-associated pneumococcal killing in AMs from ART-treated HIV-infected individuals. The authors further demonstrated that the HIV glycoprotein gp120 upregulated the myeloid leukaemia cell (Mcl) 1 thus preventing apoptosis induction of infected AMs and down regulating caspase activation despite baseline increased ROS generation (Collini et al., 2018). This observation has been observed in other disease states like chronic obstructive pulmonary disease (COPD) (Bewley et al., 2017; Preston et al., 2019).

1.9. Intracellular replication of *S. pneumoniae* within phagocytes

The success of *S. pneumoniae* to establish infection within the host is partly attributable to its ability to evade the immune responses, which includes resisting phagocyte intracellular killing. It was not only until recently that *S. pneumoniae* was reported to survive and replicate intracellular within the splenic CD169 macrophages, after establishing an “eclipse phase” early during infection (Ercoli et al., 2018). However, when anti-CD169 antibody was used to block the CD169 receptor, this resulted in diminished bacterial uptake into these cells and protected the murine against subsequent invasive disease. In other studies, it was reported to that the MRC-1 is able to influence pneumococcal endocytosis and escape of intracellular bacterial killing in Schwann and olfactory cells (Macedo-Ramos et al., 2011, 2014). Additionally, it has been shown that the MRC-1 on AMs and DCs binds pneumolysin, enabling pneumococci to invade immune proficient cells (Subramanian et al., 2019). This interaction between pneumolysin and MRC-1, inhibits the fusion of phagosome containing pneumococci with lysosomes thereby promoting its survival within the host (Subramanian et al., 2019).

Other “extracellular” gram-positive bacteria such as *S. aureus* have been also shown to have developed mechanisms of escaping intracellular bacterial killing, surviving within primary macrophages until bacterial proliferation and lysis of infected macrophages occur (Flannagan et al., 2016; Hamza and Li, 2014; Kubica et al., 2008). Bacterial intracellular replication is thought to precede the onset of host cell death and enabling their escape to the extracellular compartment where they can disseminate and propagate further(Flannagan et al., 2016).

1.10. Models for studying anti-pneumococcal immunity

For several years, scientists have been developing different models to understand the mechanisms of natural carriage, establishment of local or disseminated disease as no single model can help fully explain the various processes involved. Models developed include those of primary cells (nasal scrapes, nasal biopsies, bronchoalveolar lavage, peripheral cells), cell models line (epithelial cell, airway), animal models (mice, rats, rabbits, guinea pigs and rodents), as well as the Controlled Human Infection model (CHIM). Below I will discuss the models and their applicability in understanding the mechanism of pathogenesis of the disease, testing novel drugs or vaccines and characterising host or bacterial factors (Chiavolini et al., 2008; Ramos-Sevillano et al., 2019).

1.10.1. Primary cells and cell line models

When studying host responses to pathogens there is a tendency to focus on the cell types that comprise the biological barriers to microbes to uncover the host signalling events and virulence traits that are involved in the initial phase of carriage and disease (Duell et al., 2011). Primary cells and cell lines are the commonly used models that can define the broad range of mechanisms that underlie strategies of microbial virulence such as host receptor ligand binding and can afford critical insight into what drives host defence strategies (Duell et al., 2011). Furthermore, classical cell culture models are useful for the mechanistic and functional analysis of the one-to-one interaction between cell and microbe (Hocke et al., 2017).

During *ex vivo* cell culture, one can utilise single lineage cells (primary epithelia cells, human pharyngeal carcinoma Detroit 562 epithelial cells, human bronchial carcinoma Calu3 epithelial cells and human alveolar epithelial carcinoma A549 epithelial cells) or multiple lineage (adenoidal mononuclear cells tissue, peripheral blood mononuclear cells and bronchoalveolar lavage cells) to study pneumococcal responses in the airway and peripheral blood (Gordon et al., 2001; Jambo et al., 2011; Pido-Lopez et al., 2011; Weight et al., 2019; Zhang et al., 2011). The benefit of coculture compared to monoculture, is the capacity to better reflect the *in vivo* biology of cytokines, growth factors, and transcriptional regulators activated or repressed in response to pneumococcal carriage or disease (Duell et al., 2011). However, in the context of cytokine production and T-cell proliferation, coculturing yields results closer to the *in vivo* system than cell lines (Kasurinen et al., 2018; Tikhomirova et al., 2018).

Moreover, when comparing primary cells and cell lines; cell lines are robust, easily propagated, and may be preferable for long-term assays and where collection of primary cells is technically demanding or time consuming such as collection and processing of bronchoalveolar lavage airway samples (Duell et al., 2011; Gordon et al., 2001; Jambo et al., 2014a; Weight et al., 2019). Cell lines may also be used to understand biofilm formation, carriage and *S. pneumoniae* epithelia evasion (Marks et al., 2012; Weight et al., 2019). However, it is important to note that though *in vitro* systems are useful, they neither reflect tissue diversity at a cellular level, nor recapitulate the typical cell-cell interaction in the natural matrix (for example alveolar epithelial cell interaction with alveolar macrophage and the capillary endothelium) of the three-dimensional architecture (Hocke et al., 2017). Hence, other models like the animal models and controlled human infection models allow for the sophisticated analysis of host-pathogen interaction which is absent in primary cells or cell lines.

1.10.2. Animal models

Numerous animal models of carriage and disease caused by *S. pneumoniae* are available for clarifying mechanisms of disease pathogenesis, testing novel drugs and vaccine candidates, and characterizing the role of bacterial and host factors (Chia et al., 2005; Chiavolini et al., 2008). Animal models for pneumococcus infection and disease available include; rodents, rabbits, rats, with inbred mice strains (including BALB/c, C57BL/6, CD1, and MF1) being the most utilised (Borsa et al., 2019; Chiavolini et al., 2008; Trzciński et al., 2015; Yershov, 2005). The specificities and their importance in studying carriage and disease have been summarised in a review (Chiavolini et al., 2008). Interestingly, new porcine spleen perfusion model for bacterial sepsis which closely resembles the human spleen have gained traction, as they have comparable microanatomy and subpopulations of splenic macrophages (Chung et al., 2019; Ercoli et al., 2018). However, for many results obtained in these animal models, their translation to humans remains unclear (Mak et al., 2014). Moreover, there is a large discrepancy in the outcomes of *S. pneumoniae* infection in experimental animal models and this primarily depends on the pneumococcal strain used or its genetic background (Chiavolini et al., 2008). For example, it is widely known that certain human disease-causing isolates are poorly virulent *in vivo*, whereas other pneumococcal strains that are highly virulent in mice have less relevance for human disease (Briles et al., 1992). Despite the limitations associated with animal models, their use in clarifying the pathogenic mechanisms of disease remains an unquestionable (Chiavolini et al., 2008).

1.10.3. Controlled Human Infection Models (CHIMs)

Human challenge studies have been in existence for nearly 300 years, as a deliberate exposure of participants with an infectious agent and developing to highly controlled, ethical and accountable research studies (Darton et al., 2015). CHIMs are used in research to understand disease processes, for assessing the efficacy of vaccines or novel therapeutics developed against specific infections (Darton et al., 2015; Harro et al., 2011; Stanisic et al., 2017). Several terms have

been coined and are used to describe the challenge process. These include; experimental, artificial, or induced, in addition to controlled human infection or deliberate exposure (Darton et al., 2015). CHIMs have gained a lot of interest over the years and are being used to study the following pathogens; *Salmonella enterica* serovar Typhi, *Vibrio cholerae*, *Plasmodium falciparum*, enterotoxigenic *E. coli* (ETEC), influenza, *Giardia lamblia*, *Shigella flexneri*, *Neisseria gonorrhoeae* and *S. pneumoniae* (Cohen et al., 1999, 2002; Harro et al., 2011; Khamesipour et al., 2005; Killingley et al., 2012; McCool et al., 2002; Snyder et al., 1963; Spring et al., 2014; Stanisic et al., 2017; Wright et al., 2012).

The Experimental Human Pneumococcal Challenge model was first demonstrated in American adults in early 2000 (McCool et al., 2002, 2003) before being established in Liverpool, United Kingdom (2011) (Ferreira et al., 2011; Wright et al., 2012) and Malawi (2018) (Gordon et al., 2017; Morton et al., 2020). Current EHPC models induces carriage rather than disease and provides the opportunity to study mucosal immunity from mucosal samples such as (nasal scrapes, nasal wash, saliva and bronchoalveolar lavage fluid) and peripheral blood samples in a setting where the onset and termination of carriage events are known (Ferreira et al., 2011). EHPC can be used to study healthy individuals as well as at risk population such as the elderly, asthmatics and smokers. Pneumococcal serotypes being tested include type 6B, 23F (McCool et al., 2002; Mitsi et al., 2019; Wright et al., 2012) and recently type 3. Table 1.4. compares the differences and similarities between the EHPC and animal studies.

Table 1.4. Similarities and differences between EHPC and animal model.

Feature under study	CHIMs - EHPC	Animal model
Studying natural immunity	It can be used to study new preventative strategies that could aid in defining the mechanisms of innate and adaptive immunity to *S. pneumoniae*, which could then be targeted to enhance immunity against infection in high risk groups (Darton et al., 2015; McCool et al., 2002; Ramos-Sevillano et al., 2019).	Animal models are an essential tool for the study of mechanisms of pneumococcal disease pathogenesis, transmission dynamics, testing novel drugs and vaccine candidates, and characterizing the role of bacterial and host factors (Chiavolini et al., 2008; Hergott et al., 2015; Ramos-Sevillano et al., 2019; Reglinski et al., 2018; van Rossum et al., 2005; Zafar et al., 2017).
Number of serotypes involved	Normally used to study one serotype at a time but multiple serotypes can be studied.	This model can be used to study one or more serotypes at once.
Carriage events	Perfect model for studying short and long colonisation events and how pneumococcus interacts with other serotypes or nasal microbiome	The model can be used for studying short colonisation events and how pneumococcus interacts with other serotypes or nasal microbiome

Site of study	Blood, lung, nose	Blood, lung, nose, brain, spleen, liver
Applicability	Very applicable as one can study events, mechanisms following colonisation.	Can study mechanisms but is of more limited utility due to potential differences in immune function between species (Ramos-Sevillano et al., 2019).
Reproducibility of immune response associated with prior exposure	Immune response associated with prior exposure is reproducible (McCool et al., 2002, 2003).	Prior exposure to *S. pneumoniae* over many years is difficult to replicate in mouse models (Ramos-Sevillano et al., 2019)
Proof of concept	Provides potential correlations between effectors and host protection against disease. It provides a platform to study pathogen transmission dynamics and how evaluation of interventions transmission (Killingley et al., 2012).	Provides direct proof between a specific immune effector and protection against disease (Hergott et al., 2015; Reglinski et al., 2018; Siegel et al., 2014).
Infection endpoint	The endpoint is carriage and is detected through classical microbiology methods or *lyt*A PCR (Gritzfeld et al., 2011, 2014;	The endpoint depends on the type of study and could be diagnosis of infection (measured by a diagnostic test), carriage or infection by specific strain types, development of infection signs and

	McCool et al., 2002; Mitsi et al., 2019; Wright et al., 2012).	symptoms, or assessment of infection severity (Chiavolini et al., 2008; Darton et al., 2015; Ercoli et al., 2018; Neill et al., 2014)
Dose and route	Doses ranges between 5,000 – 160,000 colony forming units (CFU)/100μl are instilled into each nostril of a participant (Ferreira et al., 2013b; Gritzfeld et al., 2014; Jochems et al., 2018; McCool et al., 2002; Mitsi et al., 2019; Wright et al., 2012).	Doses ranges between 10^{5-7} CFU/10μl (Ercoli et al., 2018; Hergott et al., 2015; Hyams et al., 2013b; Trzciński et al., 2015; Zafar et al., 2017). The route (intramuscular, intravenous, intranasal, intraperitoneal, intratracheal, intracranial) depends on the outcome of interests; carriage, pneumonia, sepsis, meningitis, otitis media (Chiavolini et al., 2008)
Challenge strain	Common strains used for the challenge include serotype 6B (BHN418), 23F(P833) and type 3 (Wright et al., 2012)	Several strains have been used in animal models depending on the outcome of interest and these including serotypes 1, 2 (D39), 3, 4 (TIGR), 6B, 14, 19A, 19F, 23F, 35B (Ercoli et al., 2018; Neill et al., 2014; Trzciński et al., 2015). Pneumococci mutants have also been used as challenge strains.

1.11. Prevention of disease

With the rise and wide spread of antibiotic resistance, pneumococcal infections augmented with deficits of active antibiotics, vaccination against *S. pneumoniae* has become the primary option for controlling this pathogen and preventing disease (Gupalova et al., 2019). The current vaccination against *S. pneumoniae* is safe, effective and is being implemented in many developing countries through the GAVI, the Vaccine Alliance initiative (Kalata et al., 2019). Despite being efficacious, the vaccines are relatively expensive to produce and require modification due to the immunological changes of the epidemic strains (Gupalova et al., 2019). Furthermore, all vaccines in use are injectables and so do not provide optimal mucosal protection at the port of pathogen entry (nasopharynx) and the lower airway (lungs) (Gupalova et al., 2019). In this section, I will discuss the vaccines and antimicrobials in use and those in the pipeline for controlling the pneumococcus burden.

1.11.1. Vaccines

There are two main types of vaccines in use and these are the pneumococcal conjugate vaccine (PCV) and pneumococcal polysaccharide vaccine (PPSV23). These vaccines comprise of a polysaccharide or polysaccharide covalently attached to protein (glycoconjugate vaccine) and induce a polysaccharide-specific IgG which confers protection against infection with extracellular bacteria by mediating complement-dependent- opsonophagocytosis (Mitchell et al., 2014; Pollard et al., 2009). These vaccines have saved millions of lives but also have some limitations related to the heterogeneity of capsular polysaccharides of the *S. pneumoniae* strains and short T-independent immunological memory.

1.11.1.1. Polysaccharide vaccines

The PNEUMOVAX23® (PPSV23) is a 23-valent pneumococcal polysaccharide vaccine for 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), developed in 1983 (Pollard et al., 2009).

The vaccine is in widespread use in >75 countries and is approved for use in the elderly (>65 years) and persons aged ≥2 years who are at risk of pneumococcal disease (Pollard et al., 2009; Wang et al., 2018). In children below 2-years of age, PPSV23 has been demonstrated to elicit poor protective antibody responses (Wang et al., 2018).

Capsular polysaccharides are known to be T-cell-independent antigens, and generally stimulate short-lived B-cell responses by cross-linking the B-cell receptors, which drives the differentiation of B cells to plasma cells to produce antibodies (McCool et al., 1999; Mitchell et al., 2014; Obukhanych and Nussenzweig, 2006; Pollard et al., 2009). The production rate of antibodies against 23-valent serotype vaccine is not constant though more than 80% of adults demonstrate the presence of antibodies 2-3 weeks post vaccination (Chang and Woo, 2016). Immune responses to polysaccharide antigens is characterized by high levels of IgM and IgG antibodies primarily of the IgG3 subclass in mice and IgG2 in humans (McCool et al., 1999). However, the levels of antibodies that correlate with protection against pneumococcal disease remain to be clarified.

It has been shown that during vaccination with polysaccharide vaccines, new memory B cells are not produced; instead, terminal differentiation of memory B cells to plasma cells occurs, leading to the depletion of memory B-cell pool, and causing hypo-responsiveness to subsequent vaccine doses (Kelly et al., 2006). Memory B cells have been shown to increase in frequency with age and are inversely proportional to the prevalence of *S. pneumoniae* infections, whereas the elderly have lower numbers of memory B cells and this may in part contribute to their increased susceptibility to pneumococcal disease (Jha and Janoff, 2019; Khaskhely et al., 2012; Kruetzmann et al., 2003). Furthermore, young children under 2-years of age are capable of producing antibodies of the IgG1 subclass, but have limited ability to produce the IgG2 subclass and this potentially predisposes them to pneumococcal pneumonia, bacteraemia, and meningitis due to their inability to mount responses to PPSV23 (Jha and Janoff, 2019). This renders PPSV23 ineffective among the those aged <2-years. High PPSV23 antibody titres

generated have been shown to be protective against IPD for a period of 3-5 years post vaccination amongst healthy adults, but the protective immunity wanes faster in individuals with the underlying diseases (Andrews et al., 2012; Chang and Woo, 2016; Remschmidt et al., 2016). Revaccination is recommended in those aged 65 years and above, at least 5 years after the last vaccination (Kawakami et al., 2016, 2018). Amongst the HIV infected individuals on ART, significantly higher antibody levels against pneumococcal vaccine antigens are observed; however, memory B cells still diminish over time with the exception of activated memory and isotype-switched memory B cells (Papadatou et al., 2019; Tsachouridou et al., 2015). A study conducted in Uganda that followed a cohort of HIV-infected individuals vaccinated with PPSV23 and placebo demonstrated that the risk of pneumonia was actually higher in PPSV23 recipients than in controls, and this difference persisted for at least six years post vaccination with rate of survival favouring vaccination particularly those with a CD4 cell counts of 200 – 500µl/ml cells at enrolment (French et al., 2000; Watera et al., 2004).

1.11.1.2. Pneumococcal Conjugate Vaccines

The frequency of invasive pneumococcal disease (IPD) has dramatically decreased throughout the world as a result of the introduction of pneumococcal conjugate vaccine (PCV) (van Aalst et al., 2018; Bar-Zeev et al., 2015; Harboe et al., 2014; Iroh Tam et al., 2017; Riaz et al., 2018; Troeger et al., 2017). The PCVs are licenced for immunisation of children aged between 6-weeks and 5 years and also approved for use in adults >50 years. Importantly, the beneficial effects have been observed amongst the vaccinated and unvaccinated age groups but not in the elderly as observed in other countries (Galanis et al., 2016; Swarthout et al., 2020). PCVs are immunogenic in the infants and became available in many developed countries in 2000, initially with 7-valent Prevnar (4, 6B, 9V, 14, 18C, 19F, and 23F), subsequently being switched to 10-valent Synflorix® (1, 4, 5, 6B, 7F, 9V, 14, 18C,19F, and 23F) or 13-valent Prevnar13® (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F) (Cohen et al., 2017b; Swarthout et al., 2020). Despite the beneficial effects of the PCVs, some countries have started seeing a dramatic rise

in the prevalence of the non-vaccine serotype carriage and disease (Kwambana-Adams et al., 2017; Ladhani et al., 2018; Obolski et al., 2018; Swarthout et al., 2020). PCVs have been unsuccessful in reducing carriage and disease burden associated with certain vaccine serotypes such as type 3, 19F and 23F (Azarian et al., 2018; Iroh Tam et al., 2017; Swarthout et al., 2020). Also, the elimination of vaccine serotypes amongst healthy carriers might create profound changes in the global pneumococcal population structure within a community, as different strains likely coevolve as a result of reciprocal adaptation and counter-adaptation between interacting strains (Galanis et al., 2016).

Conjugation of the capsular polysaccharides to protein carriers was first reported in the 1920s and 1930 and has been shown to require a constant increase in the number of polysaccharides included in the vaccine due to the rise of non-vaccine serotypes within the population (Croucher et al., 2011; Gladstone et al., 2012; McCool et al., 1999). All the PCV13 capsular polysaccharides serotypes are conjugated to a non-toxic *Corynebacterium diphtheria* CRM_{197} protein, whereas the PCV10 uses protein D (derived from non-typable *H. influenzae*) as the carrier for eight of the serotypes, one serotype (type 18C) is conjugated to tetanus toxoid and another (type 19F) is conjugated to diphtheria toxoid protein (Cohen et al., 2017b). The PCV7 has since been phased out and most countries are using the PCV10 and PCV13 in their infant immunisation schedules.

Although the immunogenicity of pneumococcal vaccines is increased by protein conjugation of pneumococcal polysaccharides, thus leading to T-cell recruitment, strategies for boosting IgG antibody production and the generation of memory B cells are still needed (Abudulai et al., 2019; Snapper, 2016). When vaccinated with the PCVs, infants are able to generate robust IgG1 subclass responses to the pneumococcal polysaccharide capsule , which provides more effective protection against IPD (Black, 2011; Jha and Janoff, 2019). In immunocompromised children, circulating numbers of $CD27^+$ memory B cells, rather than $CD4^+$ T cells have been shown to predict the effective PCV responses (Hoshina et al., 2016). A previous study demonstrated that naïve infants aged <12 months had no pre-existing

switched memory B-cells at baseline, suggesting that the amount of pneumococcal carriage experienced during the first year of life may not be sufficient to maintain detectable levels of switched memory B-cells against pneumococcus serotype-specific rates of colonisation in this age group (Clutterbuck et al., 2008). It was further demonstrated by the same authors, that at least 2 doses of PCVs are required in infants to generate memory B-cell frequencies and antibody class switching comparable to those seen in adults (Clutterbuck et al., 2008). Contrary to infants, vaccine-naïve adults demonstrate pre-existing pneumococcal serotype-specific memory B-cells prior to vaccination with PCVs (Papadatou et al., 2019). Another study, demonstrated that HIV infected individuals vaccinated with PCV13/PPSV23 show a rise in polysaccharide antibodies from baseline but a low baseline $CD4^+$ T-cell count had significant negative effects on the magnitude of the change in antibody kinetics (Farmaki et al., 2018). Even though, PPSV23 receipt 12 months after PCV13 receipt improved PCV13 immunogenicity, a reduction in IgM^+ memory B cells was also observed after PPV23 demonstrating that PPVS23 may have a depleting effect on PCV13-associated immunological memory (Farmaki et al., 2018). Data from a randomised trial showed that despite PCV13 being more effective than the PPV23 in the HIV infected, their IgG antibody responses remain lower than those in healthy individuals (Abudulai et al., 2019; Crum-Cianflone et al., 2010; Lee et al., 2014). Another earlier study conducted amongst the elderly population (50 – 70-year olds), demonstrated that PCV7 induced significant increases in serotype-specific memory B-cell populations in peripheral blood whilst immunization with PPV23 resulted in a decrease in memory B-cell frequency (Clutterbuck et al., 2012). Table 1.5. summarises the differences of the licenced capsular polysaccharide vaccines available on the market.

Table 1.5. Differences in current licenced Pneumococcal vaccines

	PPSV23	PCV10	PCV13
Vaccine type	Capsular polysaccharide	Glycoconjugate capsular polysaccharide	Glycoconjugate capsular polysaccharide
Valency	23	10	13
Serotypes contained	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F	1, 4, 5, 6B, 7F, 9V, 14, 18C,19F,23F	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F
Carrier protein	None	Protein D, Tetanus toxoid, Diphtheria toxoid	*C. diphtheria* CRM_{197} protein
Immunity induced	Antibody and B-cell immunity.	Antibody, B-cell and T-cell. In immunocompromised infants, high levels of circulating $CD27^+$ memory B cells are protective.	Antibody, B-cell and T-cell. In immunocompromised infants, high levels of circulating $CD27^+$ memory B cells are protective
T-cell memory	No immunological memory established	Immunological memory established.	Immunological memory established
Target population	The elderly and ≥2 years at risk	Healthy infants	Healthy infants, individuals at risk and the HIV-infected
Manufacturer, Country	Merck, USA	GlaxoSmithKline, UK	Pfizer, USA

1.11.1.3. Next generation of pneumococcal vaccines

Several studies in Europe, United Kingdom, Malawi, Taiwan and the United States of America have shown that, the implementation (vaccine schedules or vaccine coverage) of PCVs has resulted in carriage and IPD serotype redistribution and this may potentially affect the evolution of pneumococcal epidemiology (Galanis et al., 2016; Kwambana-Adams et al., 2017; Ladhani et al., 2018; Lee and Kuo, 2019; Levy et al., 2019; Obolski et al., 2018; Ouldali et al., 2018; Swarthout et al., 2020). Moreover, *S. pneumoniae* has a higher propensity to acquire new genetic materials via natural transformation and recombination thus, enabling it to evolve with its host environment (Donati et al., 2010; Masomian et al., 2020). PCVs and PPSV23 only covering a limited range serotypes, are unable to protect against non-vaccine serotypes or unencapsulated *S. pneumoniae* and this has led to a rapid increase in antibiotic-resistant non-vaccine serotypes (Lee and Kuo, 2019; Masomian et al., 2020). Following vaccine introduction, pneumococcal capsular switching was reported to occur more frequently (Chaguza et al., 2017; Chang et al., 2015).

Despite the reduction in IPD burden after the introduction of PCVs and PPSV23; the cost of manufacturing PCVs remains high and the vaccines have insufficient serotype coverage for new emerging clinical serotypes (Croucher et al., 2011; Hsu et al., 2009; Xu et al., 2015). There is therefore an urgent need to develop novel pneumococcal vaccines with broad serotype coverage that induce mucosal and systemic immunity, and hinder primary intranasal carriage and invasive disease (Masomian et al., 2020). Below, I summarise the different types of vaccine candidates that are in the pipeline, developmental and clinical trial phase, focusing on newer PCVs, protein and whole cell vaccines (Table 1.6.).

Table 1.6. Summary of pneumococcal vaccine candidates in the pipeline and developmental stages

Antigen /Vaccine	Carrier	Serotypes	Route	Clinical trial phase	Immunogenicity	Correlates of protection	Protection	Reference
PCV15	*C. diphtheria* CRM197 protein	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, 33F	Intramuscular	2 Humans	Mucosal Systemic	Ab in sera	Carriage Sepsis Pneumonia	(Stacey et al., 2019)
PCV20	*C. diphtheria* CRM197 protein	1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C,	Intramuscular	1 Humans	Mucosal Systemic	Ab in sera	Carriage Sepsis Pneumonia	(Thompson et al., 2019)

		19F, 19A, 22F, 23F, 33F						
PspA	DNA vector	6B	Intraperitoneal	Mice	Systemic	Ab in sera	Sepsis	(Ferreira et al., 2006)
PSPF	Live *E. faecium*	-	Intranasal	Mice	Mucosal	Ab in sera, Ab in nasal washes	Pneumonia	(Gupalova et al., 2019)
PspA	Plasmid	-	Intramuscular	Mice	Systemic	Ab sera	Sepsis	(McDaniel et al., 1997)
PspA3	DNA vector	6B	Intraperitoneal	Mice	Systemic	Ab in sera	Sepsis	(Ferreira et al., 2008)
Lipoproteins MetQ, PnrA, PsaA, and DacB	Cholera toxin subunit B	-	Intranasal	Mice	Mucosal Systemic	Carriage load Ab in sera, Cytokines in NALT	Carriage Sepsis	(Voß et al., 2018)

γ-radiated whole bacteria	-	2 (D39)	Intranasal	Mice	Mucosal Systemic	Carriage load nasal wash and nasopharyngeal tissue, Ab in sera, cytokines – splenocytes	Carriage Pneumonia Sepsis	(Babb et al., 2016)

NALT - Nasopharyngeal-Associated Lymphoid Tissue, Ab – antibody

1.11.2. Preventative antimicrobials

Present vaccination strategies pose challenges related to increase in non-vaccine serotypes, selection and spread of antimicrobial resistant strains such as serotype 14, 19A and 15A (Cho et al., 2014; Kwambana-Adams et al., 2017; Ladhani et al., 2018; Yahiaoui et al., 2018; Zhao et al., 2017). On the contrary, before Algeria introduced PCVs into their child immunisations programme, the country had already started experiencing antimicrobial resistance within vaccine serotypes such as 6B, 14, 19A and 19F associated with pneumococcal disease (Ramdani-Bouguessa et al., 2015). Therefore, this rise in antimicrobial resistance within *S. pneumoniae* pre- and post-vaccination is a topical issue that remains unresolved. Pneumococcal diseases are treated with b-lactams, fluoroquinolones, macrolides, and vancomycin (Mandell et al., 2007; Metlay et al., 2019). As vaccines are not 100% effective in the prevention of pneumococcal infections, children (≤ 5 years) and the elderly (>65 years) with spleen dysfunction (asplenia, hyposplenia) are recommended to take oral penicillin as prophylaxis (Bonanni et al., 2017; Chang and Woo, 2016; Salvadori and Price, 2014). Amongst individuals with anaphylactic-type reaction to penicillin, erythromycin is a recommended alternative (Salvadori and Price, 2014). However, in some settings chemoprophylaxis may not be useful because of the increased penicillin and erythromycin resistant strains (Chang and Woo, 2016).

1.12. The problem

HIV infected adults, even those on ART, are at an increased risk of pneumococcal pneumonia and have high pneumococcal carriage rates than their HIV-uninfected counterparts (Glennie et al., 2013; Sepako et al., 2014; Swarthout et al., 2020; Wolter et al., 2014). To date, the immunological basis for the increased propensity to pneumococcal pneumonia in HIV-infected adults is still unclear. Previous studies failed to identify evidence of impaired pneumococcal-specific airway $CD4^+$ T-cells, reduced anti-capsular polysaccharide or protein IgG antibodies in the lung or poor AM binding and internalisation of pneumococcus (Collins et al., 2013; Eagan et al., 2007; Gordon et al., 2000, 2001, 2013; Jambo et al., 2011; Peno et al., 2018) in adults from a high disease burden low income setting. In murine models of pulmonary infection, AMs are known to clear bacteria up to a defined threshold without overt features of pneumonia (Dockrell et al., 2003a). But their failure in controlling these subclinical infections has been shown to result in the recruitment of inflammatory cells which give rise to inflammation and if not controlled leads to pneumonia (Aberdein et al., 2013; Dockrell et al., 2003a, 2012; Duan et al., 2012). **I, therefore, hypothesise that HIV infection is associated with impaired airway alveolar phagocyte killing function, leading to survival and propagation of pneumococci (see Figure 1.11)**.

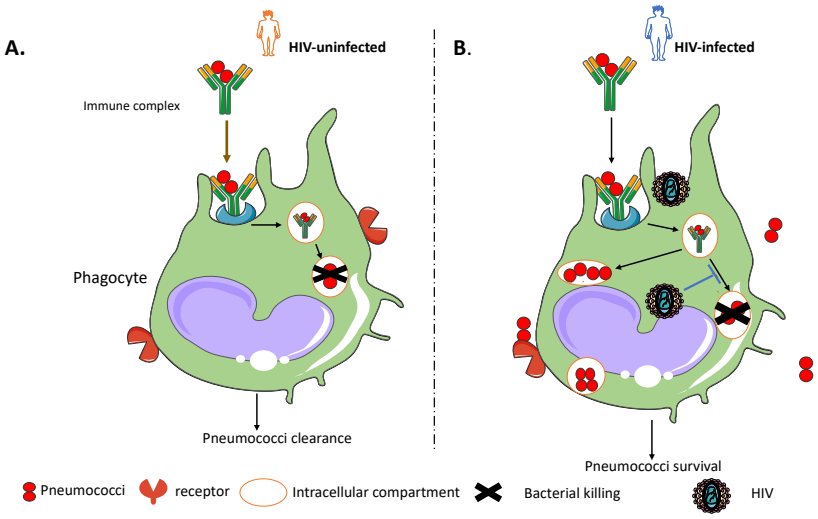


***Figure 1.11*. The model summarises the hypothesis for the thesis. A.** Alveolar phagocytes from HIV-uninfected individuals internalise opsonised pneumococci through their Fc portion resulting in pneumococcal killing and clearance from the intracellular and extracellular compartments. **B.** Alveolar phagocytes from HIV-infected individuals internalise opsonised pneumococci through their Fc portion but HIV infection impairs their ability to kill internalised pneumococci from the intracellular compartments, leading to its survival. Abbreviations: AMs – alveolar macrophage, HIV – human immunodeficiency virus. This figure contains some artwork produced by Servier Medical Art (http://smart.servier.com), under creative commons license 3.0.

1.13. Aims and Objectives

1.13.1. Overall aim

To investigate airway phagocyte immune responses against *S. pneumoniae* in HIV-infected adults compared to asymptomatic healthy controls.

1.13.2. Specific objectives

a. To determine airway phagocyte pneumococcal binding/internalisation capacity in HIV-infected adults compared to asymptomatic healthy controls.
b. To determine alveolar macrophage intracellular killing capacity of *S. pneumoniae* in HIV-infected adults compared to asymptomatic healthy controls.
c. To determine airway cell control of *S. pneumoniae* extracellular outgrowth *ex vivo* in HIV-infected adults compared to asymptomatic healthy controls.

CHAPTER 2

2.0. Materials and Methods

This chapter provides the clinical and laboratory experimental methodologies used to generate the data which are presented in later chapters.

2.1. Study design and population

Chapters 4, 5 and 6 summarise results from a comparative cross-sectional study enrolling asymptomatic HIV-uninfected and asymptomatic HIV-infected adults on antiretroviral therapy (ART) for <3-months and ART for >3-years.

2.2. Study site

The study discussed in this thesis was conducted at Malawi-Liverpool Wellcome Trust Clinical Research Programme (MLW) and the Queen Elizabeth Central Hospital. The asymptomatic HIV-infected adults on ART were recruited from health centres around Blantyre (Gateway and Ndirande), whilst the asymptomatic HIV-uninfected were recruited the communities surrounding the health centres.

2.3. Ethical approval

The study presented was approved by the College of Medicine Research Ethics Committee (COMREC) (Blantyre, Malawi) and the Liverpool School of Tropical Medicine Research Ethics Committee (LSTM REC) (Liverpool, United Kingdom) (see Table 2.1.; Appendix 1-6). The studies were compliant with Good Clinical Practice and the principles set out in the Helsinki Declaration (World Medical Association, 2013).

Table 2.1. Ethical approval reference information for the studies

Study	Ethics body	Reference	Chapters
Characterisation of HIV-associated changes in alveolar immune environment that favour the growth of *Streptococcus pneumoniae*.	COMREC LSTM REC	P.01/18/2335 18-007	4, 5 and 6

2.4. Participant recruitment and consent

All participants volunteering to be part of the studies provided written informed consent. The process of consenting and disseminating information to the participants was done in both Chichewa (local language) and English (see Appendix 7-10). Participants were first screened according to the inclusion and exclusion criteria presented in Table 2.2. and were frequency aged matched with apparently healthy controls. Both inclusion and exclusion criteria were designed to minimise variability within the group of participants being recruited.

Participants were enrolled from the 3 clinical phenotypes described below.

1) Healthy, HIV-uninfected individuals with no clinical evidence of active respiratory disease.

2) Asymptomatic, HIV-infected individuals on ART for not more than 3-months or ART-naïve individuals (irrespective of CD4 count) with no clinical evidence of active respiratory disease. For the purpose of this thesis, HIV-infected individuals on ART<3-months will be termed as HIV-infected on short-term ART and this may be used interchangeably.

3) Asymptomatic, HIV-infected individuals on ART for at least 3 years, with no clinical evidence of active respiratory disease or progression of HIV infection and CD4+ T cell counts of >350 cells/mm^3. Plasma HIV viral load results were not be available at recruitment for most participants but based on data from the HIV clinic at QECH, approximately 90% of adults on ART for > 3years have undetectable plasma HIV viremia. For the purpose of this thesis, HIV-infected individuals on ART > 3-years will be termed as HIV-infected on long-term ART and this may be used interchangeably.

Inclusion criteria

- Male or female participants aged ≥ 18 years of age.
- HIV infected or HIV-uninfected.
- Asymptomatic to respiratory infection.

- HIV ART naïve, irrespective of CD4+ T-cell count.
- HIV-infected individuals on ART for not more than 3-months with a CD4+ T cell count ≥350 cells/mm^3.
- HIV-infected on ART for more than 3-years with a CD4+ T cell count ≥350 cells/mm^3.

Exclusion criteria

- Refusing HIV testing.
- Showing symptoms of a respiratory tract infection.
- Having had a lower respiratory infection in the last 6-months.
- An individual with anaemia (Hb <8g/dl).
- An individual suspected or known to be pregnant.
- A known smoker or previous smoker within past 1 year.
- An individual with suspected or known to have chronic obstructive pulmonary disease (COPD).
- Having the following contraindications; unstable cervical spine, unresponsive hypoxia, unstable angina, bleeding diathesis, malignant cardiac arrhythmia.

2.5. Clinical procedures

This section provides the sequential details of all the clinical procedures involved in collecting samples from our participants.

2.5.1. Clinical materials

All the details pertaining the clinical materials I used in collecting samples from the participants are all listed in Table 2.3. together with manufacturer and product code information.

Table 2.3. General clinical consumables

Name	Manufacturer	Product code
FLOQSwabs™	Copan	516CS01
Rapid influenza A+B test kits	Coris BioConcept	K-1512
Venous blood sample tubes, Sodium Heparin, plastic	Becton Dickinson	KFK279
Venous blood sample tube, K_2EDTA, plastic	Becton Dickinson	KFK042
Lidocaine hydrochloride	Hameln Pharmaceutical	PL01502/0021R
Universal transport media	Copan	330C
50ml polypropylene conical tubes	Corning	352070
50ml Syringe	BD plasticpack	300866
IV cannula	Biomatrix Healthcare	IT0101-20
Neovac IV cannula with wings and injection port	Neovac	
20ml Disposable syringe		

Abbreviations: IV – intravenous, EDTA – dipotassium ethylenediaminetetraacetic acid

2.5.2. Nasopharyngeal sample collection and influenza screening

This procedure is used to detect for the presence of influenza viruses (Flu A and Flu B) in nasopharyngeal swabs collected from study participants. The participant had their head tilted back at an angle of 70° and a FLOQSwabs™ swab was inserted into the nostril (the swab had to reach a depth equal to distance from nostril to outer ear's opening). Once the swab reached the nasopharynx, the swab was left in place for 2-3 seconds and then slowly removed whilst rotating it (this was repeated in the other nostril). One swab was collected from each nostril. The first swab to be collected was cut into 2, with one half used to test for the presence of influenza virus immediately whilst the other half was inserted into a tube containing skimmed milk tryptone glucose and glycerine media (STGG) (see

figure 2.5.2). The second full swab collected was put into universal transport media, chilled on ice and transported to the laboratory for long-term storage.

For influenza screening, a rapid influenza immunochromatographic test cassette was first labelled with the participant identifier. This was followed by placing the swab into a small semi-rigid tube (that comes with the kit) and 18 drops of dilution buffer added, stirred and swab pressed firmly against the sides of the tube before being removed and discarded into a biohazard container. The sample was mixed thoroughly, and a dropper inserted into the semi-rigid tube, the tube was inverted and slowly 3 drops of the diluted sample were added into cassette well. Results of the test were read between 15-20 minutes and recorded to the participant's electronic case report form. The collection and screening of influenza illustrated in Figure 2.1.

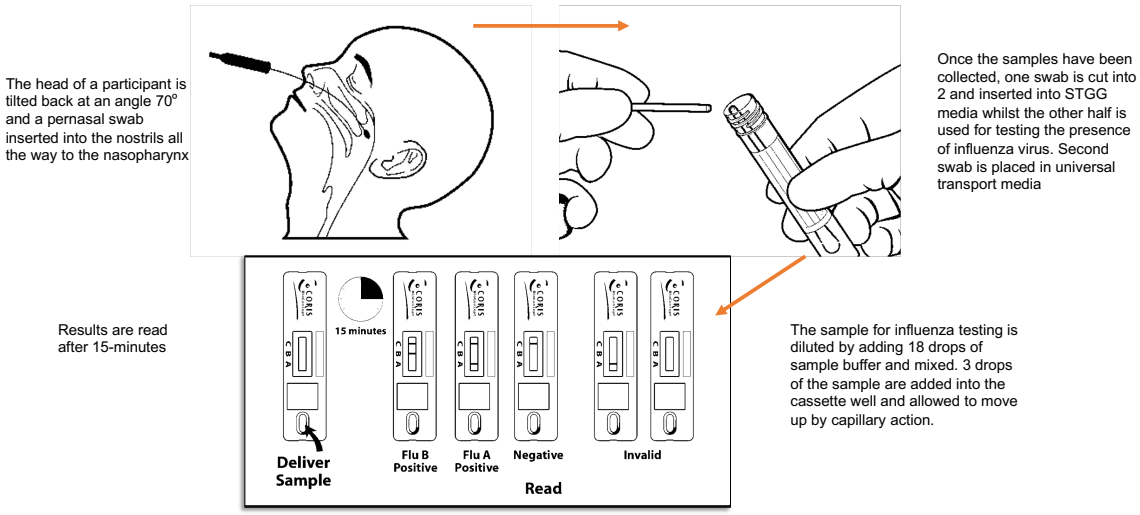


Figure 2.1. Pernasal swab collection and influenza testing procedure.

2.5.3. Peripheral blood collection

Participants were prepared for venous blood collection by swabbing the skin around the antecubital fossa in circular motion with an alcohol wipe. Blood was collected using a vacutainer system and universal precautions were observed. Blood was collected according to samples required and the following blood samples were collected; serum (2-mls clot activated SST tubes) and plasma (2-mls K_2EDTA tubes). The labelled blood sample tubes were gently inverted 5-6 times as

to allow the anticoagulant or clot activator to mix well with the blood and placed on ice and transported in a cooler box to the MLW laboratories. Once the samples reached the diagnostic laboratory blood in clot activator was allowed to clot at room temperature before the tube being spun at 2,000*g* for 10 minutes in a refrigerated centrifuge. Serum was harvested and stored at -80°C. Peripheral $CD4^+$ counts were measured from K_2EDTA tubes upon reaching the laboratory.

2.5.4. Bronchoalveolar lavage collection

Participants were prepared for bronchoscopy by taking blood pressure measurements, heart rate, pulse oximetry and respiratory rate. Topical lignocaine spray was applied to the nasal and pharyngeal mucosa of semi-recumbent participants. A fibre-optic bronchoscope was passed down trachea and 4% lignocaine applied to the vocal cords and 2% lignocaine used in the larger airways. The bronchoscope was passed down to the level of sub-segmented bronchus of the right middle lobe to achieve a seal. Four 50ml aliquots of sterile normal saline at 37°C was instilled and lavage fluid removed by using gentle hand suction. The aspirated bronchoalveolar lavage (BAL) fluid was placed in four 50ml falcon tubes and transported to the laboratory on ice within 30-minutes of collection. All participants were reviewed before discharge for any immediate complications and returned back within 72-hours for further review and clinical examination.

2.6. Laboratory procedures

This section provides details of the laboratory procedures and experiments conducted that were used to generate data presented in the preceding chapters.

2.6.1. Laboratory consumables

All the details pertaining the laboratory materials and consumables used in processing the samples from the participants are all listed in the appendix 2. The sequence of tables below Table 2.4. to Table 2.7. present the list of laboratory consumables, manufacturer and product codes used in the study.

Table 2.4. General laboratory consumables

Name	Manufacturer	Product code
15 ml Falcon tubes	Corning	352097
50 ml Falcon tubes	Corning	352070
Pasteur pipettes	ELK laboratory products	127-P503-STR
Nunc 1.8ml Cryovials	ThermoFisher Scientific	368632
Formaldehyde solution	Sigma-Aldrich/Merck	F1635
Blood Agar Base	Oxoid	CM0055
Skimmed milk	Oxoid	LP0031
Tryptic Soy Broth	Sigma-Aldrich/Merck	-------
Glucose	Prolabo	G/0500/53
Glycerine	Fisher Scientific	--------
Yeast Extract	Sigma-Aldrich/Merck	Y1625
Sodium azide (NaN_3)	Sigma-Aldrich/Merck	S2002
Costar Stripette 10ml	Costar	4499
Costar Stripette 25ml	Costar	4489
Frosted microscope slides	------	7105

Table 2.5. Consumables for airway infections

Name	Manufacturer	Product code
Sterile treated 6 well plates	Corning	353046
Sterile treated 12 well plates	Corning	353043
Sterile treated 96 well plates (Greiner)	Corning	3799
16cm Scrappers	SARSTEDT	83.1832
Hank's Balanced Salt Solution	Sigma-Aldrich/Merck	H9394
Gentamicin	Sigma-Aldrich/Merck	1405-41-0
Saponin	Sigma-Aldrich/Merck	47036
RPMI 1640	Sigma-Aldrich/Merck	R8758
Anti-pneumococcal capsule Reference Serum	NIBSC	007SP
IVIG	NIBSC	04/140
Distilled water	Invitrogen	10977-035
Dulbecco's PBS $CaCl_2$/$MgCl_2$ (-/-)	Invitrogen	20012-019
CellTracker™ Red CMTPX	Invitrogen	C34552

Table 2.6. Consumables for tissue culture

Name	Manufacturer	Product code
Glycerol	Fisher Scientific	G/P450/08
Trypan blue 0.4%	Gibco	15250-061
Foetal Bovine Serum	Sigma-Aldrich/Merck	F9665
Amphotericin B	Gibco	15290-026
Bovine Serum albumin	Sigma-Aldrich/Merck	A9414
100µm nylon cell strainer	Corning	352360
Glasstic Slide with Grid	KOVA	87144

Table 2.7. Consumables for immunophenotyping

Name and Fluorochrome	Host species	Clone	Manufacturer	Product code
anti-human CD206 FITC	Mouse	15-2	BioLegend	321104
anti-human CD163 BV421	Mouse	GHI/61	BioLegend	333612
anti-human CD66b APC	Mouse	G10F5	BioLegend	305118
anti-human CD19 PE	Mouse	SJ25C1	BioLegend	363004

2.6.2. *S. pneumoniae* culture and quantitation

An invasive strain of *S. pneumoniae* serotype 3 (ST3) and sequence type 700 isolated from a 43-year-old male with meningitis from Blantyre, Malawi in 2017 was used in the *ex vivo* infections. The ST3 was first grown into single colonies on sheep blood agar (SBG) supplemented with 5mg/L gentamicin overnight (Figure 2.2). Ten distinct single colonies were then resuspended in 10mls of Todd Hewitt broth supplemented with 0.5% yeast (THY) extract. Hourly growth curves were constructed by measuring the optical density (OD) of the THY at 490nm using a microplate reader (EZ Read 400, Biochrom, Massachusetts, USA) and the number of colony forming units accurately quantified per ml in parallel using the modified method of Miles and Misra (Miles et al., 1938). Twenty-four serial dilutions $\log_{10}$ of the suspension were made by diluting 100µl of the suspension with 100µl of sterile phosphate buffered saline (PBS) in a 96-well microtitre plate before mixing thoroughly using a 200µl pipette. Using a 20µl graduated pipette, three 10µl drops of each dilution were then placed onto the 8 equally divided sectors on the SBG plate. The drops were allowed to dry and then incubated at 37°C in a 5% CO_2 incubator (MIDI 40, Thermo scientific™, USA) overnight for 18 hours (Figure 2.2). The number of viable colonies of ST3 were then counted and quantified using the Equation:

CFU per ml = number of colonies per sector/3 x 100 x dilution factor x titre

Both the ODs and Miles and Misra technique were used to determine the mid-log growth phase of ST3. Thereafter, the broth was centrifuged and the sedimented ST3 was aliquoted and immediately frozen at -80°C in 1ml vial of 15% THY glycerol suspension. All the work was done in a containment level 2 laboratory (CL2).

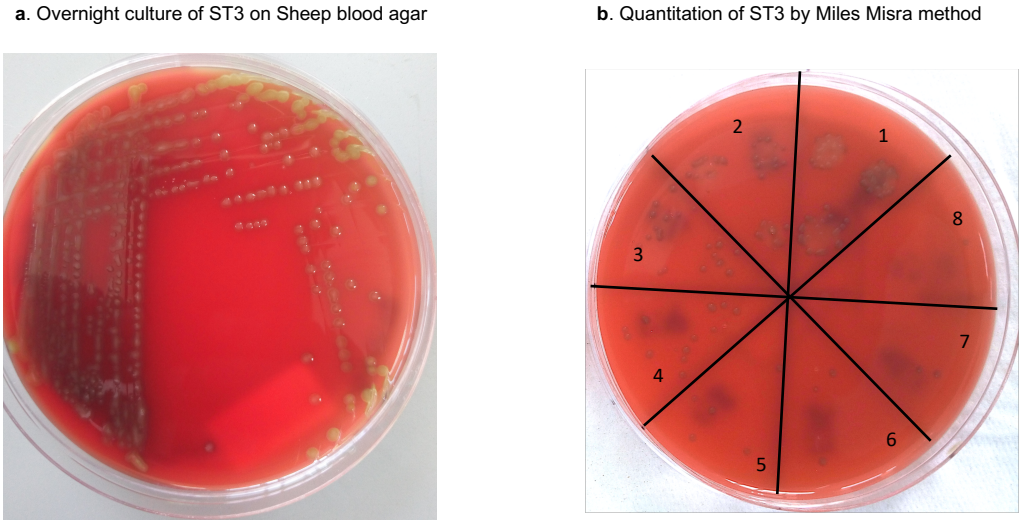


Figure 2.2. **Overnight culture plates of *S. pneumoniae* serotype 3. A.** Plate cultured to obtain pure single colonies of pneumococci-ST3 **B.** Overnight bacterial quantitation plate using the Miles Misra method.

2.6.3. Opsonisation of *S. pneumoniae* bacteria

Frozen aliquots of ST3 were thawed at room temperature, centrifuged 5 times at 4,000*g* to obtain a pure pellet. Pellets of ST3 (~1x10^8 cfu) were then opsonised with 25% of 007SP human anti-pneumococcal capsule reference serum and 10% intravenous immunoglobulin (IVIG) in a final volume of 200µl infection medium. Infection medium was prepared by supplementing RPMI with 10% heat inactivated foetal bovine serum (FBS). The ST3 suspension was then incubated for 30 minutes in a horizontal shaking incubator (ThermoFisher Scientific, USA) at a temperature of 37°C and a speed of 170rpm. The suspension was centrifuged at 4,000*g* for 5 minutes and then washed with 1% heat inactivated FBS-RPMI 1640 at 4,000*g* for 5 minutes. Miles and Misra viability bacteria count was performed in

parallel for each *ex vivo* infection conducted to confirm the multiplicity of infection (MOI).

2.6.4. Fluorescent tagging of *S. pneumoniae* suspension

To quantify the proportion of cells associated ST3 via binding and/or internalisation, frozen aliquots of ST3 were thawed at room temperature, centrifuged 5 times at 4,000*g* to obtain a pure pellet and stained with CellTracker™ Red CMTPX. The CellTracker Red CMTPX ($C_{42}H_{40}ClN_3O_4$) contains a chloromethyl group that reacts with thiol groups on the surface of *S. pneumoniae* forming cell-impermeable reaction products that are retained in living cells through several generations. The ST3 pellet (~$1x10^8$ cfu) in a 15-ml falcon tube was then fully resuspended in 250µl of 10µM CellTracker™ Red CMTPX solution and was wrapped in aluminium foil since Red CMTPX is light sensitive and incubated at 37°C for 1 hour, whilst gently shaking at 170rpm to ensure even staining of bacteria. Once stained, the tube containing the bacteria was centrifuged twice at 4,000*g* for 5 minutes to concentrate the stained ST3 pellet. The pellet was then resuspended in 2mls of Hanks balanced salt solution (HBSS) in 0.2% bovine albumin serum and washed twice at 4,000*g* for 5 minutes. After the final wash, stained ST3 was resuspended in 100µl infection media. Opsonisation of bacteria then proceeded using the previously described method (see 2.6.3). To confirm staining quality, the bacteria suspension was centrifuged at 4,000*g* for 5 minutes, fixed in 3% paraformaldehyde (Sigma-Aldrich, Saint Louis, USA) for 5 minutes in the dark. The suspension was washed, and the fluorescent tagging confirmed using the LSR Fortessa (BD Bioscience, California, USA) flow cytometer. Forward and side scatter flow plots were used to identify the ST3 population and at least 5,000 events were analysed for CMTPX expression with 405nm violet laser and the 610/20 filter (Figure 2.3).

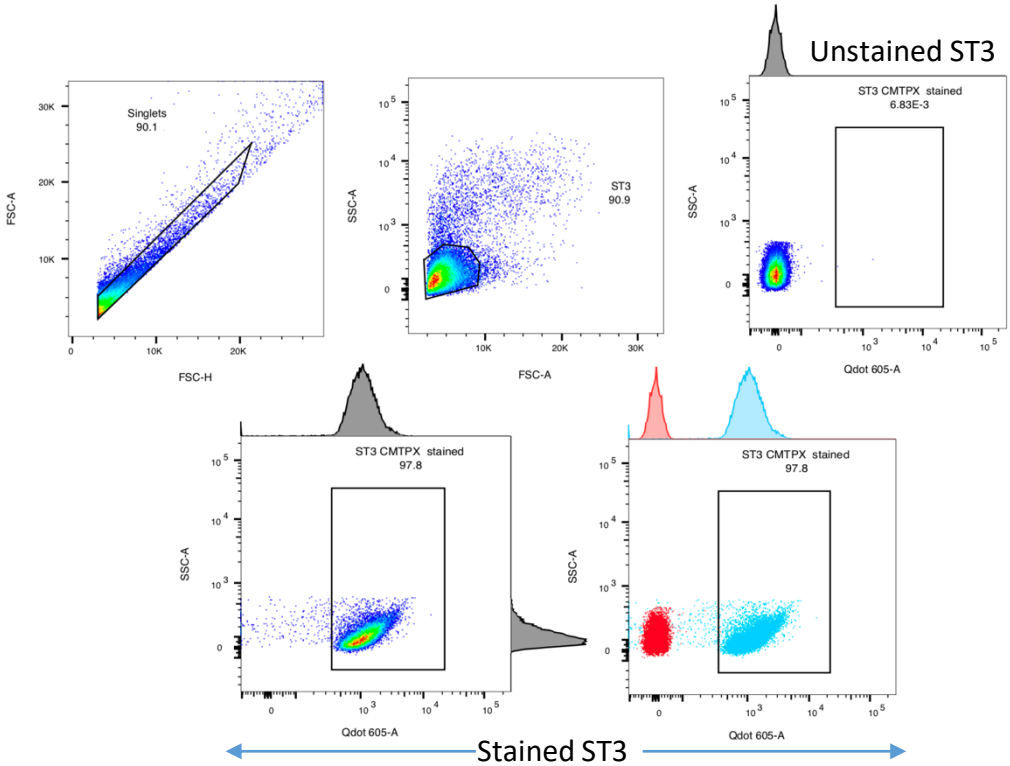


Figure 2.3. CMTPX stained *S. pneumoniae* serotype 3
Red – unstained pneumococci; Blue – stained pneumococci

2.6.5. Bronchoalveolar lavage processing and resting of cells

Bronchoalveolar lavage (BAL) fluid from three 50ml falcon tubes obtained as stated in section 2.5.4 were filtered using a 100µm cell strainer to remove any potential mucus plugs before pooling into three sterile 50ml falcon tubes that were centrifuged at 500*g* at 4°C for 8-minutes. After centrifugation, one tube of the 50ml supernatant was stored and the rest decanted. The remaining cell whole pellet was resuspended in 50ml of cold PBS and centrifuged at 500*g* at 4°C for 8-minutes. After washing, the cell pellet was further resuspended in 5ml of infection media and cell count performed using the KOVA® disposable counting chamber under bright field microscope (Olympus, Tokyo, Japan). To distinguish between non-viable and live BAL cells, trypan blue was prepared by diluting 1-part of trypan blue with 9-parts of PBS. A 1:1 ratio of cell suspension to diluted trypan blue was used for cell counts after which the airway cells were apportioned for the various experiments. The airway cell suspension was centrifuged, supernatant discarded, and the cell density adjusted to $1x10^6$ cells/ml by adding appropriate

amount of infection media. One millilitre of the airway cells was then transferred into each of the 12-wells of a culture plate and incubated for 3-hours at 37°C under 5% CO_2 to allow adherence of alveolar macrophages before the onset of ST3 infections. The collection, processing and resting of cells are illustrated in Figure 2.4.

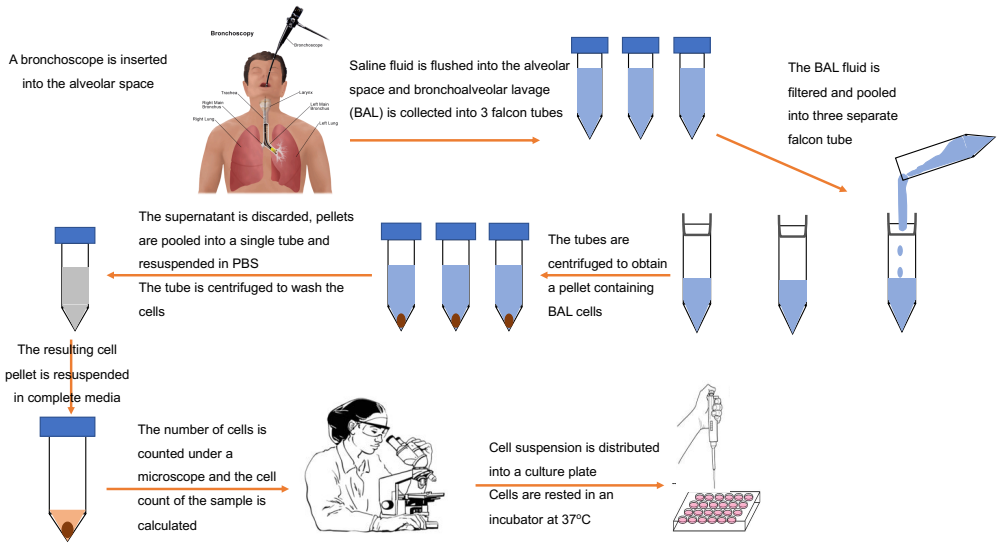


Figure 2.4. Sequential collection and bronchoalveolar lavage processing.

2.6.6. Airway cell *ex vivo* infections

a. Alveolar phagocytes and ST3 association assay

To determine the number of alveolar phagocytes (macrophages, monocytes, neutrophils) associated with *S. pneumoniae* ST3, rested BAL cells (see 2.6.5) were first infected with opsonised fluorescent tagged ST3 at MOI 50 and MOI 10 for 1-hour. Thereafter, the plates were centrifuged for 3 minutes at 1800rpm to initiate an immediate contact between the phagocytes and the ST3. Plates were then incubated at 37°C in 5% CO_2 with gentle horizontal shaking 50rpm. One-hour post infection, the supernatant was aspirated together with the non-adherent cells and transferred to FACS tubes. The supernatant was washed twice by centrifugation at 500*g* for 8 minutes and then fixed in 4% PFA for 5 minutes in the dark. As for the adherent cells, cold PBS was added to the well and the 12-well plate was incubated on ice buckets for 20 minutes. This facilitated the adherent cells to come off from their bound surfaces. Cells were scraped and transferred to FACS

tubes and centrifuged at 500*g* for 8 minutes at 4°C. The adherent cells were fixed for 5-minutes in 4% PFA and combined with their corresponding supernatants before the cells underwent immunophenotyping (see 2.6.7). The airway cell infections *ex vivo* experiments are summarised in Figure 2.6.

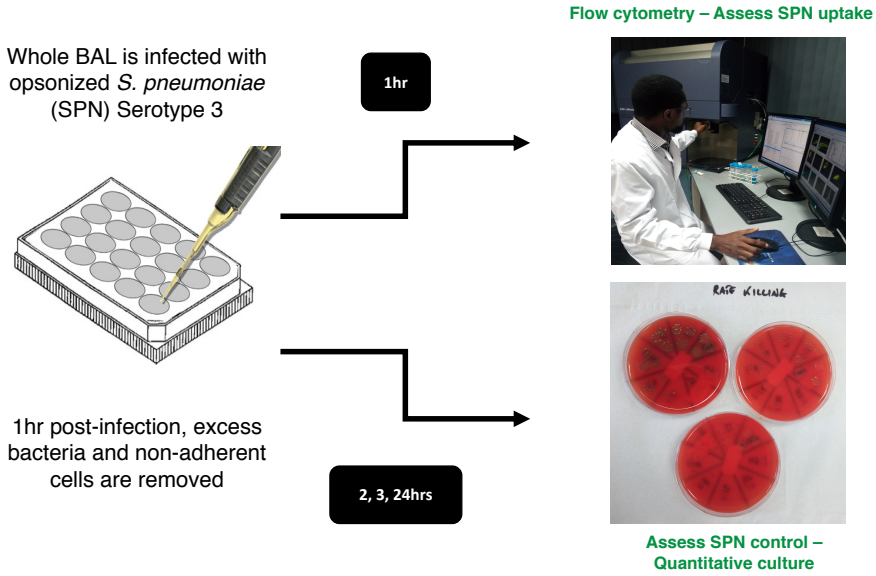


Figure 2.6. The airway cell infections *ex vivo* experiments.

b. Gentamicin protection assay experiments

To determine the number of viable intracellular ST3 in alveolar phagocytes (macrophages), rested BAL cells (see 2.6.5) were first infected with opsonised ST3 at MOI 50. Thereafter, the plates were centrifuged for 3 minutes at 1800rpm to initiate an immediate contact between the phagocytes and the ST3. Plates were incubated at 37°C in 5% CO_2 with gentle horizontal shaking 50rpm. One-hour post infection, the supernatant was removed, and the wells washed twice with fresh PBS. 450µl of infection media was added to the wells with adherent cells followed by 100µg/ml of gentamicin. Plates were further incubated for 30 minutes at 37°C in 5% CO_2 with gentle horizontal shaking 50rpm. Thirty minutes post gentamicin exposure, the wells were washed twice to remove any traces of gentamicin and viable extracellular bacteria, leaving phagocytes with internalised ST3.
Supernatant harvested from the gentamicin protection assay was centrifuged at

4,000*g* for 5-minutes, decanted and to the remaining pellet 1ml of PBS was added and any viable ST3 was quantified using the Miles and Misra technique (see 2.6.2).

To the adherent cells in the wells of the cell culture plates, fresh infection media with no antibiotics was added and incubation continued for 24-hours. At times 1, 2, 3- and 24-hours post infection, supernatant was removed and stored. Wells corresponding to the different time intervals were washed twice with PBS and 750µl of 2% saponin solution added. The well/s were incubated with saponin solution for 1-minute. The saponin lysed phagocytic cells to release phagocytosed ST3. Afterwards, 3mls of PBS was added to the well/s to dilute the effect of the saponin. The liquid in the wells was aspirated into a 15ml falcon tube and centrifuged for 5-minutes at 4,000*g*. Supernatant was decanted and 1ml of PBS added to the pellet, vortexed and viable intracellular ST3 quantified using the Miles and Misra technique (see 2.6.2).

c. Bacterial outgrowth assay

To determine the growth inhibition of extracellular ST3 by airway cells, rested airway cells (see 2.6.5) were infected with opsonised ST3 at MOI 1:50 or 50 bacteria per phagocyte in infection medium. The infections were done in a class IIAB laminar flow biohazard hood. Thereafter, the plates were centrifuged for 3 minutes at 1800rpm to initiate intimate contact between the phagocytes and the ST3. Plates were incubated at 37°C in 5% CO_2 for up to 24-hours with gentle horizontal shaking 50rpm. After each hour and for three continuous hours post initial phagocyte and bacteria contact, plates were taken out and the supernatant for the matching hour was removed, and wells washed twice with PBS after which fresh infection media was added before the cell culture plate was re-incubated (Figure 2.5). For the condition under test, the supernatant and cell wash supernatant were combined and centrifuged for 5 minutes at 4,000*g*. Supernatant was then discarded, and 1ml of fresh PBS added to the pellet and viable outgrowth ST3 quantified using the Miles and Misra technique (see 2.6.2). This was done for time points 1, 2,3- and 24-hours post infection.

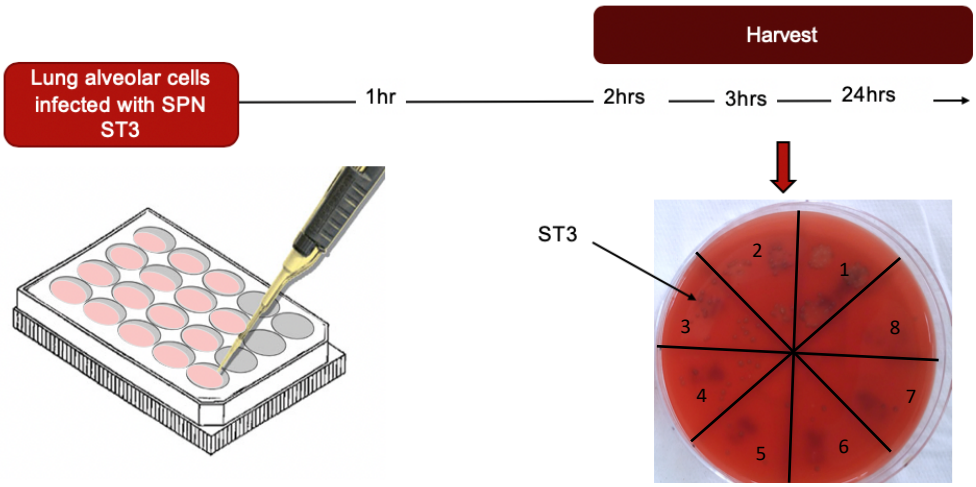


Figure 2.5. *Ex vivo* airway infections and ST3 quantification

2.6.7. Immunophenotyping

Flow cytometry-based immunophenotyping was used to characterise cells associated *S. pneumoniae*. Firstly, cells were adjusted to $1x10^6$/50µl and transferred into FACS tubes. A cocktail of antibodies was prepared using optimum antibody concentration. Antibodies were added to appropriate FACS tube and incubated in the dark for 15mins. After incubation, 1ml of cold PBS was added to resuspend cells. The tubes were then spun in the centrifuge at 500*g* for 8mins and supernatant gently poured off after centrifugation. Cells were gently resuspended and fixed in 0.5% paraformaldehyde and acquired on a BD Fortessa flow cytometer (Beckman Dickinson, USA).

2.6.8. Gating strategies

All gating strategies used in the protocols described in this thesis have been described in the methods section associated with the result chapter.

2.6.9. Cell sorting

Harvested airways cells from apparent health HIV-uninfected individuals were allowed to rest and incubated at 37°C in a 5% CO_2 incubator for 3-hours before the supernatant containing non-adherent cells was removed. This was done only for experiments which required highly pure airway cells depleted of lymphocytes.

Lymphocytes were purified from the whole non-adherent fraction through cell sorting (BD fluorescence activated cell sorter ARIAIII), followed by reseeding of lymphocyte depleted fraction to its cognate 12-well plate and incubated for a further 3-hours at 37°C, 5% CO_2. Following incubation, rested airway cells were infected with CMTPX stained *S. pneumoniae* ST3, harvested, immunophenotyped and acquired on the BD Fortessa flow cytometer as described previously in this chapter (2.6.6a and 2.6.7).

2.6.10. Culturing nasopharyngeal swabs for *S. pneumoniae*

The swab frozen in STGG media was thawed and vortexed for 30 seconds. 100µl of the mixture was pipetted onto the SBG agar and was streaked and incubated overnight at 37°C in a 5% CO_2 incubator. Pneumococci produce pneumolysin which breaks down haemoglobin into a green pigment that can be observed around the colonies. The following day, a few well isolated green and alpha haemolytic colonies were picked and streaked onto a new SBG and an ethyl hydrocupreine hydrochloride (Optochin) disk of concentration 5µg/ml was placed onto the streaked area. All pneumococci are sensitive (zone of inhibition ≥14 mm) to optochin and helps distinguish *S. pneumoniae* from other streptococci that are all optochin resistant.

2.6.11. Preparation of slide for confocal microscopy

The slides with fixed airways cells (experimental infection) in 4% paraformaldehyde were first washed off with PBS three-times to remove any dust that could have accumulated during storage. After washing, the tissue (airway cells) were then incubated and stained with 200µl of 0.5% wheat germ agglutinin (Alexa Flour® 633 WGA, Thermo scientific), to stain the cell membranes (magenta, AF633) of the airway cells and the bacteria. Alexa Fluor® 633 WGA binds to sialic acid and N-acetylglucosaminyl residues. The slide was then washed 3-times with PBS to remove excess WGA before permeabilising the airway cells with 100µl of 0.1% triton for 10 minutes. This was followed by washing off the triton 3-times

with PBS. Fixed airway cells were further incubated for an hour with pneumococcus strain serotype 3 capsule antiserum (SSI Diagnostica, Denmark) diluted in 5% goat serum. Following incubation, the slides are washed 3-times with PBS to remove excess pneumococcus antisera and then incubated for a further 45 minutes with secondary antibody (to detect bacteria) conjugated with the fluorochrome (anti-rabbit AF488, Thermo Scientific). For slides requiring AMs to be identified, anti-human CD206, AF568 (Thermo Scientific) was co-incubated with bacterial secondary conjugated antibody. AMs stained red with anti-CD206. The slide was then washed for the last time with PBS and once in distilled water. Finally mounting media containing 4',6-diamidino-2-phenylindole (DAPI) was added to the slide before mounting with a coverslip. DAPI stains the nucleus blue. Nail polish was used to seal the slide before analysis on the confocal or fluorescent scanning microscope. The slides were used to detect for the presence of intracellular *S. pneumoniae* within AMs. The confocal slide images were acquired using an Olympus FluoView 1000 confocal laser scanning (inverted) microscope. Z-stacks were recorded at 1-mm intervals at either 20X and 60X oil objectives, and ImageJ (https://imagej.nih.gov/ij/) was used for processing the images. 3D images were reconstructed using Huygens Essential deconvolution software version 16 (Scientific Volume Imaging) and viewed in Imaris 3D reconstruction software 9.4 (Bitplane).

2.7. Sample size

A total of 150 participants were screened with 76 being recruited into study. These comprised of 31 healthy HIV-uninfected; 29 asymptomatic HIV- infected on short-term ART (n=27; <3-months) or ART naïve (n=2); and 16 asymptomatic HIV-infected on long-term ART ≥3-years. The target sample size was 90, with each group having an estimated sample size of 30 participants. As this study was a pilot in nature, a sample size of 30 was decided upon, as recommended for pilot studies which range between 10-40 individuals per group (Johanson and Brooks, 2010; Julious, 2005; Leon et al., 2011; Viechtbauer et al., 2015). However, our

target was not met due to time constraints and bronchoscopy equipment challenges.

2.8. Data management and statistical analysis

a. Data management

All names of participants recruited were de-identified and assigned a unique study participant identifier. All clinical and research data generated from the MLW laboratory were merged and anonymised. Results for the participants were reported using the unique study identifier. Consent forms and were kept under lock and key at MLW and access to these documents was restricted to the principal investigator and data manager. Upon completion of data entry and cleaning, original forms were stored under lock and key at MLW until final analyses and reports were prepared. All paper documents will be destroyed after 5-years according to MLW policy and prevailing regulations in Malawi.

b. Statistical analyses

All statistical analyses used in this thesis have been described in the methods section associated with the result chapter.

CHAPTER 3

3.0. Assay development and adaptation

3.1. Alveolar infection control model for *S. pneumoniae*

This chapter provides the experimental methods developed, adapted and optimised to generate the data used in the subsequent chapters. Some of the methods have previously been published as cited in the sections that follow.

3.2. Specific objectives

a. To establish pneumococcal serotype 3 (ST3) growth kinetics.
b. To generate a fluorescent labelled-pneumococcal ST3 for use in a flow cytometry-based binding and internalisation assay.
c. To optimise opsonisation conditions for pneumococcal ST3.
d. To standardise and quality control the pneumococcal inoculum dose for the *ex vivo* infection.

3.3. Establishing pneumococcal serotype 3 (ST3) growth kinetics

3.3.1. Pneumococcus ST3 growth curve and quantitation

The pneumococcus serotype 3 (sequence type 700) optimised in these assays and used in subsequent experiments was described elsewhere (2.6.2). The growth curve assay is a fundamental technique used in microbiology to determine the bacterial growth kinetics (lag phase, log/exponential phase and stationary and death phase) and aids in determining the bacteria's physiological and metabolic functions as well as quantifying the actual numbers of the bacteria. The growth curve principle is based on the fact that when bacteria replicate, they divide asexually through binary fission yielding two daughter cells.

In standardising the ST3 for the infections experiment, the bacteria were cultured and quantified as described in section 2.6.2. Of note ST3 was quantified every 2-hours during the first 6-hours and every 3-hours thereafter up to a maximum of 24-hours. This was done after observing the growth kinetics of the bacteria. Serial

plate dilutions were done and the number of *cfu* present quantified on SBG as previously described (2.6.3). Figure 3.1. shows the ST3 24-hour growth curve generated, showing the growth kinetics of ST3 from lag phase through to the death phase were the autolytic enzymes of the bacteria are activated. The viability of ST3 was not quantified beyond 24-hours as all *ex vivo* infection experiments had a maximum limit of 24-hours.

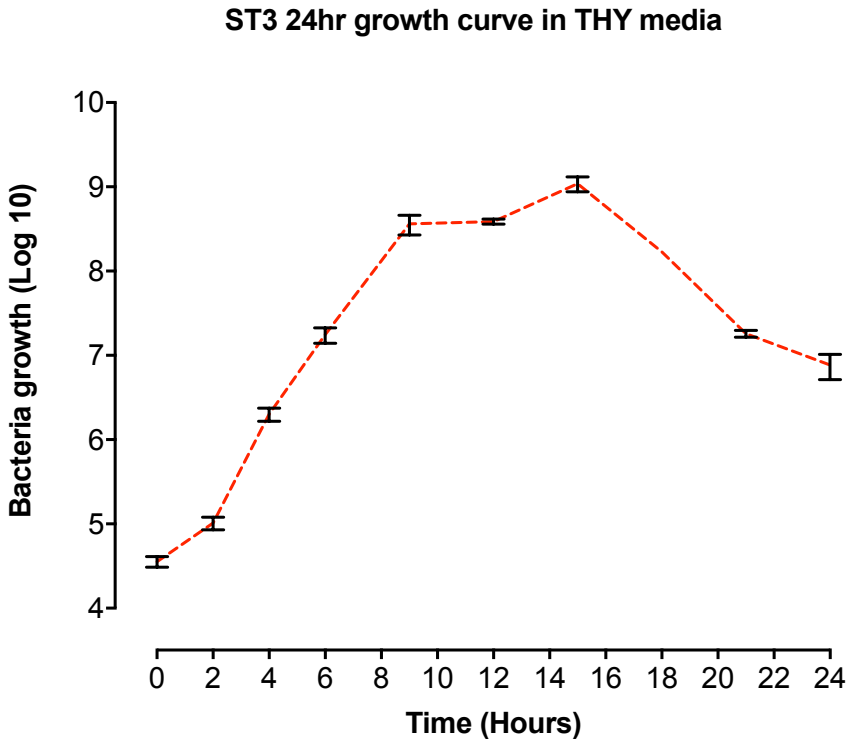


***Figure 3.1. S. pneumoniae* ST3 growth curve following incubation in Todd Hewitt Broth supplemented with 5% yeast extract**. One colony of pneumococcus ST3 was inoculated into 10mls of pre-warmed broth and incubated at 37°C in 5% CO_2. The total colony forming units were quantified every 2-hours during the first 6-hours and every 3-hours thereafter using the Miles and Misra technique on sheep blood agar supplemented with 5mg/L gentamicin. Average number of replicates at each time point n=3, error bars show standard error of the mean (SEM).

3.3.2. Viability of pneumococcus ST3 growth in infection media and RPMI

Next we determined whether RPMI alone or infection medium (RPMI +10% FBS) best maintains ST3 growth. This was an important component of assay development as the media was meant to support the viability of ST3 the first 6-hours of the *ex vivo* infection and thereafter the ST3 decayed exponentially as the media failed to maintain further bacterial growth. In standardising the viability of ST3 for the infections experiment, $5x10^7$ colony forming units (actual infections bacterial dosage) of ST3 were grown in 1-ml of liquid culture infection media or RPMI alone and quantified as a function of time as described (2.6.2). Of note, ST3 was quantified every 3-hours over 24-hours. The viability and decay of ST3 in infection media is shown in Figure 3.3.1. It was observed that the infection media was supportive of the ST3 survival for the first 6-hours and then went through a rapid decay until it reached the assay's limit of detection between 15 and 18-hours (Figure 3.2).

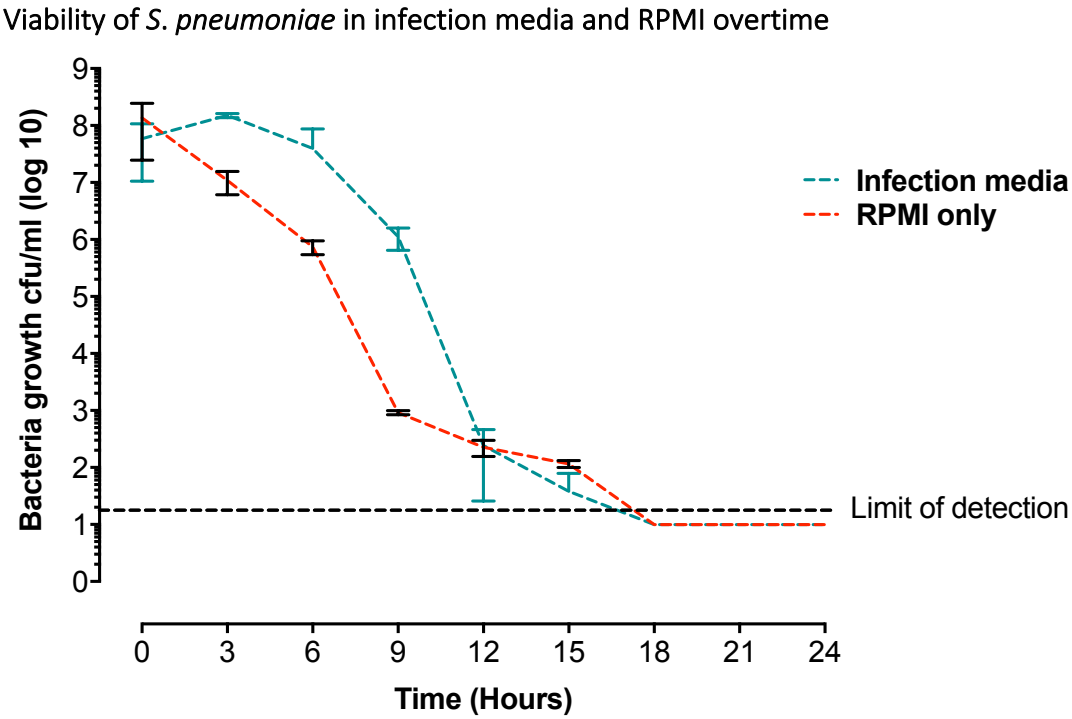


Figure 3.2. S. pneumoniae-ST3 growth kinetics in RPMI or infection medium (RPMI +10% FBS). $5x10^7$ colony forming units of pneumococcus ST3 were inoculated into 1ml of pre-warmed broth and incubated at 37°C in 5% CO_2. The total colony forming units were quantified every 3-hours for

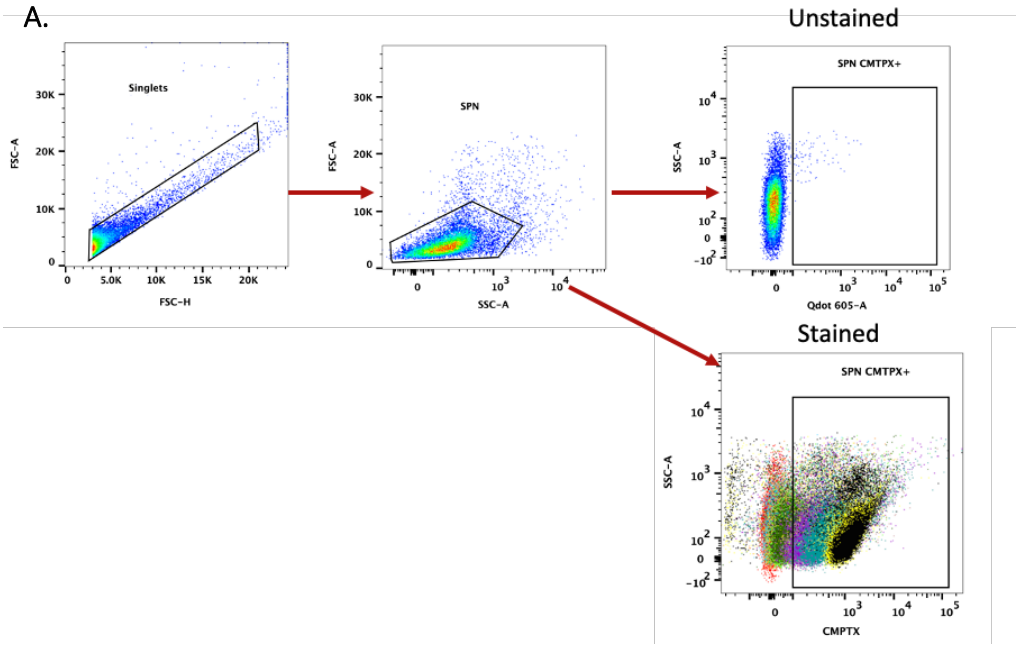
24 hours using the Miles and Misra technique on sheep blood agar medium supplemented with gentamicin with 5mg/L gentamicin. Abbreviation: cfu – colony forming units, SPN – pneumococcus. Replicates at each time point n=2, error bars show SEM.

3.1. To produce a fluorescent labelled-pneumococcal-ST3 for use in a flow cytometry-based binding and internalisation assay.

3.1.1. Optimum staining concentration of pneumococci-ST3 with CellTracker™ Red CMTPX dye

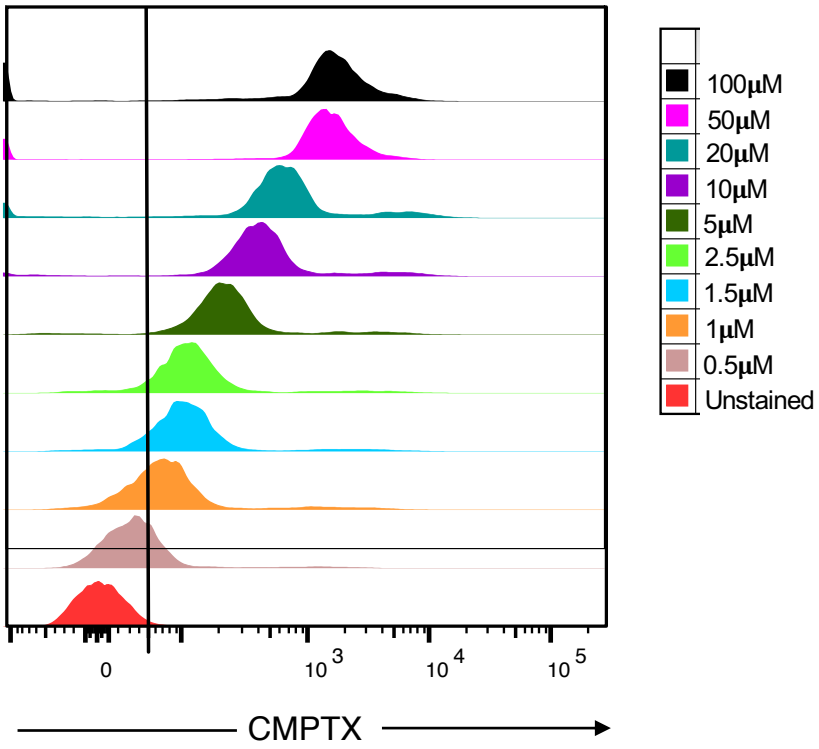
Having confirmed that the infection medium maintains the growth of pneumococci-ST3, I further determined the optimum concentration of CMTPX dye to use in staining the bacteria. Frozen aliquots of pneumococci ST3 bacteria were thawed to room temperature, centrifuged 5 times to obtain a pure pellet. The pneumococci-ST3 pellet (~1x10^8 *cfu*) was then fully resuspended in 250µl of various concentrations of Red CMTPX (0, 0.5µM, 1µM, 1.5µM, 2.5µM, 5µM, 10µM, 20µM, 50µM, 100µM) and bacteria tagged and acquired as described in 2.6.4. 10µM CMTPX of was found to be the optimum staining concentration for the pneumococci as higher concentrations were not cost effective.

Gating strategy for the CMTPX stained bacteria



Histograms of pneumococci staining intensity at various CMTPX concentrations

B.



C.

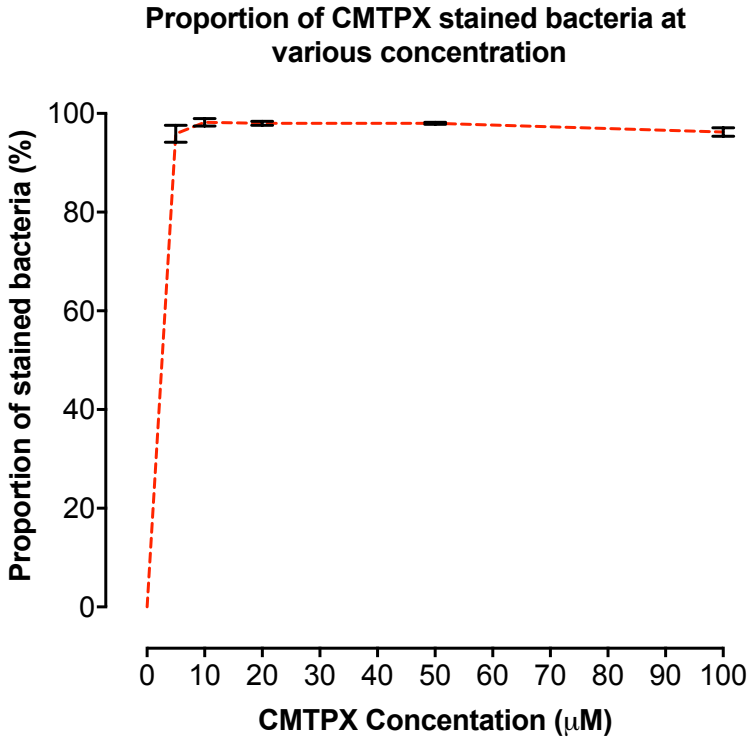


Figure 3.3. **Optimum pneumococci CellTracker™ Red CMTPX. A**. The gate for unstained CMTPX pneumococci-ST3 was used to identify pneumococci positive for CMTPX**. B.** Histograms were used

to compare the staining intensity of pneumococci-ST3 to the different concentrations of CMTPX **C.** Proportion of CMPTPX stained bacteria at various concentrations (average number of replicates at each concentration n=3), error bars show SEM. Abbreviations: FSC-A – forward scatter area, FSC-H – forward scatter height, SSC-A – side scatter area.

3.1.2. Stability of CMTPX stained pneumococci-ST3 over time.

To determine the stability of CMTPX stained pneumococci-ST3 over time, the pneumococci ST3 pellet (~5x10^7 *cfu*) was resuspended in 250µl infection media and 10µM CellTracker™ Red CMTPX. The 15ml falcon tube was wrapped in aluminium foil and incubated at 37°C for 1 hour, whilst gently shaking at 170 rpm to ensure even staining of bacteria. Bacterial staining was done as described earlier (2.6.4). To determine the stability of CMTPX stained pneumococci, the bacteria was further resuspended in infection media alone for 0, 30, 60 and 120 minutes, then pelleted by centrifuging and prepared for acquisition as described in 2.6.4 The CMTPX stained bacteria was observed to be stable for up to 2-hours post staining and this was crucial since bacteria are exposed to airway cells for approximately 2-hours prior to infection to acquisition.

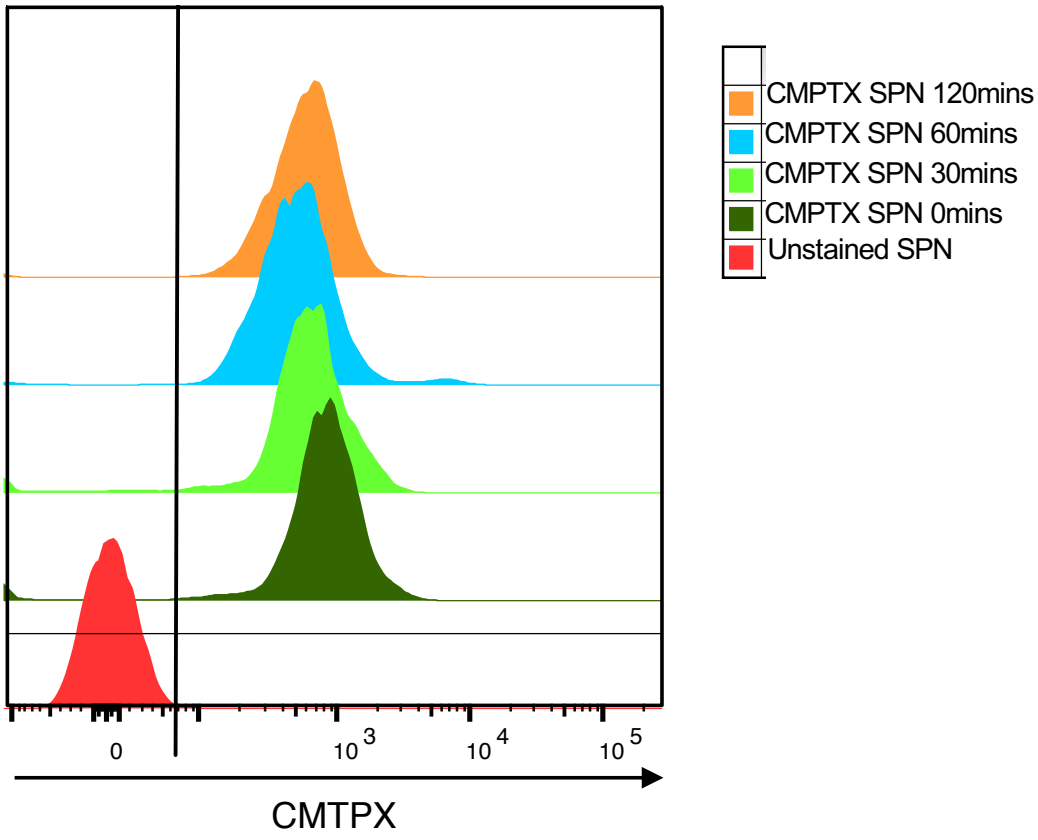


Figure 3.4. **Stability of CellTracker™ Red CMTPX stained pneumococci overtime.** Histograms were used to compare the stability of the CMTPX stained bacteria over time (0, 30, 60 and 120 minutes). Abbreviations: SPN – *S. pneumoniae*.

3.2. To optimise opsonisation conditions for pneumococci-ST3.

3.2.1. Defining optimum IgG for pneumococcal opsonisation and uptake.

Having established the optimum staining concentration for CMTPX and its stability on stained bacteria, I then determined the to optimum volumes of IgG 007SP human anti-pneumococcal capsule reference sera and the IgG intravenous immunoglobulin (IVIG) individually and in combination (Goldblatt et al., 2017; National Institute for Biological Standards Control, 2008). I decided to use 007SP as it is pooled serum from 287 healthy volunteers following vaccination with 23-valent pneumococcal polysaccharide vaccine (PneumovaxII®) that has antibodies capable of binding to the pneumococcus serotype 3, thus facilitating its internalisation through FcγRI/CD64, FcγRII/CD32, FcγRIII/CD16. Intravenous immunoglobulin is a collection of IgG antibodies of varying specificities to a wide range of antigens (including *S. pneumoniae*) and super antigens facilitating binding and internalisation by phagocytes (Takei et al., 1993). Pneumococci bacteria were stained as described (2.6.4.) and opsonised with varying IgG immunoglobulin volumes (IVIG - 10µl, 20µl, 40µl; 007SP - 25µl, 50µl, 100µl) or in combination (IVIG-10µl +007SP-50µl) as described (2.6.3). The 007SP human anti-pneumococcal sera contains an estimated 1.45µg/ml of IgG antisera against pneumococcal serotype 3, whereas 1ml normal IVIG (5% IgG, w/v). Rested airway cells (see 2.6.5) were infected with opsonised pneumococci-ST3 at MOI 50 in infection medium (2.6.6a). The cells were processed, fixed and acquired on the flow cytometer. A combination of IVIG-10µl and 007SP-50µl was found to be the best ratio resulting in a higher association of pneumococci-ST3 to AMs. This is summarised in Figure 3.5.

Flow plots comparing different IgG volumes for pneumococci opsonisation and uptake

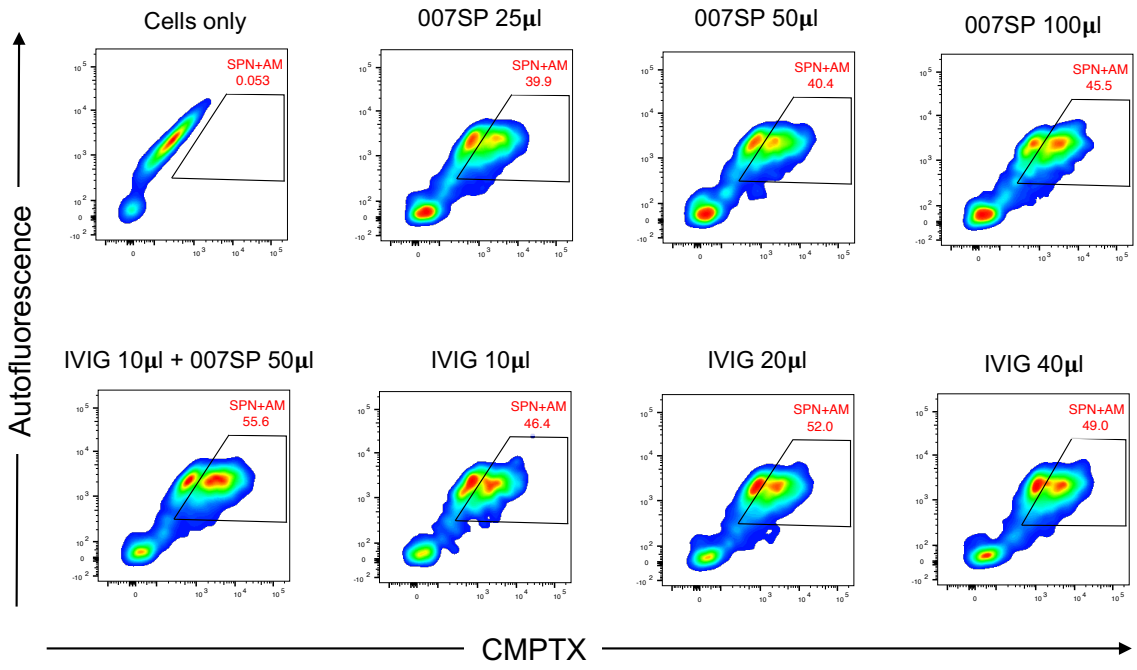


Figure 3.5. **Representative flow plots comparing different IgG volumes for pneumococci-ST3 opsonisation and uptake.** Uninfected cells were used as the standard gate to identify the proportions of airway phagocytes associated pneumococci-ST3.

3.2.2. Unopsonised pneumococci-ST3 bacteria exhibited increased binding and association with AMs

Next, I investigated whether the frequency of AMs associated with opsonised IgG (IVIG + 007SP) or unopsonised pneumococcus-ST3 was similar at MOI 1, MOI 10 and MOI 50. Airway cells from asymptomatic HIV-uninfected individuals were infected *ex vivo* with IgG opsonised and unopsonised CMTPX stained pneumococcus-ST3 at various MOI. In this study, the binding index was defined as the proportion of pneumococcus associated with each individual cell. The binding index for AMs was calculated using equation 2 below. AMs infected with CMTPX stained pneumococcus-ST3 and non-infected cells were stained with antibody fluorochrome conjugated monoclonal antibodies specific to the cell of interest and then acquired on the flow cytometer. The proportion of AMs associated with pneumococci-ST3 was increased in the unopsonised pneumococci-ST3 conditions (MOI 11, p=0.0039; MOI 50, p=0.039) compared to the IgG opsonised pneumococci-ST3. Furthermore, unopsonised pneumococcus-ST3 exhibited increased binding to AMs with a 1.02-fold median increase, (n=11, p=0.0078) at MOI 10 when compared to opsonised pneumococci-ST3; at MOI 50 a 2.59-fold median increase, (n=11, p=0.0039) was observed (Figure 3.6). MOI 1 was not included in the final analysis as the number of individuals were fewer and binding index below 0.01. Taken together these findings indicate that unopsonised pneumococcus-ST3 exhibit higher binding and association with AMs than IgG-opsonised pneumococcus-ST3.

Equation 2 – Alveolar macrophages

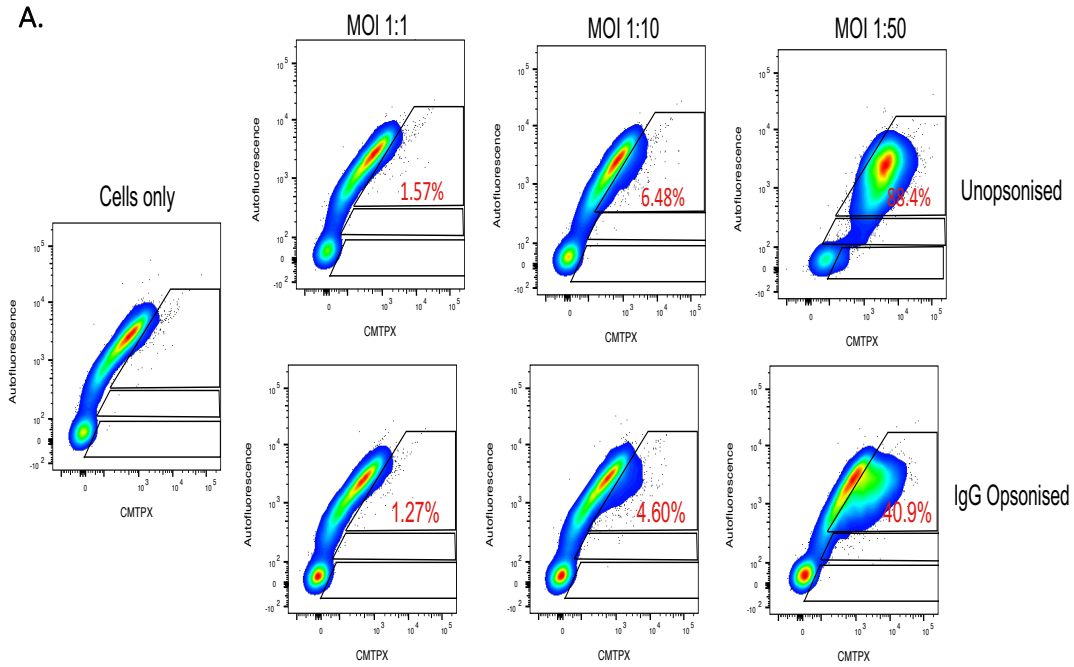
$$\text{Binding index} = \frac{\text{MFI (infected AMs*CMTPX SPN}^{+}\text{)- MFI (uninfected AMs*CMTPX SPN}^{-}\text{)}}{\text{2 x rSD (uninfected AMs*CMTPX SPN}^{-}\text{)}}$$

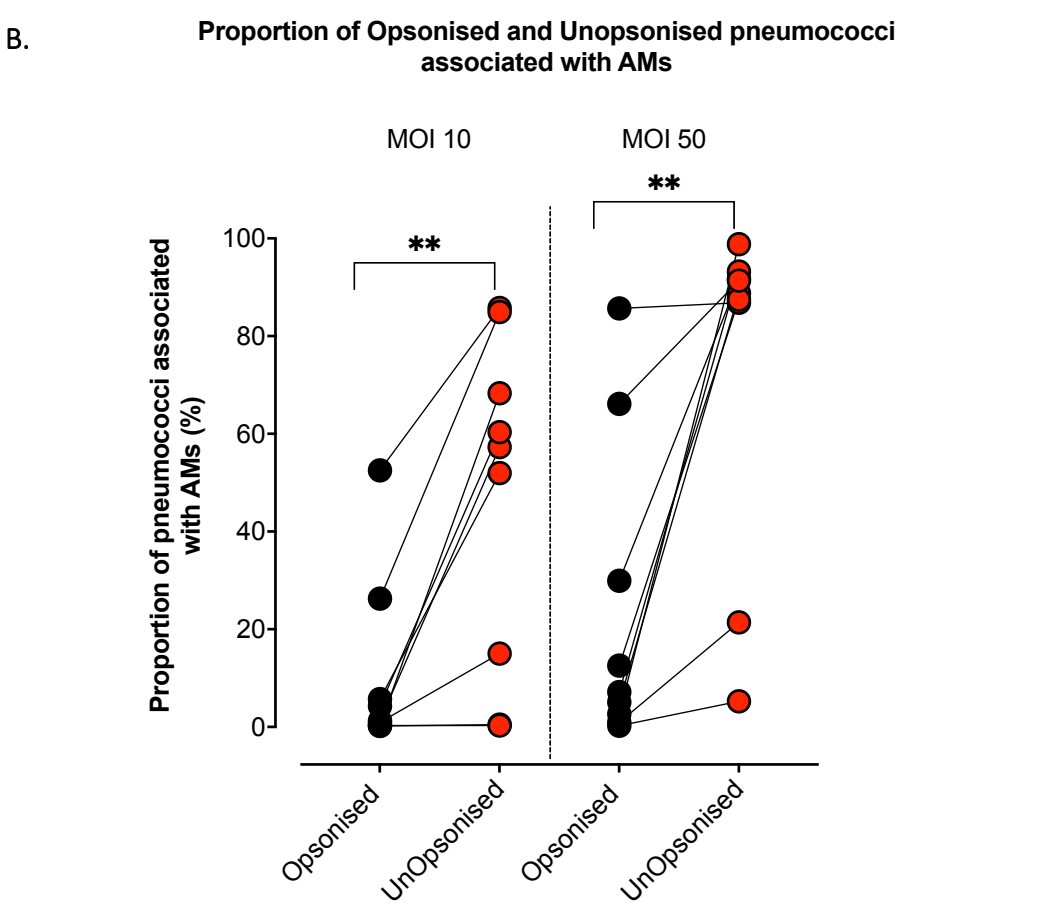
Equation 3 – Neutrophils

$$\text{Binding index} = \frac{\text{MFI (infected neut *CMTPX SPN}^{+}\text{)- MFI (uninfected neut*CMTPX SPN}^{-}\text{)}}{\text{2 x rSD (uninfected neut*CMTPX SPN}^{-}\text{)}}$$

Abbreviations: AMs – Alveolar macrophages, MFI – median florescent index, neut – neutrophils rSD – robust standard deviation, SPN - pneumococcus-ST3.

Flow plots showing proportional association of pneumococci by AMs





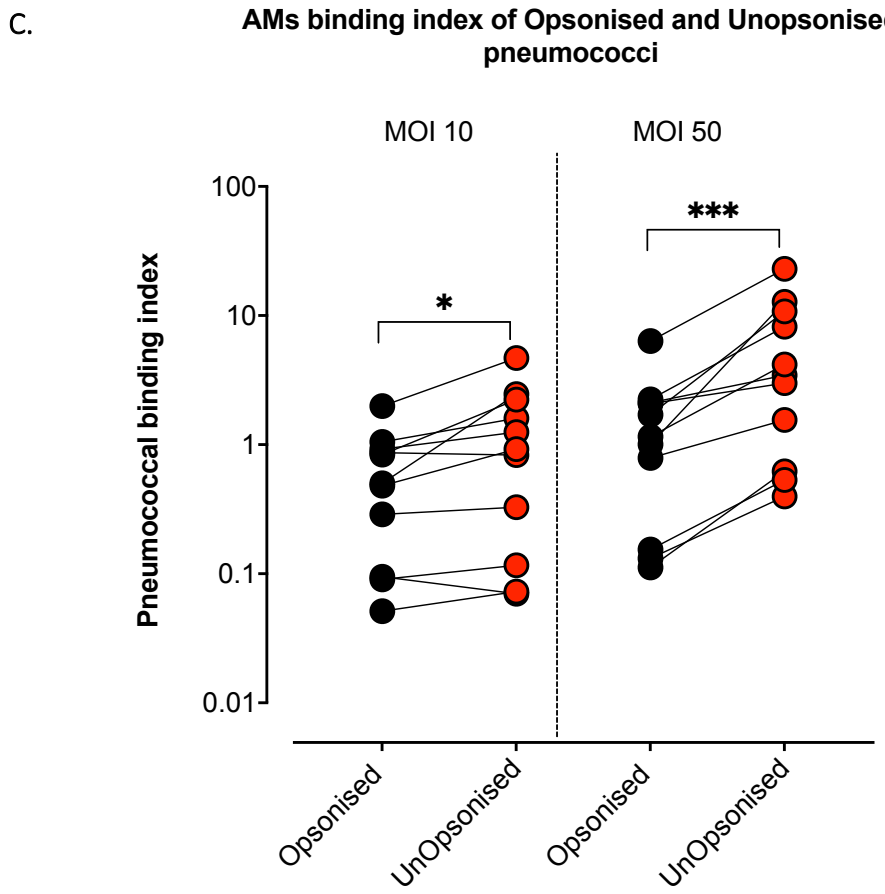
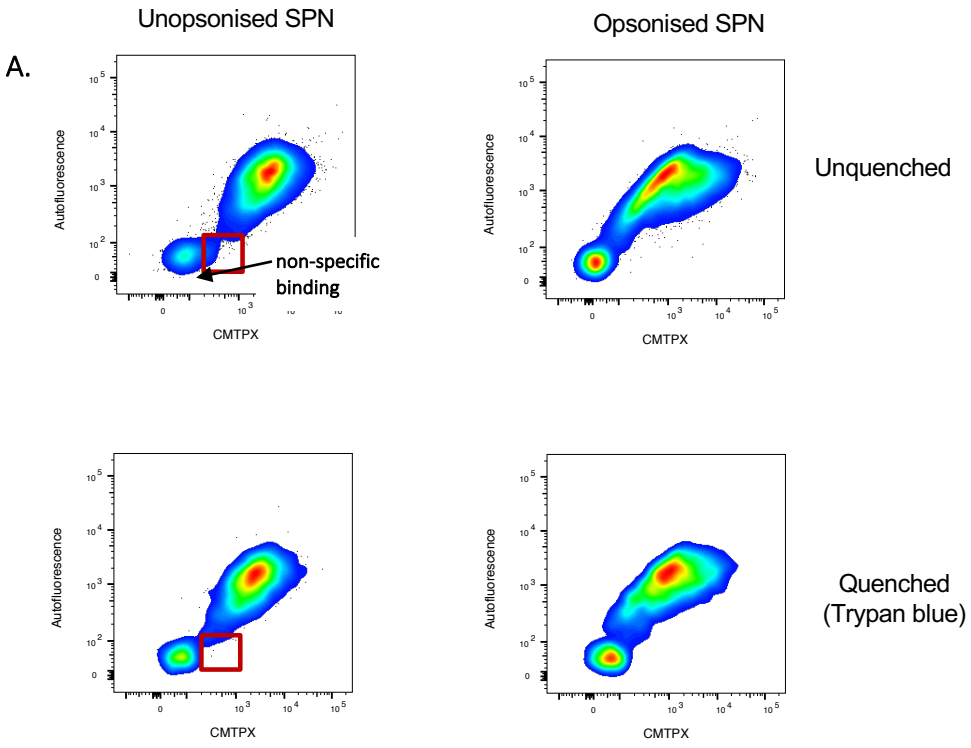


Figure 3.6. **Differential association of pneumococci-ST3 with AMs in IgG opsonised and unopsonised conditions**. **A.** Representative flow plots comparing different MOI of opsonised and unopsonised pneumococci-ST3 and their association with airway cells. **B.** Proportion of AMs associated with IgG opsonised and unopsonised pneumococci-ST3 post infection *ex vivo* in asymptomatic HIV uninfected individuals (n=11) at MOI 10 and MOI 50 by Wilcoxon signed-rank test, ** p=0.0039, **p=0.0070. **C**. Pneumococcal binding index per AM associated with IgG opsonised and unopsonised pneumococci-ST3 post infection *ex vivo* in asymptomatic HIV uninfected individuals (n=11) at MOI 10 and MOI 50 by Wilcoxon signed-rank test, ** p=0.0078, **p=0.0039. Abbreviations: AMs – alveolar macrophages, MOI – multiplicity of infection.

3.2.3. Quenching inhibits the non-specific binding of pneumococci onto airway cells.

This experiment was designed to identify whether the binding/internalisation of unopsonised pneumococci-ST3 was true internalisation and not just some non-specific extracellular association. Using MOI 50, I infected the airway cells with opsonised and unopsonised pneumococci-ST3. Following harvesting of the airway cells, I quenched the extracellular pneumococcus with trypan blue at a dilution of 10µl trypan blue in 990µl of PBS for 5 minutes. Following quenching, trypan blue was washed off with 1ml PBS and cells centrifuged and discarded the supernatant. Airway cells were then fixed in 3% paraformaldehyde, then further washed in PBS and resuspended in PBS before acquisition. Unopsonised pneumococci were found to associate non-specifically to airway phagocytes and lymphocytes as shown in (Figure 3.7). In summary, unopsonised pneumococci associates with more airway phagocytes resulting in less internalisation.

Gating showing pneumococci non-specific binding to lymphocytes



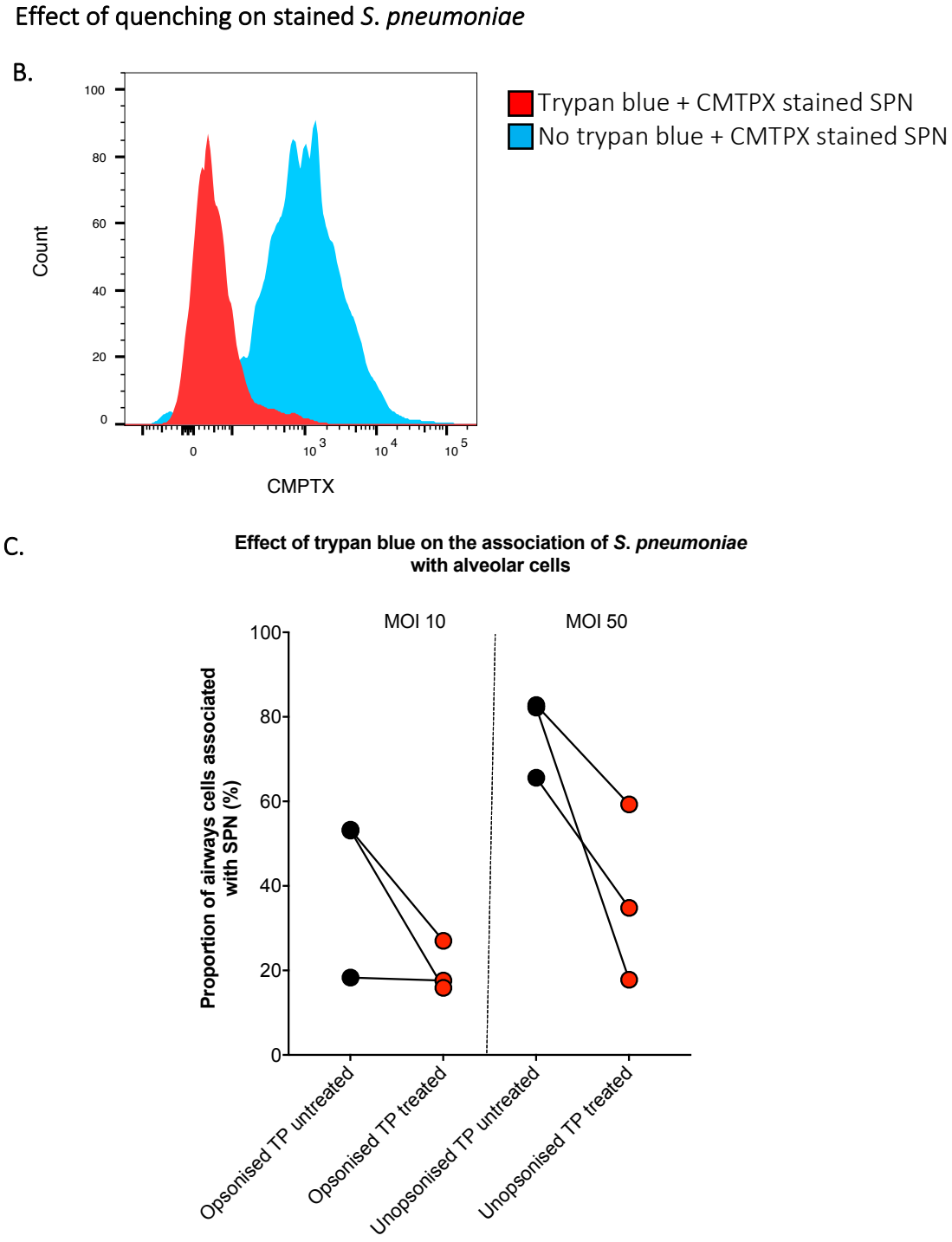


Figure 3.7. Quenching inhibits the non-specific binding of pneumococci on airway cells. A. Representative flow plots comparing airway with IgG opsonised and unopsonised pneumococci-ST3 after quenching. B. Representative histogram comparing the effects of quenching on AMs. C. Comparison of pneumococci association with alveolar cells before and after quenching with trypan blue (n=3 HIV-uninfected individuals) by Wilcoxon signed-rank test at MOI 10 or MOI 50. Abbreviations: MOI – multiplicity of infection, SPN – *S. pneumoniae*, TP – trypan blue.

3.2.4. Defining the correlation between pneumococci-ST3 binding index and proportional association

I further investigated the relationship between AMs pneumococcal binding index and proportional association in IgG opsonised conditions at MOI 10 and MOI 50. I observed a moderate relationship between the proportion of AMs associated with pneumococcus-ST3 and the binding index (Figure 3.8).

Correlation between pneumococci binding index and proportional association with AMs

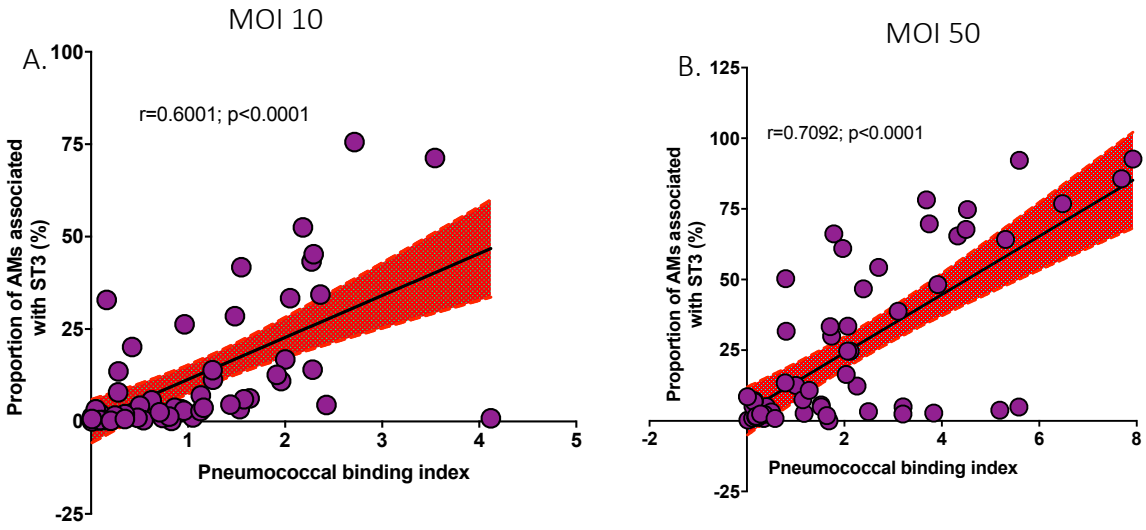


***Figure 3.8*. Relationship between pneumococcal binding index and the proportion of AMs associated with pneumococcus-ST3.** Average number of pneumococcus bound to each AMs (n=56 individuals) is shown to be positively correlated to proportion of AMs associating with *S. pneumoniae at* **A.** MOI 10 and **B.** MOI 50**.** Individual subjects are shown, and the strength of the relationship is denoted by the Pearson correlation test (r), linear regression line with 95% confidence interval are shown.

3.3. Reproducibility of inoculum dose for the *ex vivo* infection.

The last experiment optimised was the inoculum dose used for the *ex vivo* infections. The standard inoculum dose for the experiments is $5x10^7$ cfu/ml. I thawed the frozen batch of pneumococcus, resuspended it in infection media and cultured it using the Miles and Misra for viable bacteria count to confirm the multiplicity of infection (MOI) and its viability. I also did 6 replicates of the inoculum doses to demonstrate proficiency and reproducibility of the Miles and Misra techniques. The median inoculum density was 4.8 (IQR; 4.5 – 5.1) $x10^7$ *cfu*/ml with a coefficient of variation of 6.43% (Figure 3.9, Table 3.1.).

Reproducibility of *S. pneumoniae* inoculum dose

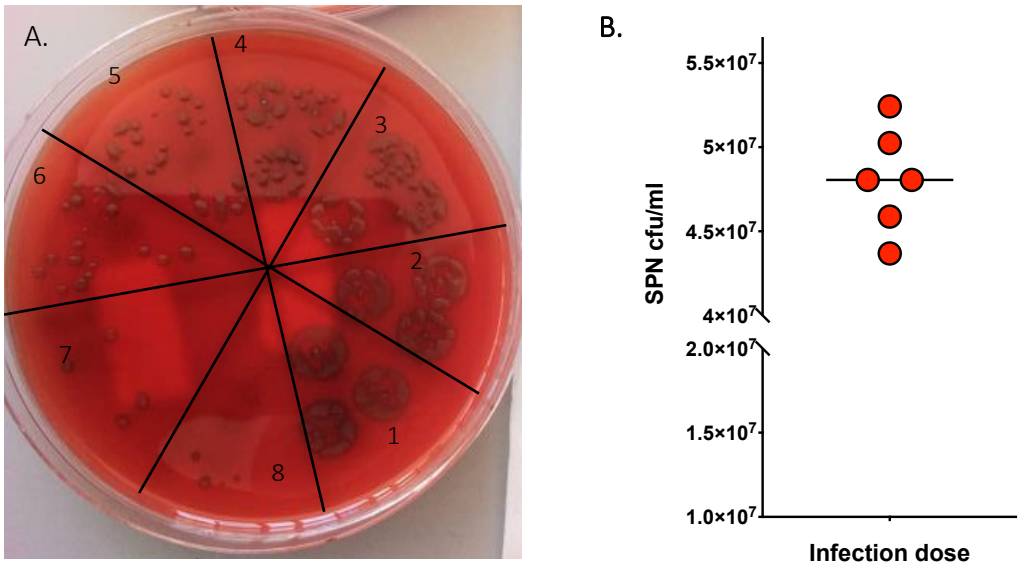


Figure 3.9. **Reproducibility of inoculum dose for the *ex vivo* infection. A**. Pneumococcus inoculum serially diluted 1:2 was plated anticlockwise on a sheep blood agar plate starting with the highest dilution at position 1 and finishing with the lowest dilution at position 8. **B**. Distribution of pneumococcus inoculum dose results after plating on sheep blood agar (n=6 replicates), middle line represents median inoculum density ($4.8x10^7$).

Table 3.1. Concentration of inoculum dose for the *ex vivo* infection

Concentration of Infection dose	
Replicate	Pneumococcus cfu/ml
1	$5.02x10^7$
2	$4.59x10^7$
3	$4.81x10^7$
4	$4.81x10^7$
5	$4.37x10^7$
6	$5.24x10^7$
Median/mean	$4.81x10^7$
Coefficient of variation	6.43%

3.4. Discussion

To investigate the cellular immune response against pneumococci in the lung, there was need to develop and optimise *ex vivo* infection assays that would aid understanding of immune control in the airway.

The effectiveness of the pneumococcal conjugate vaccine (PCV)13 to pneumococci serotype 3 remains uncertain and conflicting. Little reduction in serotype 3 disease has been observed globally compared to disease due to other vaccine serotypes (Azarian et al., 2018). Unpublished data from Malawi demonstrates that anti-capsular antibodies against pneumococcus serotype 3 do not increase with age, as do those for other common serotypes. Surveillance data from Malawi has also reported that serotype 3 is among the commonest serotypes in pneumococcal carriage. I therefore decided to study pneumococcal serotype 3 using airway cells, in order to understand the lung infection dynamics. Firstly, I developed a 24-hour growth curve to establish its kinetics of propagation. The growth curve results obtained with serotype 3 were similar to those obtained with other serotypes of pneumococci (Restrepo et al., 2005; Tóthpál et al., 2019). Furthermore, the growth curve results helped me to define the different stages of pneumococci growth and in particular, the log phase. The pneumococci used in this study for infection studies were all grown to the log phase, which is characterised by a higher number a surviving bacterium with a constant doubling capacity. I also evaluated the growth potential of pneumococci in RPMI medium compared to infection media (RPMI + 10% foetal bovine serum). This was critical for the infection studies as the pneumococci were to be maintained in culture medium conducive for both bacterial and immune cell survival for 24-hours. This enabled ascertainment of the early and late killing kinetics of pneumococci serotype 3 in experimental conditions containing airway cells.

AMs are highly auto fluorescent and tend to fluoresce at the same wavelength as green fluorescent protein (GFP), a common fluorescent protein used in tagging bacteria (Margolin, 2000; Phillips, 2001; Southward and Surette, 2002). An

alternative dye was optimised to stain the bacteria, and Cell Tracker Red CMTPX was capable of staining the bacteria and was stable for 2-hours. CMTPX has been used to stain pneumococci before, in an experiment to demonstrate that dendritic cells can transport *S. pneumoniae* to stromal follicular dendritic cells which are important for antibody production as they interact with B cells (Heesters and Carroll, 2016). I was able to demonstrate that CMTPX stained pneumococci-ST3 is stable for at least 2 hours, as the bacteria is exposed to airway cells for approximately 2-hours from infection to acquisition in the binding and internalisation assays.

Airway phagocytes can internalise pneumococci using a number of cell surface receptors and depending on the receptor system engaged, the ingested pneumococci is dealt with differently. In this thesis, I had to optimise the IgG immune sera used for pneumococci opsonisation and bacteria internalisation via Fc receptors of phagocytes. The combination of IVIG and 007sp gave optimum binding and internalisation of at least 30% when using MOI 50. Other authors have reported using IVIG and 007SP in *ex vivo* infection experiments (Gordon et al., 2013; Mitsi et al., 2017, 2019). I observed that unopsonised pneumococci associated with airway cells non-specifically and this was supported by the observation that quenching of CMTPX-stained pneumococci with trypan blue reduced the signal on non-phagocytic cells.

Finally, to generate robust data in the *ex vivo* pneumococcal infection studies, it was critical that the inoculum dose had to be reproducible across experiments, amongst participants and over a period of time. I was able to reproduce and optimise the pneumococcal-ST3 inoculum dose for the infection studies. The coefficient of variation was 6.43% from 6 different experiments, with all the inoculum infection dose within the same log. This was important as it suggests that the variation in the data to be produced is due to the individual host factors and not due to variations in bacterial fitness.

3.5. Conclusion

In summary, this chapter has shown the development and optimisation of important assays for understanding airway cell control of pneumococcal infection. Having accomplished this, the appropriate assays were subsequently used in Chapters 4, 5 and 6.

CHAPTER 4

4.0. Airway phagocyte binding and internalisation of pneumococcus serotype 3 in asymptomatic Malawian adults.

4.1. Introduction

S. pneumoniae remains the leading cause of pneumonia, meningitis and septicaemia globally, resulting in significantly high morbidity and mortality in the under-fives , the elderly and HIV infected individuals (Aston et al., 2019; Iroh Tam et al., 2017; O'Brien et al., 2009; Troeger et al., 2017; Wahl et al., 2018b). Despite the availability of pneumococcal conjugate vaccines (PCV), which are based on polysaccharide capsular antigens against common disease causing pneumococcal serotypes, the vaccines have proven less effective against pneumococcal pneumonia (Bonten et al., 2015; Cutts et al., 2005; Jacups and Cheng, 2011; Madhi et al., 2015; Prato et al., 2018). In Africa, pneumococcal pneumonia remains substantially prevalent amongst the HIV-infected individuals despite the high roll out of ART. Sub-Saharan Africa harbours the highest burden of HIV (Albrich et al., 2017).

Mucosal control of pneumococci in the lung is complex involving both innate and adaptive immunity (Wilson et al., 2015). Once pneumococci microaspirate or translocate into the lower airway they encounter phagocytes (alveolar macrophages, neutrophils, dendritic cells, monocytes) and host soluble microbiocidal and signalling factors (Dudek et al., 2016b; Smith et al., 2018). Phagocytes contribute to the first line of defence against pneumococci, by pathogen recognition, phagocytosis, bacterial killing, as well as providing a linkage between dendritic cells and T-cells (Aberdein et al., 2013; Byrne et al., 2015; Collini et al., 2018; Gordon et al., 2000; Jambo et al., 2014a; Morales-Nebreda et al., 2015). Phagocytes bind and recognise pneumococci via germline coded receptors called PRRs which bind to conserved molecular structures found on large groups of pathogens, termed PAMPs (Byrne et al., 2015; Janssens and

Beyaert, 2003; Kohler et al., 2016c; Medzhitov and Janeway, 1997; Mogensen, 2009). Phagocytes may also recognise pneumococci bound to an immunoglobulin via the fragment crystallisable (Fc) receptors present on its surface or via complement through the C3b deposited on the pneumococci (through receptors CR1, CR3 and CR4) (Dustin, 2016; Gordon et al., 2000; Guilliams et al., 2014; Walport, 2001). AMs constitutively express three classes of FcγR namely FcγRI/CD64, FcγRII/CD32, FcγRIII/CD16 and utilise these receptors to internalize antibody-opsonized pathogens, potentially leading to antibody-mediated clearance (Guilliams et al., 2014; Hunegnaw et al., 2019; Taylor et al., 2005).

AMs are the most abundant phagocytes in the lower airway lumen of a healthy human lung under homeostatic conditions (Byrne et al., 2015; Gordon et al., 2013; Jambo et al., 2014a) and are thought to be integral in defence against pneumococcal infection in the lung (Aberdein et al., 2013; Collini et al., 2018; Gordon et al., 2001; Wilson et al., 2015). Consequently, this has traditionally driven the focus to AM-mediated anti-pneumococcal immune control during HIV infection (Collini et al., 2018). In untreated HIV-infected adults, AMs exhibit normal binding and internalisation of pneumococci (Gordon et al., 2001, 2013). However, whether other airway phagocytes such as neutrophils also show similar behaviour is unclear. Furthermore, the impact of ART on anti-pneumococcal immunity in the airway is also unclear, but it has been shown that ART is associated with increased carriage and increased frequency of pneumococcal-specific Th17 cells (Glennie et al., 2013; Heinsbroek et al., 2015; Peno et al., 2018; Sepako et al., 2014). I therefore hypothesized that airway phagocyte subsets from HIV-infected adults on short-term ART are differentially impaired in their pneumococcal binding and internalisation capacity.

4.1.1. Research question

Are airway phagocytes pneumococcal-binding/internalisation capacity differentially impaired in HIV-infected adults on short-term ART?

4.1.2. Aims and Objectives

Aim

To determine whether HIV-infected adults on short-term ART possess differentially impaired airway phagocyte pneumococcal-binding/internalisation capacity

Specific objectives

1. To determine the proportions of airway phagocyte subsets in asymptomatic HIV-uninfected compared to HIV-infected adults on short-term or long-term ART.
2. To assess the pneumococcal binding and internalisation amongst airway phagocyte subsets in asymptomatic HIV-uninfected adults.
3. To determine the airway phagocyte pneumococcal binding and internalisation in asymptomatic HIV-uninfected adults compared to HIV-infected adults on short-term and long-term ART.

4.2. Methods

4.2.1. Study participants and design

This was a comparative cross-sectional study of asymptomatic adults namely, HIV uninfected, HIV-infected on short-term ART and long-term ART. Participants were recruited from the MLW's Clinical Investigation Unit at the Queen Elizabeth Hospital in Blantyre, Malawi. All participants underwent clinical assessment and a questionnaire was administered to collect information relating to previous respiratory infections and social demographic characteristics (such as high exposure to carbon/soot) that could potentially impact their alveolar cell functional response *ex vivo* before recruitment. The recruitment criteria used has been discussed elsewhere (see Chapter 2.4).

4.2.2. Sample collection and processing

Bronchoalveolar lavage and per nasal swab samples were collected from all participants and transported to the MLW laboratories within 30-minutes of collection. Sample collection and processing have been described (see Chapter 2).

4.2.3. Laboratory assays

The laboratory assays associated with this chapter on the *ex vivo* infection model of primary cells have been described in Chapter 2. In particular, for specific details on fluorescent tagging of pneumococci (2.6.5); opsonisation of *pneumococci* with IgG (2.6.4), BAL processing and resting of cells (2.6.6), BAL *ex vivo* infections (2.6.9) and per nasal swab processing and pneumococci identification (2.6.15).

4.2.3.1. Alveolar cell surface immunophenotyping

Neutrophils and AMs associated with pneumococci were characterised using immunophenotyping. In brief, after harvesting the alveolar cells as described in section 2.6.9, six tubes of opsonised, unopsonised and uninfected cells were immunophenotyped as described in section 2.6.8. Antibody used are described in Table 4.1. Acquisition was done on the BD LSR II Fortessa flow cytometer

(Beckman Dickinson, USA). The gating strategy used is shown in macrophages and neutrophils is shown in Figure 4.1.

Table 4.1. Immunophenotyping antibody panel for alveolar cells.

Cell Markers	Function	Fluorochrome	Concentration	Cells identified
CD66b	Adhesion and activation	APC	2.5:100	Neutrophils
CD163	Scavenger receptor	BV421	3:100	AMs
CD206	Mannose binding receptor	FITC	5:100	AMs
CD19	Transmembrane glycoprotein	PE	2.5:100	B-cells
CMTPX		Qdot-605		ST3

This panel was used to characterise the abundance of AMs and neutrophils from airway samples. Abbreviations: AMs – alveolar macrophages, APC – allophycocyanin, BV – brilliant violet, CD - cluster of differentiation, PE – phycoerythrin, FITC – fluorescein isothiocyanate.

a. Gating strategy for macrophages and neutrophils

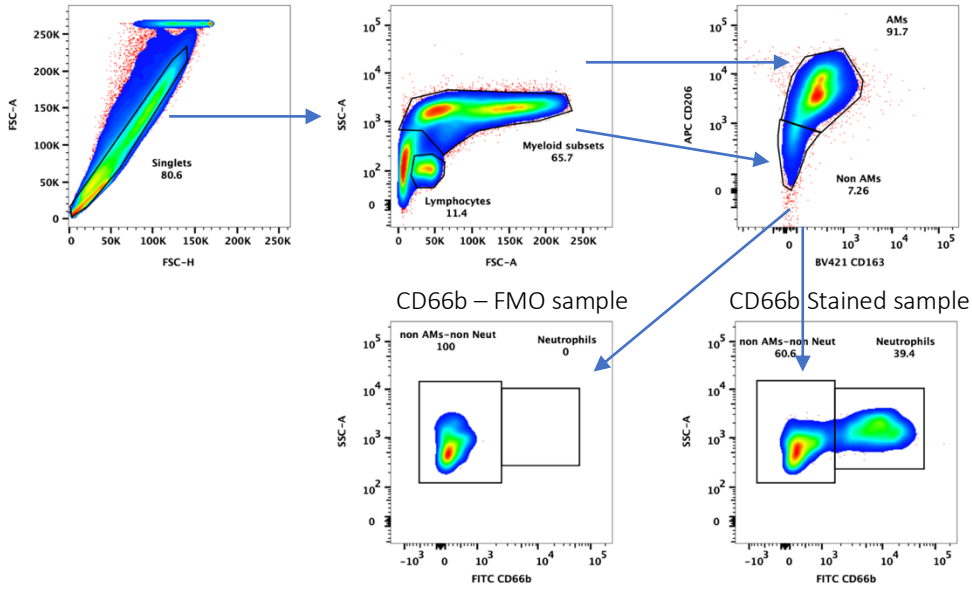


Figure 4.1. **Alveolar macrophage and neutrophil gating strategy.** Alveolar macrophages and neutrophils were identified after exclusion of doublets, debris and lymphocytes. This was after the identification of alveolar phagocytes based on side scatter area (SSC-A) and forward scatter area (FSC-A). AMs were identified as cells expressing CD206 and CD163. In the same panel, non-AMs were identified from cells expressing low levels of CD206 and CD163, and then neutrophils were identified as cells expressing high levels of CD66b. Non-AMs non-Neut were identified as cells expressing low levels of CD206, CD163 and CD66b. A fluorescent-minus-one (FMO) was used to help gate for neutrophils. Abbreviations: AMs – alveolar macrophages, APC – allophycocyanin, BV – brilliant violet, CD – cluster of differentiation, FITC – fluorescein isothiocyanate, FSC-A – forward scatter area, FSC-H – forward scatter height, Neut – neutrophils, PE – phycoerythrin.

b. Gating strategy for the binding and internalisation of pneumococci by AMs

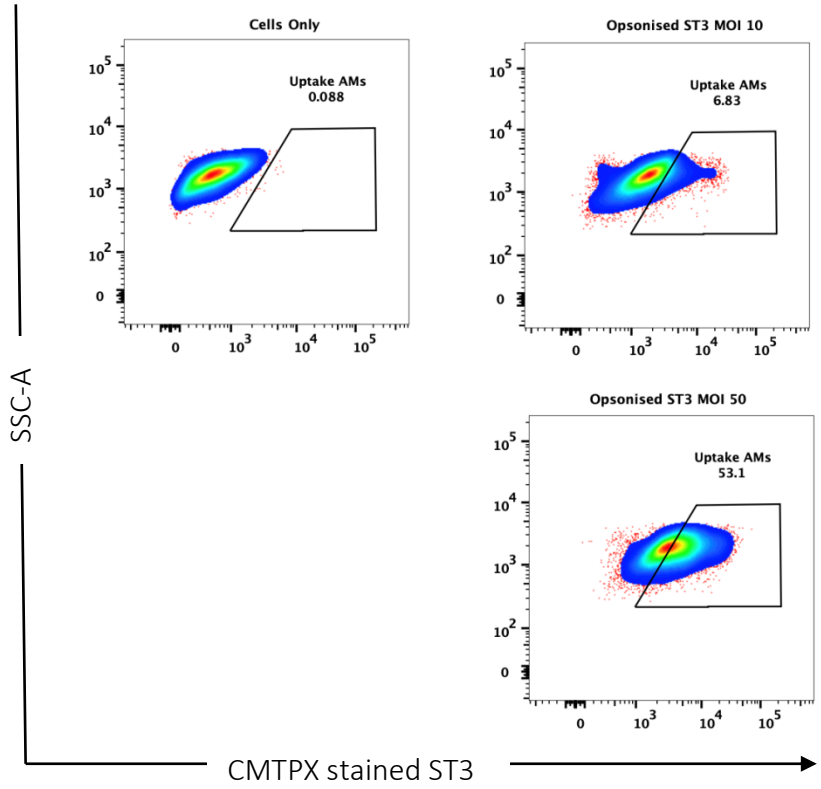


Figure 4.1b. **Gating strategy for pneumococcal-AMs binding and association.** Flow plots comparing IgG opsonised pneumococci at MOI:10 and MOI:50. AMs associated with pneumococci were identified by subtracting the gating frequency of AMs infected with CMTPX stained ST3 from the uninfected AMs. Abbreviations: MOI – multiplicity of infection, AMs – alveolar macrophages, SSC-A – side scatter area.

c. Gating strategy for the binding and internalisation of pneumococci by neutrophils

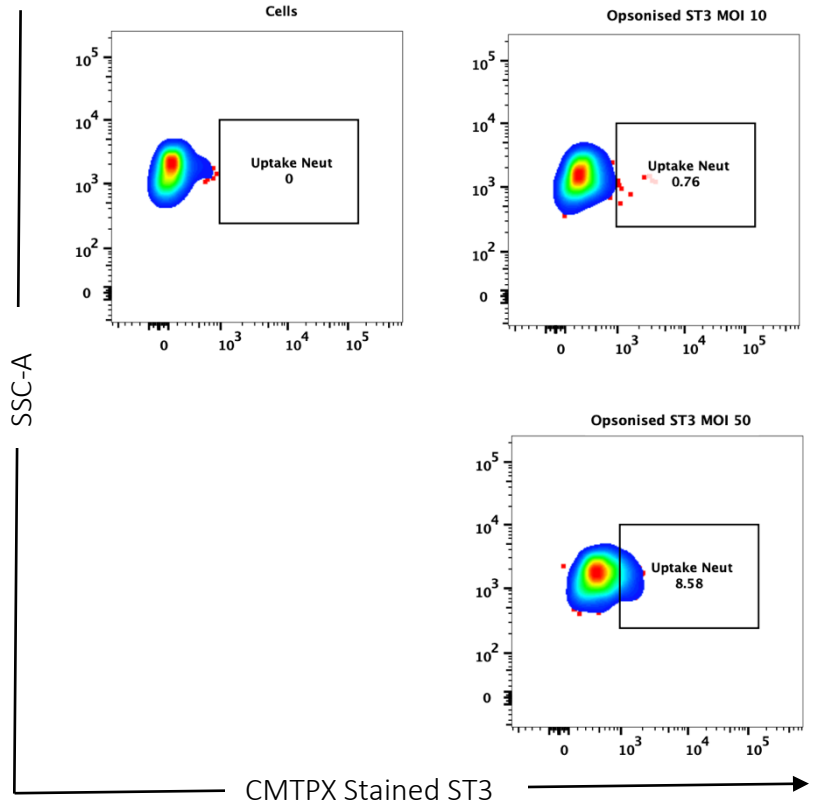


Figure 4.1c. **Gating strategy for pneumococcal-neutrophil binding and association.** Flow plots comparing IgG opsonised pneumococci at MOI:10 and MOI:50. Neutrophils associated with pneumococci were identified by subtracting the gating frequency of neutrophils infected with CMTPX stained ST3 from the uninfected neutrophils. Abbreviations: MOI – multiplicity of infection, Neut – neutrophils, SSC-A – side scatter area.

4.2.3.2. Confocal microscopy

Confocal microscopy was used to demonstrate the association between *S. pneumoniae* with airways cells after infection. The full details have been described elsewhere (see Chapter 2.6.11).

4.2.4. Statistical analysis

Descriptive statistics were used to for continuous variables by calculating medians and interquartile ranges. Groups were compared using non-parametric tests (Wilcoxon rank sum or Wilcoxon signed-rank test, Kruskal Wallis tests) depending on the distribution. For multiple pairwise comparisons the Dunn test was used to adjust for *p*-values. Categorical data were summarised as proportions and compared using the $\chi 2$ or Fisher's exact tests. All statistical tests were two-sided at α value of 0.05 ($p<0.05$*, $p<0.01$**, $p<0.001$***, $p<0.0001$****) and all statistical analyses were done using GraphPad Prism *v*9 software.

4.3. Results

4.3.1. Demographic characteristics of the population

A total of 76 healthy and asymptomatic individuals were recruited into this study, with 31 (41.3%) being HIV-uninfected, 29 (38.6%) HIV-infected and on short-term ART and 16 (21.3%) HIV-infected and on long-term ART. Only 2/29 recruited in the HIV-infected on short-term ART, were ART naïve. All participants had not received any form of pneumococcal vaccine, were negative for both influenza-like illness and influenza infection. The demographic and baseline laboratory data for the study participants are shown in Table 4.2.

Table 4.2. Demographic and laboratory characteristics of the population.

Characteristic	HIV-uninfected (n=31)	HIV+ on short-term ART (n=29)	HIV+ on long-term (n=16)	p-value
Age median (IQR)	33.0 (22 – 41)	31.5 (27.0 – 38.3)	41.0 (38.5 – 46.5)	0.021
Gender (Female) n (%)	11 (35.9)	16 (53.5)	11 (68.8)	0.0001*
CD4+ count median (IQR)	640.0 (515.0 – 829.0)	488.0 (335.0 – 564.0)	619 (399.5 – 724.0)	0.0015
**Individuals with undetectable plasma HIV viral RNA	n/a	10 (37.0)	12 (75.0)	0.0268*
BAL volume median (IQR)	122.0 (108.5 – 135.0)	130.0 (115.0 -140.0)	120.0 (105.0 -135.0)	0.3519
Pneumococcus carriage (%)	8 (25.8)	8 (27.6)	1 (6.25)	0.2210

Analysis were done χ^2- test* for gender but the rest of the analysis was done using Kruskal-Wallis. Abbreviations: ART – antiretroviral therapy, BAL – bronchoalveolar lavage, CD – cluster of differentiation, IQR – interquartile range. **Limit of plasma viral load (log_{10}) <2.42 copies/ml.

The HIV-infected on long-term ART were the oldest of the 3 groups and this was significant when compared with the HIV-uninfected (p=0.0010) and HIV-infected on short-term ART (p=0.0032). Only the HIV-infected on short-term ART had

lower $CD4^+$ counts when compared to the other 2 groups and this was considerable different with the HIV-uninfected individuals (p=0.0008). Plasma HIV viral load was undetectable in 22/43 (51.2%) of the treated HIV-infected individuals on ART, with 75% of HIV-infected on long-term having undetectable viral loads. The median bronchoalveolar lavage fluid volume collected from all participants were similar (p>0.05) amongst all the three groups. The proportions of pneumococcal carriage were similar between the 3 groups (p>0.05).

4.3.2. Proportions of AMs and neutrophils are preserved during short-term and long-term ART.

I sought to investigate the proportional distribution of AMs and neutrophils in the airway and the impact of HIV and ART on the proportions. Bronchoalveolar lavage cells collected from asymptomatic HIV-uninfected, HIV-infected on short-term ART, HIV-infected on long-term ART individuals were stained with fluorochrome-conjugated antibodies and phenotyped using flow cytometry. AMs were the most abundant phagocytes in airway (AMs vs neutrophils; median (interquartile range), 92.12% [IQR; 88.92 – 95.67] vs. 0.58% [0.18 – 1.85], p<0.0001). Furthermore, I compared the proportions of AMs between the 3 groups. I found no significant difference in the proportion of AMs in the HIV-uninfected compared HIV-infected adults on short-term (92.12% vs. 94.74%; p>0.05) or long-term ART (92.12% vs. 90.26%; p>0.05) (Figure 4.3.2). Similarly, there was no significant difference in the proportion of neutrophils in HIV-uninfected adults compared HIV-infected adults on short-term (0.86% vs. 0.49%; p>0.05) or long-term ART (0.86% vs. 0.70%; p>0.05) (Figure 4.2). Taken together these findings show that the proportions of AMs and neutrophils are relatively preserved, irrespective of ART duration.

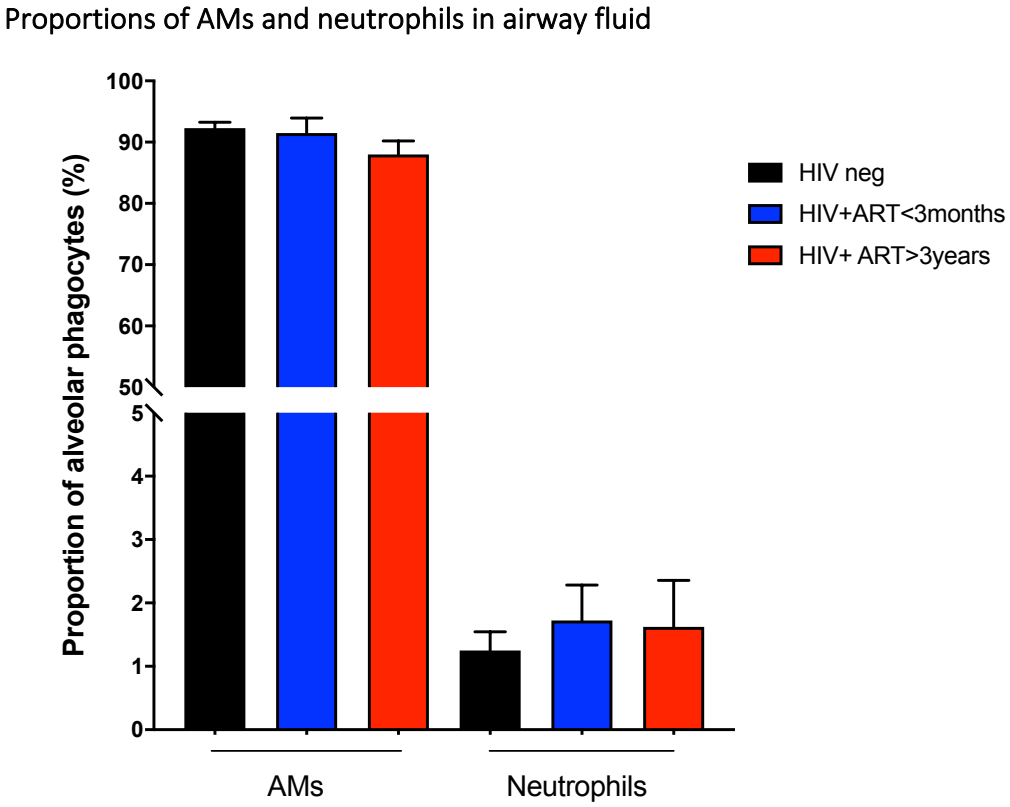


Figure 4.2. **Proportions of AM and neutrophils in BAL fluid collected from asymptomatic HIV-uninfected, HIV-infected on ART <3-months, HIV-infected ART>3-years individuals.** Proportion of AMs, and neutrophils. Bars represent median and interquartile range. All analyses were done using Kruskal Wallis and Dunn Test; p>0.05. HIV-uninfected (n=27), HIV-infected on ART <3-months (n=17), HIV-infected ART>3-years (n=12 individuals). Abbreviations: AMs – alveolar macrophages, ART – antiretroviral therapy, HIV – Human immunodeficiency virus.

4.3.3. Alveolar macrophages exhibit an increased pneumococcal-ST3 association with an increase in multiplicity of infection.

Having observed preserved proportions of AM and neutrophils in airway, even following ART initiation, I then measured the frequency of AMs associated with pneumococcal-ST3 following *ex vivo* infection with fluorescent-labelled bacteria. Human airway cells obtained from asymptomatic HIV-uninfected, HIV-infected on ART <3-months (short-term), HIV-infected ART>3-years (long-term) individuals were infected *ex vivo* with opsonised IgG CMTPX stained pneumococcal -ST3 at MOI 10 and MOI 50. An MOI 50 was associated with an increased AMs-pneumococcal binding/internalisation in all the 3 groups from baseline (MOI 10), median increase in HIV-uninfected (4.03-fold increase, n=27, p<0.0001); short-term ART (4.38-fold increase, n=17, p<0.0001); long-term ART (4.39-fold increase, n=12, p=0.0005) Figure 4.3. These results show a dose-dependent increase in the number of AMs associated with pneumococci following *ex vivo* infection.

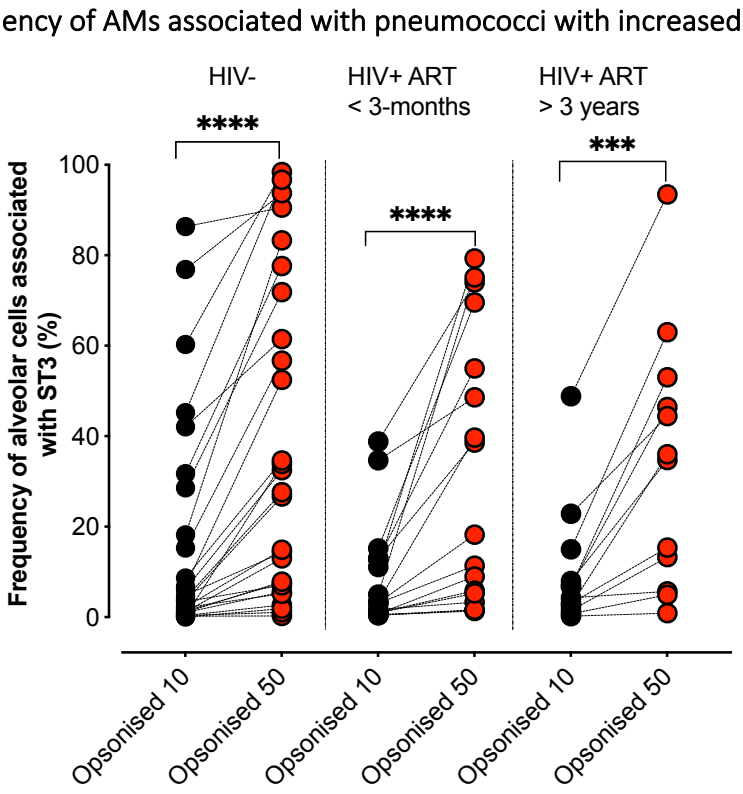
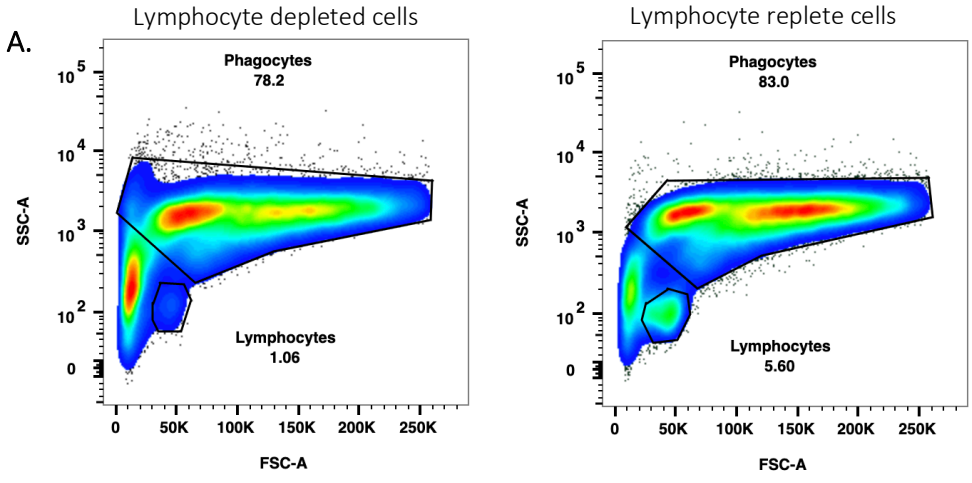


Figure 4.3. Frequency of AMs associated with pneumococcal-ST3 post *ex vivo* infection; asymptomatic HIV-uninfected (n=27), HIV-infected on ART <3months (n=17), HIV-infected ART>3-years (n=12) by Wilcoxon signed-rank test, **** p<0.0001, ***p=0.0005. Opsonised pneumococcal-ST3 bacteria at a multiplicity of infection 10 and 50 were used in all experiments. Abbreviations: AMs – alveolar macrophages, ART – antiretroviral therapy, HIV – Human immunodeficiency virus, ST3 –serotype 3.

4.3.4. Defining the contribution of lymphocytes to the binding/ internalisation of pneumococci by AMs during first hour of infection.

I further investigated the influence of lymphocytes on the binding and internalisation of pneumococci by AMs within the first 1-hour of infection. Airway cells from HIV-uninfected individuals were rested for 2-hours to allow the AMs to adhere to the 12 well plate. Non adherent cells were removed and sorted on the BD FACSAria™ III, to deplete the number of lymphocytes in the non-adherent fraction. Controls (lymphocyte replete fraction) were not passed through the sorter. After sorting, the lymphocyte depleted fraction was centrifuged, supernatant decanted and combined with its cognate adherent cell well faction. The cells were further rested for 2-hours, before being infected with CMTPX stained opsonised pneumococci at MOI 50 for an hour. Post infection, the cells were harvested, phenotyped and acquired on the LSR Fortessa flow cytometer. Depleting the airway fraction of lymphocytes did not affect the binding and internalisation of pneumococci-ST3 by AMs at MOI 10 (median, IQR; 0.8303 [0.5381 – 1.965] vs 0.9911 [0.5921 – 1.763], p>0.9999) and MOI 50 (median, IQR; 1.576 [0.8933 – 5.083] vs 2.191[1.390 – 5.425], p=0.1250) as shown in Figure 4.4. These findings suggest that lymphocyte depletion did not impact AMs binding/internalisation of pneumococci.

Flow plots comparing lymphocyte depleted and replete airway cells



B.

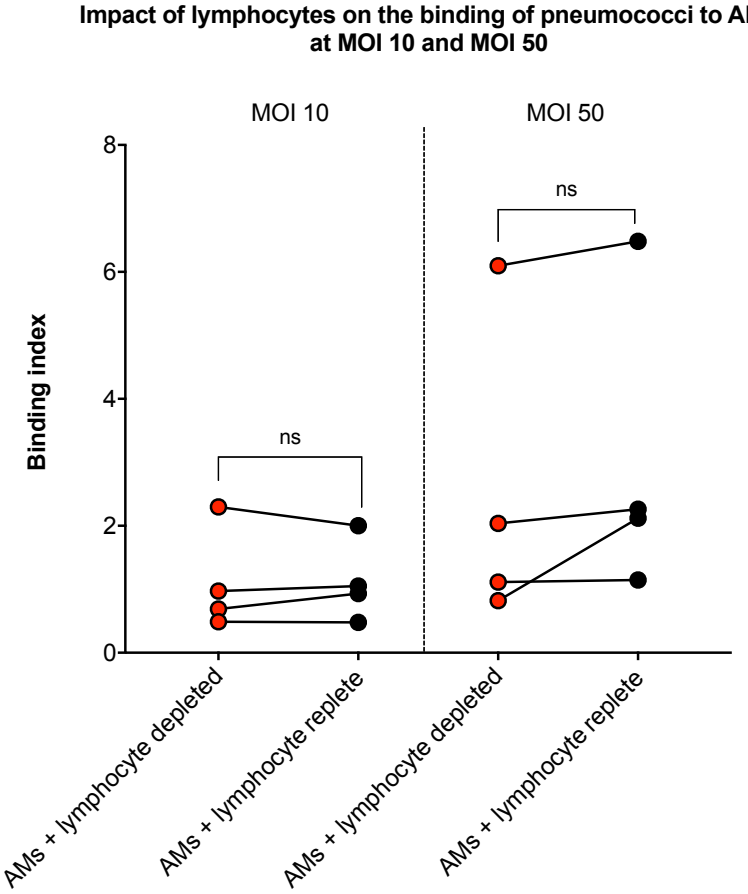


Figure 4.4. Contribution of lymphocytes to the binding of pneumococci by AMs. A. Flow plots comparing lymphocyte depleted and replete airway cells. B. Comparison of pneumococci-ST3 binding by AMs from HIV-uninfected asymptomatic individuals (n=4), by Wilcoxon signed rank test (all p>0.05). Abbreviation: AMs – alveolar macrophages, MOI – multiplicity of infection, ST3 – *serotype* 3.

4.3.5. Evidence of binding and internalisation of pneumococcal-ST3 within alveolar cells post *ex vivo* infection.

To confirm the binding and internalisation of pneumococcal-ST3 by airway phagocytic cells. Airway cells infected with opsonised CMTPX stained pneumococcal-ST3 at MOI 10 and 50 were imaged using confocal microscopy (samples from HIV-uninfected individuals). I observed low binding/internalisation at MOI 10 (Figure 4.5 A) and increased binding/internalisation at MOI 50 (Figure 4.5 B) as demonstrated earlier using flow cytometry. At higher magnification, the images showed pneumococcal-ST3 binding to the airway cells (Figure 4.5 C) and also being internalised in others (Figure 4.5 D). These findings demonstrate that pneumococcal-ST3 binds and is also internalised by airway phagocytes following *ex vivo* infection.

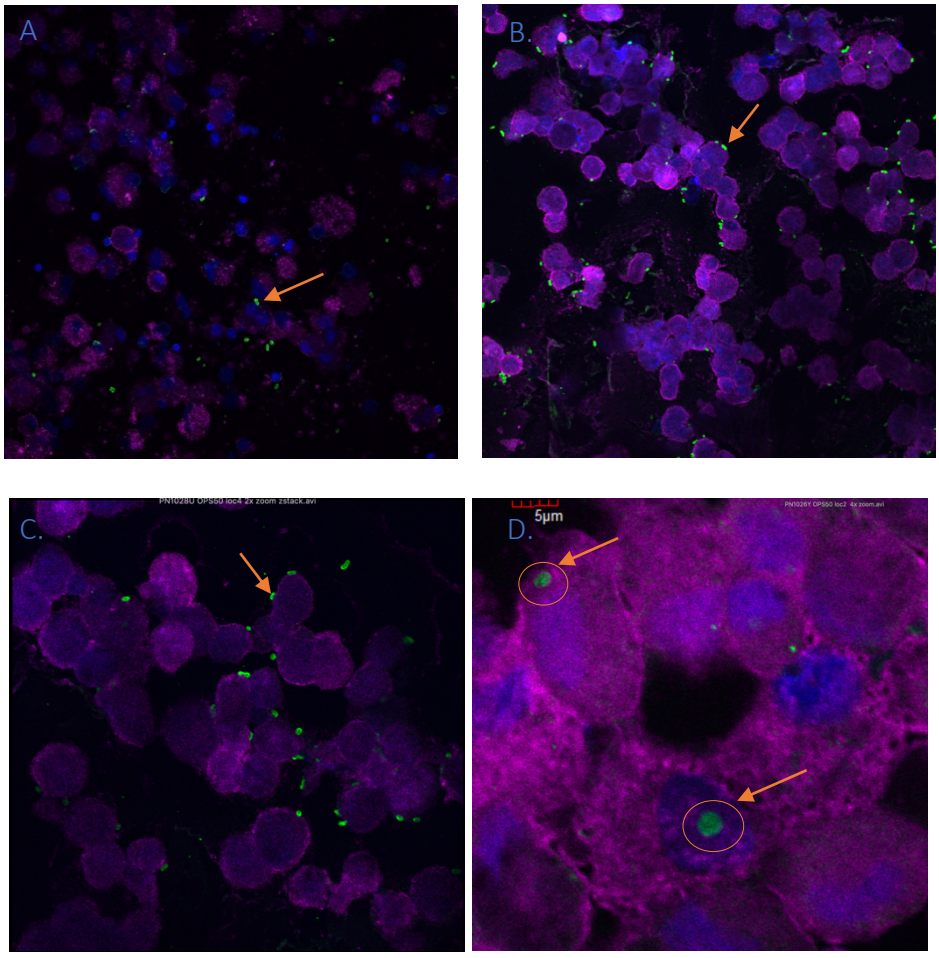


Figure 4.5. Binding and internalisation of pneumococcal-ST3 to airway phagocytes *ex vivo*.

A-B. Representative single fluorescent field images demonstrating the association of alveolar cells (WGA – purple; DAPI – nucleus) with pneumococci-ST3 (bacteria – green) at MOI 10 and 50 (at magnification x63) respectively shown with the arrow (orange). **C**. Representative single fluorescent field of pneumococcal-ST3 at MOI 50 (at magnification x126) demonstrating pneumococci-ST3 (bacteria – green) binding to AMs (WGA – purple; DAPI – nucleus) shown with the arrow (orange). **D**. Representative single fluorescent field of pneumococci-ST3 at MOI 50 (at magnification x 252), with arrow demonstrating internalised pneumococci-ST3 (bacteria – green) within AMs (WGA – purple; DAPI – nucleus) show with the arrow (orange). Abbreviations: DAPI – 4′,6-diamidino-2-phenylindole, MOI – multiplicity of infection, WGA – wheat germ agglutinin.

AMs are the predominant airway phagocytes associated with pneumococcal-ST3 following *ex vivo* infection.

To identify the airway phagocyte population that associates more with pneumococcal-ST3 following *ex vivo* infection. I compared the proportion of airway cells associated with pneumococcal-ST3 within the AMs population when compared to the neutrophil population, amongst the three study groups (Figure 4.6). A higher proportion of AMs were associated with pneumococcal-ST3 when compared to neutrophils in all the study groups and this was statistically significant ($p<0.0001$). These data show that AMs were the predominant airway phagocytes that bound/internalised pneumococcal-ST3 following *ex vivo* infection.

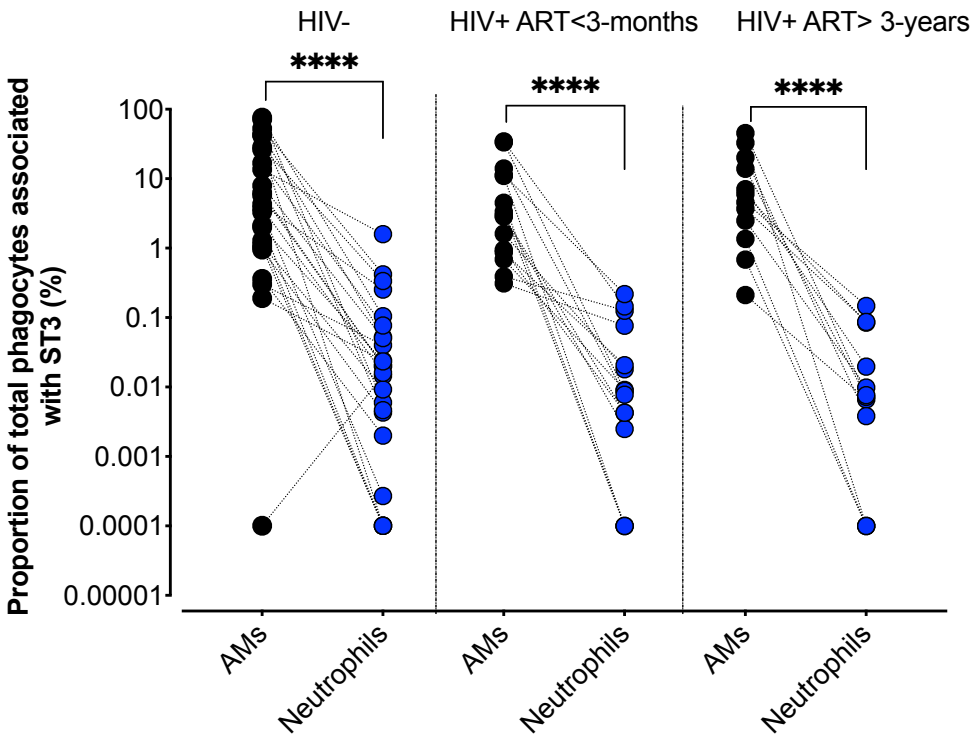
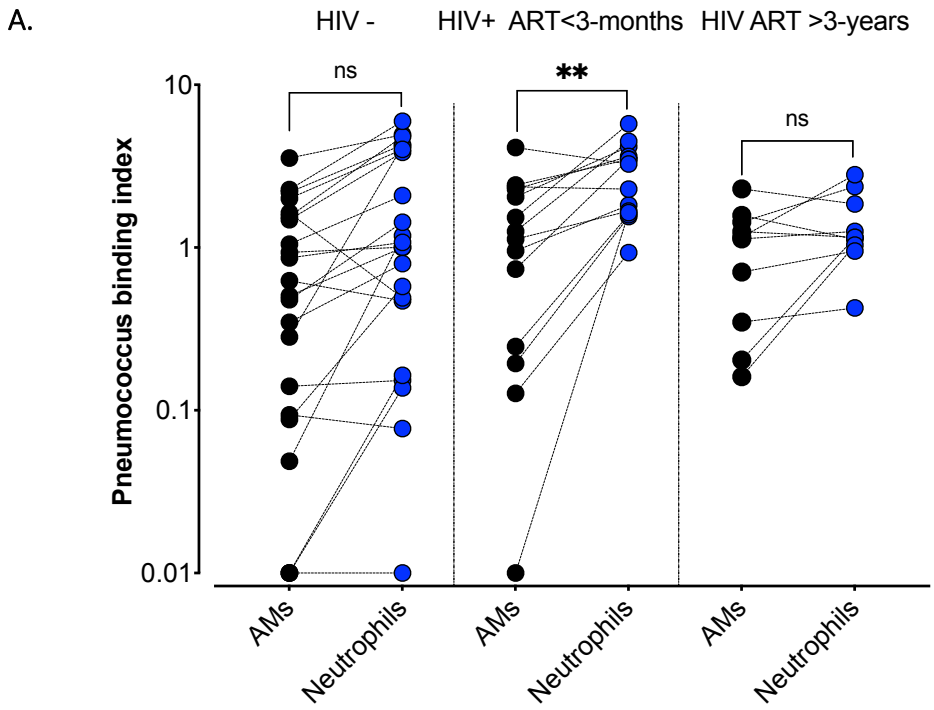


Figure 4.6. **Proportions of airway phagocyte subsets associated with pneumococcal-ST3 *ex vivo*.** Comparison of proportions of AMs and neutrophils associated with pneumococcus at MOI 10 post infection *ex vivo* in asymptomatic HIV-uninfected (n=24), HIV-infected on ART <3months (n=14), HIV-infected ART>3-years (n=11). Wilcoxon signed-rank test was used to compare the populations ****p<0.0001. Abbreviations: AMs – alveolar macrophages, ART – antiretroviral therapy, HIV – Human immunodeficiency virus, ST3 –serotype 3.

4.3.6. Neutrophils from HIV-infected adults on short-term ART exhibit increased binding to pneumococcal-ST3 compared to AMs following *ex vivo* infection.

Next, I investigated the binding index between the two airway phagocyte subsets. The binding index represents the number of bound or internalised bacteria per individual cell. I observed no statistically significant differences in the binding index at lower MOI between AMs and neutrophils (MOI 10, p=0.3224), but statistically significant differences in binding index were observed at higher MOI between AMs and neutrophils (MOI, p=0.0319) amongst the HIV-uninfected individuals (Figure 4.7. A-B). In contrast, for the HIV-infected adults on short-term ART, the pneumococcal-ST3 binding index was significantly higher in neutrophils compared to AMs (MOI 10, p=0.0079; MOI 50, p=0.0037) (Figure 4.7. A-B). Lastly, when I compared the binding index between AMs and neutrophils amongst HIV-infected adults on long-term ART, I found no statistically significant differences at lower and higher MOI (all p>0.05). These data show that neutrophils were equal and, in some cases, better at binding pneumococcus than AMs.



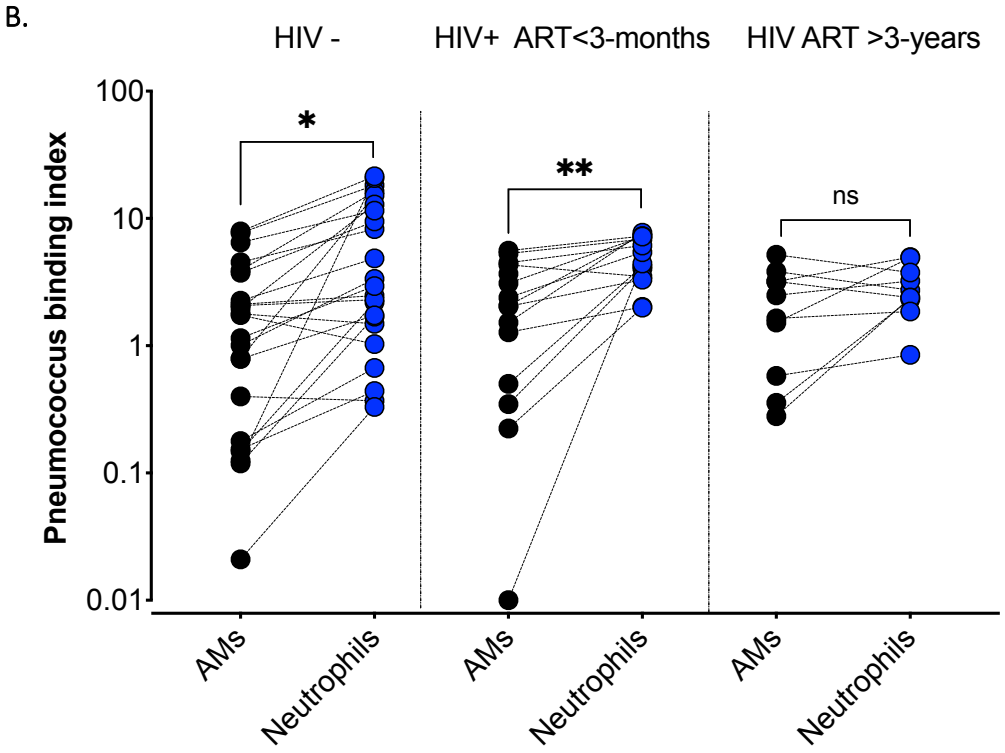


Figure 4.7. **Alveolar phagocyte subsets and pneumococcus binding index *ex vivo* at MOI 10 and 50. A.** Comparison of AMs and neutrophils pneumococcal binding index post *ex vivo* infection in asymptomatic HIV-uninfected (n=24), HIV-infected on ART<3-months (n=14), HIV-infected on ART>3-years (n=11) at MOI 10. **B.** Comparison of AMs and neutrophils pneumococcus binding index post *ex vivo* infection in asymptomatic HIV-uninfected (n=24), HIV-infected on ART<3-months (n=14), HIV-infected on ART>3-years (n=11) at MOI 50. Kruskal-Wallis and Dunn multiple comparison test were done and *p<0.05. Abbreviations: AMs – alveolar macrophages, ART – antiretroviral therapy, MOI – multiplicity of infection.

4.3.7. Proportions of AMs and neutrophils associated with pneumococcal-ST3 are not altered in HIV-infected adults during ART following *ex vivo* infection.

Having demonstrated that pneumococcal-*ST3* associates with alveolar phagocyte either by binding or internalisation, I next investigated whether alveolar phagocytes interaction with pneumococcal-ST3 was altered in HIV-infected adults on ART. I compared the proportion of AMs or neutrophils associated with pneumococcal-ST3 in HIV-uninfected compared to HIV-infected adults on short-term ART or long-term ART. There were no statistically significant differences in the proportion of AMs associated with pneumococcal-ST3 in HIV-uninfected compared to HIV-infected adults on short-term ART or long-term ART at lower or higher MOI (MOI 10, p=0.7036; MOI 50 p=0.7157) (Figure 4.8 A-B). Similarly, there were no statistically significant differences in the proportion of neutrophils associated with pneumococcal-ST3 in HIV-uninfected compared to HIV-infected adults on short-term ART or long-term ART at lower or higher MOI (MOI 10, p=0.5758; MOI 50, p=0.7998) (Figure 4.8). These findings demonstrate that the proportion of AMs or neutrophils associated with pneumococcal-ST3 following *ex vivo* infection is not altered in HIV-infected adults on ART, irrespective of duration.

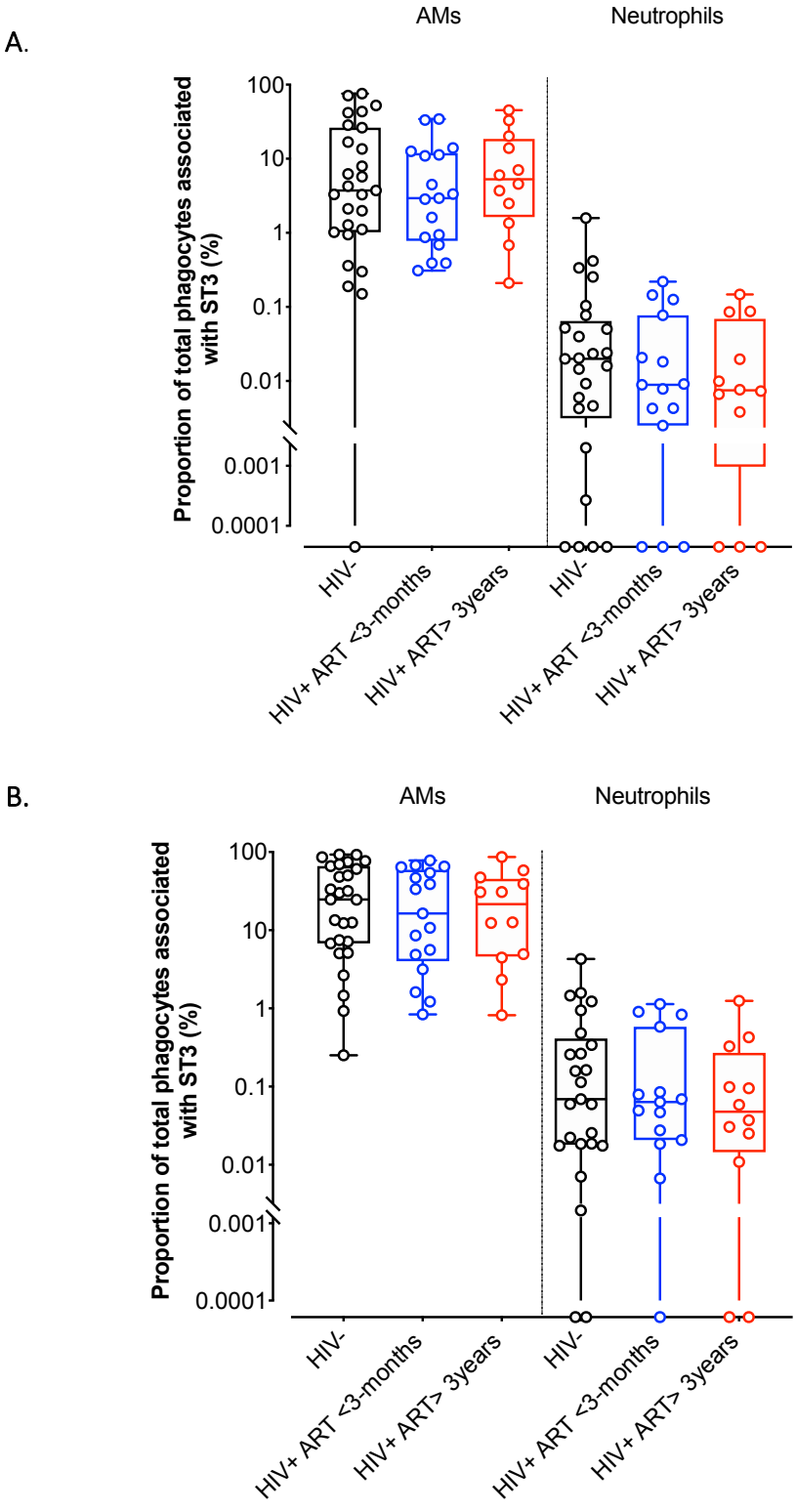
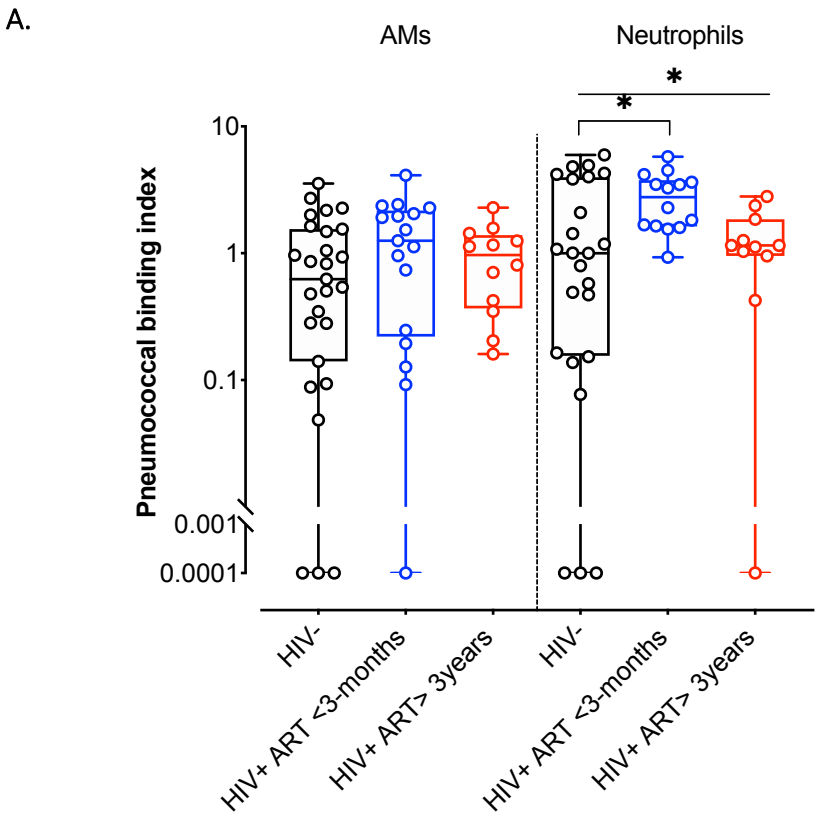


Figure 4.8. **Proportions of alveolar phagocyte subsets associated with pneumococcal-ST3 *ex vivo*.** **A.** Proportion of AMs and neutrophils associated with opsonised pneumococcal-ST3 at MOI 10 post *ex vivo* infection in asymptomatic HIV-uninfected (n=27), HIV-infected on ART <3-months (n=17), HIV-infected ART>3-years (n=12). **B.** Proportion of AMs and neutrophils associated with opsonised pneumococcal-ST3 at MOI 50 post *ex vivo* infection in asymptomatic HIV-uninfected

(n=27), HIV-infected on ART <3-months (n=17), HIV-infected ART>3-years (n=12). Boxplots represent the median (centre line) and interquartile range (box), minima and maxima (whiskers). Kruskal-Wallis and Dunn multiple comparison test were done and p>0.05 in all analysis done. Abbreviations: AMs – alveolar macrophages, ART – antiretroviral therapy, MOI – multiplicity of infection, ST3 –serotype 3.

4.3.8. Neutrophils from HIV-infected adults on short-term ART exhibit increased binding to pneumococcal-ST3 following *ex vivo* infection.

Lastly, I compared the pneumococcal-ST3 binding index of AMs or neutrophils amongst the 3 groups, to observe if it was altered in HIV-infected adults on ART. The AMs binding index was not significantly different between HIV-uninfected and HIV-infected adults on long-term ART at lower MOI and higher MOI (all p>0.05) as shown in Figure 4.9. In contrast, statistically significant differences in binding indices were observed at a lower MOI (MOI 10; 1.77-fold increase, p=0.0378) between HIV-infected adults on short-term ART compared to HIV-uninfected individuals (see Figure 4.9 A). At a higher MOI, there were no statistically significant differences in the neutrophil binding index between HIV-uninfected and HIV-infected adults on short-term or long-term ART (p>0.05). These findings demonstrate differential impact of HIV and ART on neutrophils compared to AMs.



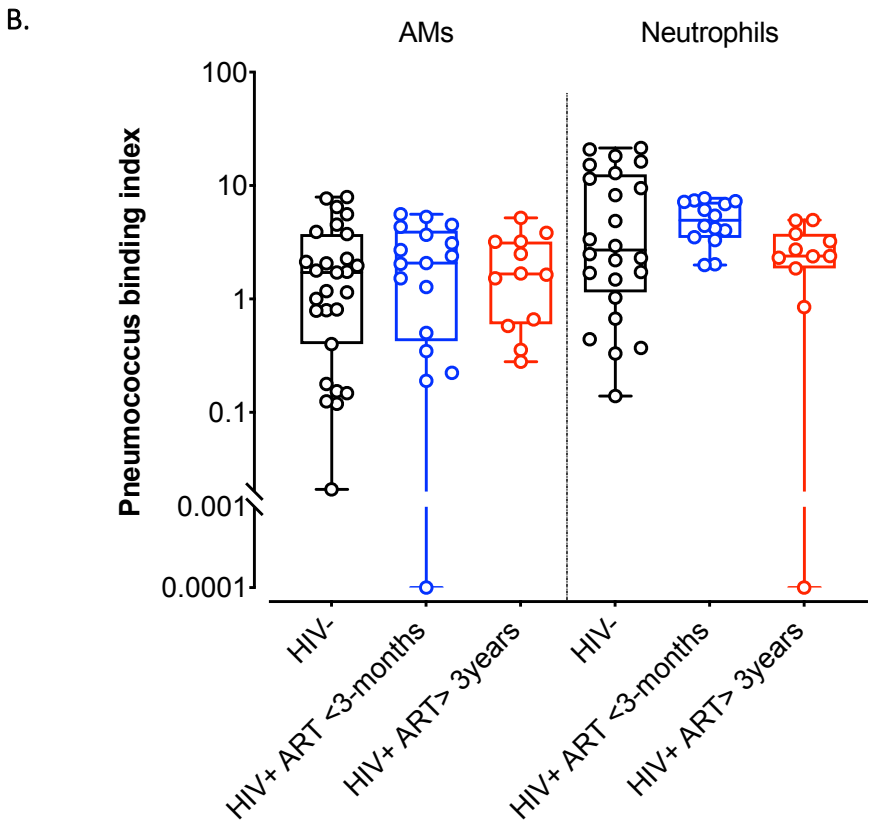


Figure 4.9. **Alveolar phagocyte subsets and pneumococci-ST3 binding index *ex vivo* at MOI 10 and 50. A.** Comparison of alveolar phagocyte subset pneumococcal-ST3 binding index at MOI 10. **B.** Comparison of alveolar phagocyte subset pneumococci-ST3 binding index at MOI 50. Boxplots represent the median (centre line) and interquartile range (box), minima and maxima (whiskers). Asymptomatic HIV-uninfected (n=24), HIV-infected on ART <3months (n=14), HIV-infected ART>3-years (n=11). Kruskal-Wallis and Dunn multiple comparison test were done and *p<0.05, *p=0.0258. Abbreviations: AMs – alveolar macrophages, ART – antiretroviral therapy, HIV – Human Immunodeficiency virus.

4.4. Discussion

In this study, using an *ex vivo* infection model of primary cells, I made several findings regarding the interaction of pneumococcal-ST3 and airway phagocytes. Firstly, I demonstrated that AMs are the principal phagocytic cell in the airway and the major cell associated with IgG-opsonised pneumococcal-ST3 following *ex vivo* infection. Secondly, AMs from HIV-infected adults on short-term or long-term ART do not exhibit impaired binding and internalisation of IgG-opsonised pneumococcal-ST3 with AM. Lastly, I demonstrated differentially enhanced binding of IgG-opsonised pneumococcal-ST3 to neutrophils, but not to alveolar macrophages, in HIV-infected adults on short-term ART.

AMs are known to be critical in the control and clearance of pneumococci in the airway during early infection in mice models, with the efficacy of AMs mediated control being affected by the pneumococci inoculum size or prior respiratory viral infections (Bansal et al., 2018; Camberlein et al., 2015; Ghoneim et al., 2013; Knapp et al., 2003; Mitsi et al., 2019). Consistent with previous findings, AMs were the predominant (~90%) phagocytic cell population, while neutrophils were less abundant but consistently detectable in airway cells of asymptomatic adults (Jambo et al., 2014a; Mwale et al., 2018). AMs were the predominant airway phagocyte associated with binding and internalisation of pneumococcal-ST3 following *ex vivo* infection likely due to their abundance in airway, but neutrophils were also able to bind and internalise pneumococci. Although neutrophils were present at significantly lower frequencies in the airway of asymptomatic adults, their ability to bind and internalise IgG opsonised pneumococcal-ST3 per cell was higher than AMs in HIV-infected adults on short-term ART. This trend was similar in the HIV-uninfected and HIV-infected on long-term ART. During active infection AMs trigger the induction of pro-inflammatory cytokines and chemokines, such as IL-8, at the site of infection resulting in neutrophil recruitment into the lung and causing their activation and degranulation (Domon et al., 2016; Jhelum et al., 2018; Silva and Correia-Neves, 2012). This observed association of pneumococcal-ST3 with AMs and neutrophils supports previous suggestions that these airway

phagocytes play a role in the immune response against pneumococcal lung infection in humans (Byrne et al., 2015; Camberlein et al., 2015; Gordon et al., 2000).

Untreated HIV infection is not associated with impaired AMs binding and internalisation of pneumococci (Gordon et al., 2001, 2013). This is consistent with observations in this study showing similar binding and internalisation of pneumococci by AMs between HIV-uninfected and HIV-infected adults on short-term or long-term ART. This is in agreement with observations that AMs proportions are preserved during HIV infection as shown in this study and others (Hunegnaw et al., 2019; Jambo et al., 2014a). Unlike T cells, AMs are long-lived and are relatively resistant to the cytopathic effects of HIV (Boliar et al., 2019; Collini et al., 2018; Kumar and Herbein, 2014). These results suggest that similar to other AM-mediated processes, surface Fc receptors on AMs responsible for binding and internalisation IgG-opsonised pneumococci are likely less impacted by HIV infection in ART treated individuals.

Interestingly, the pneumococcal-ST3 binding index on neutrophils was higher in the HIV-infected adults on short-term ART than in HIV-uninfected individuals. Enhanced binding and internalisation of neutrophils could be attributed to immune activation. Basal hyperactivation has been observed in peripheral blood neutrophils in HIV-infected individuals on ART (Campillo-Gimenez et al., 2014). Activation of neutrophils either by infection or inflammatory cytokines (TNF, IFN-γ or granulocyte colony stimulating factor) is known to drive an upregulation of Fc receptors (CD64 and CD32A) leading to an efficient binding of IgG-opsonised bacteria (Alemán et al., 2016; Guyre et al., 1990; Schiff et al., 1997; Wang and Jönsson, 2019). On the other hand, previous studies have shown that HIV impairs several functions of peripheral neutrophils including chemotaxis, down regulation of the FcγRIII/CD16, phagocytosis, bactericidal activity and oxidative burst (Campillo-Gimenez et al., 2014; Elbim et al., 1994; Heit et al., 2006; Lazzarin et al., 1986). Whether the phagocytic and killing potentials of these tissue-associated

neutrophils is preserved or altered by HIV remains to be explored and warrants further investigation (Hensley-McBain and Klatt, 2018).

This study had some limitations, the lungs are complex organs with various cells and antimicrobial proteins involved in the clearance of pneumococci. Current findings were only limited to AMs and neutrophils airway phagocytes and might not perfectly reflect the interaction of pneumococcus and alveolar phagocytes *in vivo*. Second, although these findings were exclusively based on experiments with *S. pneumoniae*-ST3 from the over 96 pneumococcal serotypes, but they are likely to have broad impact and important implications on other pneumococcus serotypes. Third, these findings are derived from a fairly young healthy adults, who are "not at high risk" of pneumococcal disease and might not reflect the changes in high risk groups such as elderly or children under the age of 5 years, but have a broader impact on pneumococcal responses in treated HIV-infected individuals. Lastly, it would have been ideal to include a population of ART-naïve HIV-infected adults, however due to the HIV Test and Treat policy, it was extremely challenging to recruit this group.

4.5. Conclusion

In conclusion, these findings suggest that AMs and neutrophils are not differentially impaired in their pneumococcal binding capacity in HIV-infected adults on short-term or long-term ART. Neutrophils were however associated with enhanced binding and internalisation capacity during short-term ART. The preserved *S. pneumoniae* binding and internalisation capacity of AM provides a potential cell to target for vaccine development or immunological boosting in the control of pneumococcus in the lung. Nonetheless, binding and internalisation is the first step in control against extracellular pathogens, the next step is intracellular killing of the internalised bacteria, and this could also be a potential step that could be impacted by HIV infection. In the next chapter, I will investigate AM-mediated intracellular killing of pneumococci and whether this is different in HIV-uninfected compared to HIV-infected adults on ART.

CHAPTER 5

5.0. Alveolar macrophage intracellular killing and within cell survival of *S. pneumoniae* in asymptomatic adults residing in Malawi.

5.1. Introduction

HIV infection is known to infect and impair a variety of cell types including $CD4^+$ T-cells, dendritic cells, macrophages and monocytes, and it's also known to affect bystander cells (Benichou and Bouchet, 2018; Boliar et al., 2019; Collins et al., 2015; Doitsh and Greene, 2016; Richard et al., 2018; Wong et al., 2019). Evidence from asymptomatic HIV-infected individuals living in high transmission settings show that pneumococcal-specific antibodies and pneumococcal-specific $CD4^+$ T cells are preserved in the airway (Collins et al., 2013; Eagan et al., 2007; Gordon et al., 2003; Jambo et al., 2011; Peno et al., 2018). Untreated HIV infection has been shown not to impair the binding and internalisation of *S. pneumoniae* by alveolar macrophages (AMs) (Gordon et al., 2001, 2013). I have shown in the previous chapter that AMs and neutrophils are not differentially impaired in their pneumococcal binding and internalisation capacity in HIV-infected adults on short-term or long-term ART (Chapter 4).

AMs phagocytosis and clearance of *S. pneumoniae* from the lungs is enhanced by the opsonisation of bacteria by complement and antibody (Cole et al., 2014; Dockrell et al., 2003b; Gordon et al., 2000). Internalisation of opsonised *S. pneumoniae* facilitates intracellular killing bacterial (Aberdein et al., 2013; Collini et al., 2018). In murine models of pulmonary infection, AMs clear *S. pneumoniae* to a defined threshold without overt features of pneumonia but when AMs fail to control these subclinical infections this results in the recruitment of neutrophils and other inflammatory cells to aid in controlling the infection (Aberdein et al., 2013; Dockrell et al., 2003b; Duan et al., 2012). On the other hand, uncontrolled inflammation in the lung results in the clinical syndrome of pneumonia, and this

highlights the role of AM-mediated killing threshold as an important factor in protection against pneumococcal pneumonia.

In humans, HIV persists in the lung even during suppressive ART (Costiniuk et al., 2018; Honeycutt et al., 2017). It has also been shown that HIV gp120 impairs the intracellular late killing of *S. pneumoniae* by AMs in HIV-infected adults on ART in a low pneumococcal transmission setting (Collini et al., 2018). It has been reported that *S. pneumoniae* is able to replicate within $CD169^{+}$ metalophillic splenic macrophages, after an eclipse period from the blood (Ercoli et al., 2018). AMs express CD169 (Morrell et al., 2018) and have been shown to harbour pneumococci for months following human experimental challenge (Mitsi et al., 2019). However, the extent to which HIV persistence impacts the AMs threshold of *S. pneumoniae* control in high pneumococcal transmission setting remains unclear. Furthermore, whether pneumococci evasion of AM intracellular killing is enhanced in HIV-infected adults on ART is still unclear. Therefore, **I hypothesised that pneumococci evade AM intracellular killing leading to their intracellular survival, and this is exacerbated during HIV infection even following ART initiation.**

5.1.1. Research question

Does *S. pneumoniae* evade alveolar macrophage killing leading to their sustained intracellular survival, and is this exacerbated by HIV infection?

5.1.2. Aims and Objectives

Aim

To determine whether HIV infection is associated with impaired AM killing of *S. pneumoniae* leading to intracellular bacteria survival.

Specific objectives

1. To determine the human AMs intracellular killing kinetics of internalised *S. pneumoniae ex vivo* in HIV-uninfected adults.
2. To compare the human AMs intracellular killing kinetics of internalised *S. pneumoniae* between HIV-uninfected individuals and HIV-infected adults on short-term or long-term ART.
3. To investigate the impact of active pneumococcal carriage on human AM control of *S. pneumoniae ex vivo.*

5.2. Methods

This section covers some of the methods used in chapter 4 as the same group of participants were used though the analysis done was different.

5.2.1. Study participants and design

This was a comparative cross-sectional study of asymptomatic HIV uninfected, HIV-infected on ART<3-months and ART>3-years. Participants were recruited from the Malawi-Liverpool Wellcome-Trust's (MLW) Clinical investigation unit at the Queen Elizabeth Hospital in Blantyre, Malawi. All participants completed a specific questionnaire relating to their previous clinical history and social demographic characteristics that could potentially impact their alveolar cell functional response *ex vivo* before recruitment. The recruitment criteria used has been discussed elsewhere (see Chapter 2.4).

5.2.2. Ethical approval and study clearance

The study received ethical approval from both College of Medicine Research Ethics Committee (COMREC), Malawi and Liverpool School of Tropical Medicine Research Ethics Committee (LSTMREC), United Kingdom and this together with the recruitment criteria used have previously been described (see Chapter 2.3).

5.2.3. Sample collection and processing

Bronchoalveolar lavage and per nasal swab samples were collected from all participants and transported to the MLW laboratories within 30-minutes of collection. Sample collection and processing have been described (see Chapter 2).

5.2.4. Laboratory assays

The laboratory assays associated with this chapter have been described in Chapter 2. In particular for specific details *S. pneumoniae* growth inhibition (2.6) and gentamicin protection assay (2.6); BAL processing and resting of cells (2.6.6), BAL *ex vivo* infections (2.6.9) and per nasal swab processing and *S. pneumoniae* identification (2.6.15), identification of intracellular *S. pneumoniae* through confocal microscopy (2.6).

5.2.5. Statistical analysis

Descriptive statistics were used to for continuous variables by calculating medians and interquartile ranges. Groups were compared using non-parametric tests (Wilcoxon rank sum or Wilcoxon signed-rank test, Kruskal Wallis tests) depending on the distribution. For multiple pairwise comparisons, the Dunn test was used. Categorical data were summarised as proportions and compared using the $\chi 2$ tests. The relationship between within AMs pneumococci and extracellular pneumococci during the late killing phase was estimated using the Pearson correlation. All statistical tests were two-sided and were deemed statistically significant at an α value of 0.05 ($p<0.05$*, $p<0.01$**, $p<0.001$***, $p<0.0001$****) and all analyses were done in GraphPad Prism *v*9 software.

5.3. Results

5.3.1. Intracellular survival of *S. pneumoniae* in *ex vivo* infected alveolar macrophages from HIV-uninfected individuals.

As earlier demonstrated in Chapter 4, AMs are the main phagocytic population associated with *S. pneumoniae* following *ex vivo* infection. I sought to investigate the human AMs intracellular killing kinetics of internalised *S. pneumoniae* in asymptomatic adults (n=31) following addition of gentamicin (100μg/ml), 1-hour post infection (the dose shown to kill extracellular pneumococci). As expected, I found a significant reduction of intracellular bacteria burden 3-hours post infection (1hr vs 2hrs post infection, 3267[IQR; 500 – 15644] vs 84 [IQR; 10 – 419] colony forming units (cfu)/ml, $p<0.0001$; 1hr vs 3hrs post infection, 3267 [IQR; 500 – 15644] vs 10 [IQR; 10 – 83] cfu/ml, $p<0.0001$) (Figure 5.1 A). However, no significant difference was observed in intracellular bacteria counts between 2-hours and 3-hours post infection ($p>0.05$). This demonstrates that AM-mediated intracellular killing of *S. pneumoniae* is rapid and happens predominantly within the first three hours following bacterial internalisation.

It was observed that the 3hr time point was the "eclipse phase" with 61.2% (n=18) of the individuals having no microbiologically detectable *S. pneumoniae* within their AMs (limit of detection 33 cfu/ml). However, I observed an outgrowth of intracellular *S. pneumoniae*-ST3 bacteria in 41.9% (n=13) of the individuals 24-hours post-infection. Individuals with *S. pneumoniae* present within their AMs 24-hours post-infection, were termed "persisters" and those that completely cleared *S. pneumoniae*-ST3 within their AMs as "non persisters". I then decided to investigate if the early killing kinetics represented by the area under the curve (AUC) for the time points (1 – 3hrs) could help in distinguishing between persisters and non-persisters. Persisters had a higher AUC value compared to the non-persisters (7339 [IQR; 2022 – 9696] vs. 972.5 [IQR; 146.8 – 2494], $p=0.0118$; Figure 5.1B), demonstrating that persisters tend to kill intracellular *S. pneumoniae* at a slower rate compared to the non-persisters. These findings demonstrate that slower early killing kinetics of intracellular *S. pneumoniae* in AMs is associated

with intracellular bacterial survival, which could render AM as potential reservoirs for pneumococcal infection.

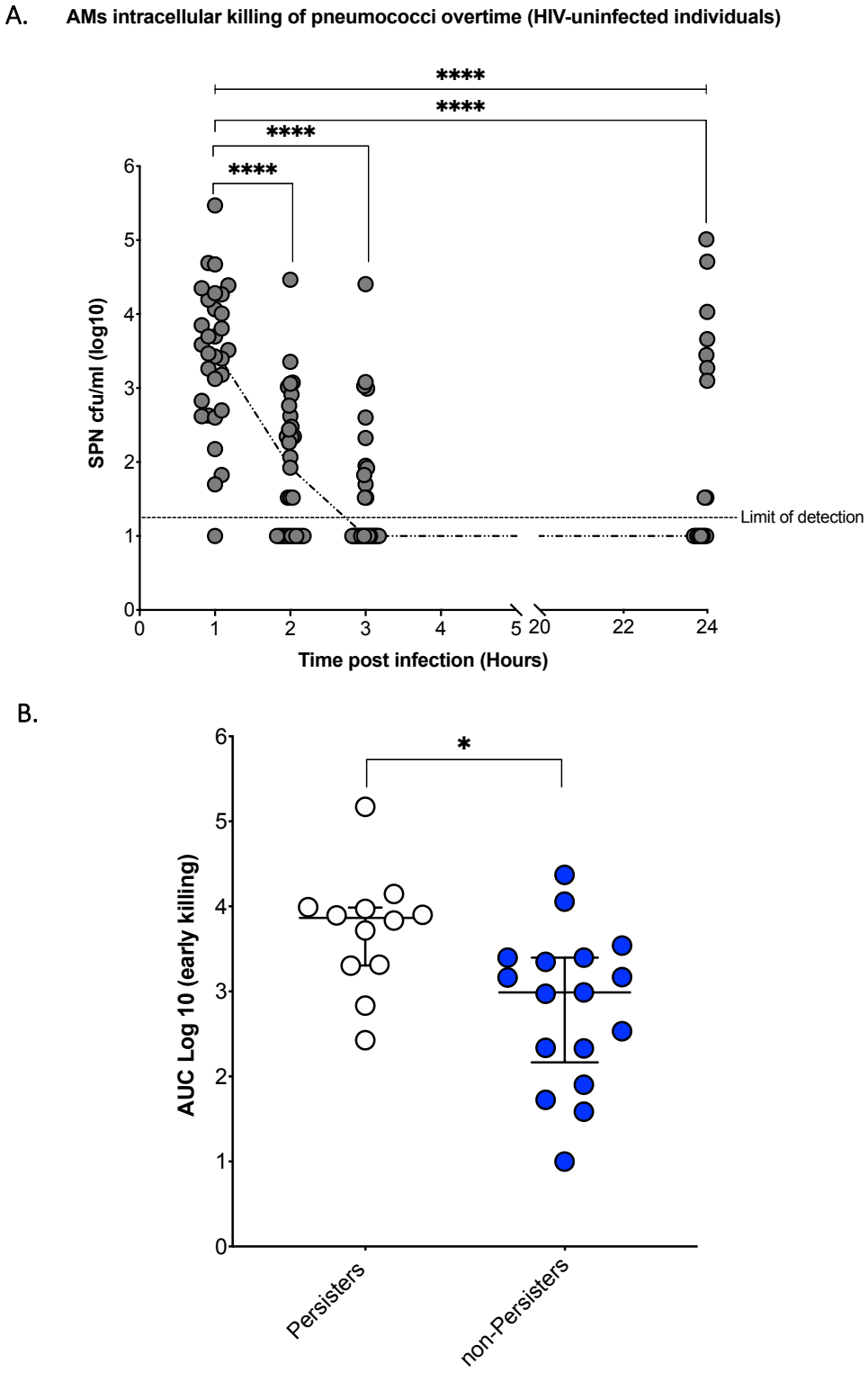


Figure 5.1. AMs *S. pneumoniae*-ST3 intracellular killing kinetics in HIV-uninfected individuals (n=31). A. Comparison of the reduction in AMs intracellular *S. pneumoniae*-ST3 overtime between

1-hour and 24-hours post infection; by Kruskal Wallis and Dunn multiple comparison test, ****p<0.0001, ***p=0.0002. **B.** Comparison of the early killing kinetics of persisters and non-persisters as demonstrated by the area under the curve (AUC), by Wilcoxon rank sum test, *p=0.0118. Abbreviations: AUC – area under the curve, cfu – colony forming units, SPN – *S. pneumoniae*, HIV – Human immunodeficiency virus.

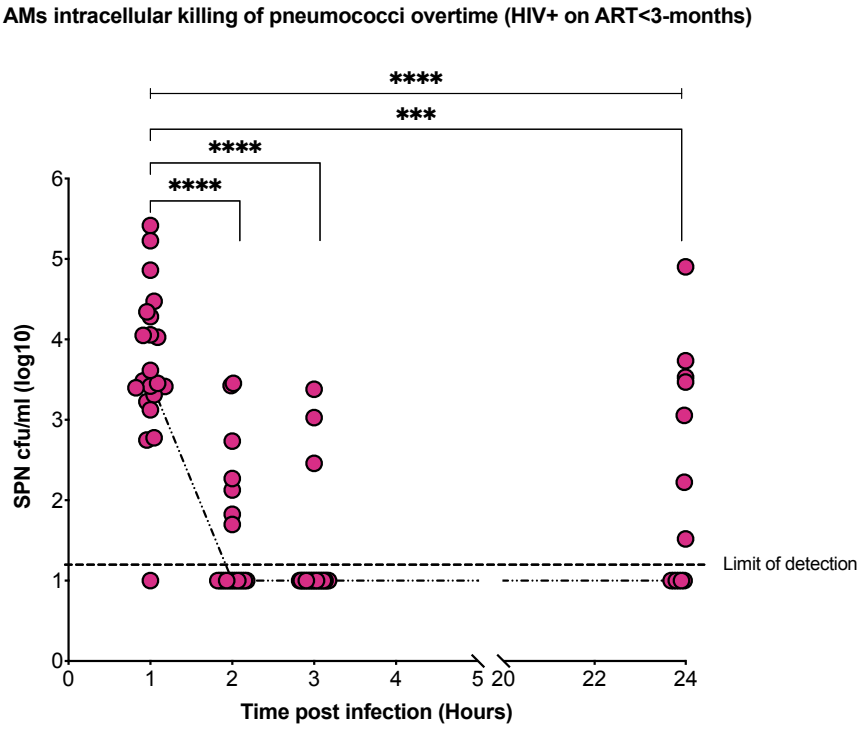
5.3.2. Intracellular survival of *S. pneumoniae* in *ex vivo* infected alveolar macrophages of HIV-infected adults on short-term and long-term ART.

To investigate the human AMs intracellular killing kinetics of internalised *S. pneumoniae* in asymptomatic HIV-infected adults on short-term and long-term ART. AMs were infected with *S. pneumoniae*-ST3 and then exposed to gentamicin (100μg/ml) 1-hour post infection as above. Among short-term ART participants, I found a significant reduction of intracellular bacteria burden 3-hours post infection, (1hr vs 2hrs post infection, [3039 (IQR; 1862 – 20622) vs 10 (IQR; 10 – 100.5) cfu/ml, p<0.0001; 1hr vs 3hrs post infection 10 [IQR; 10 – 2400] cfu/ml, p<0.0001, Figure 5.2A). However, no difference was observed in intracellular bacteria counts between 2-hrs and 3-hrs post infection (p>0.05). Between 2-hours and 3-hours post infection, 67% and 86% of the individuals did not have any microbiologically detectable *S. pneumoniae* within their AMs, respectively. In those on long-term ART, I observed a significant reduction intracellular bacterial burden 3hrs post infection, (1hr vs 2hrs post infection, 1677 [IQR; 133 – 7200] vs 10 [IQR; 10 – 62.25] cfu/ml, p<0.0214; 1hr vs 3hrs post infection, 1677 [IQR; 133 – 7200] vs. 10 [IQR; 10 – 10] cfu/ml, p=0.0050; Figure 5.2B). Between 2hrs and 3hrs, I did not observe any significant differences in viable intracellular *S. pneumoniae* load post infection. At 2hrs and 3hrs post infection, 70% and 100% of the individuals had no microbiologically detectable *S. pneumoniae* within their AMs, respectively.

Furthermore, similar to earlier observations of persisters within the HIV-uninfected group, I also observed an outgrowth of intracellular *S. pneumoniae*-ST3 bacteria, in 43% (n=9) of AMs from individuals on short-term ART, and 30% (n=3) in individuals on long-term ART, 24-hrs post-infection. I further compared the early killing kinetics (AUC) for the time points (1-hr – 3-hrs) between the persisters and non-persisters. In contrast to the HIV-uninfected group, I did not observe any difference between the early killing kinetics of persisters and non-persisters during short-term ART (2230 [IQR; 785.3 – 60528] vs 1491 [IQR; 880.5 –

9392], p=0.4639) or long term ART (2429 [IQR; 1112 – 6952] vs 1062 [IQR; 528.5 – 2624], p=0.2667) see Figure 5.2 C. Taken together, these findings demonstrate that survival of *S. pneumoniae* within AMs 24-hours post infection is a common phenomenon among the 3 participant groups.

A.



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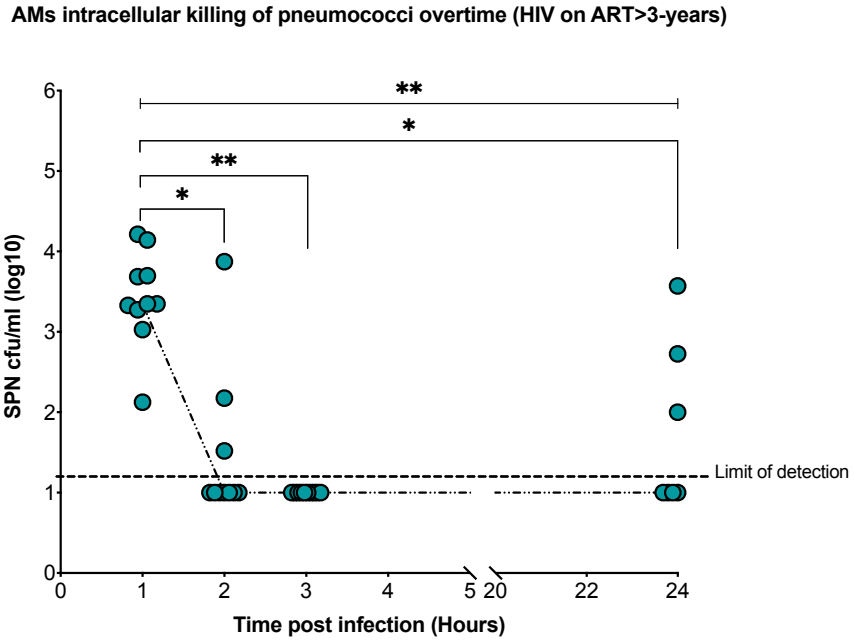


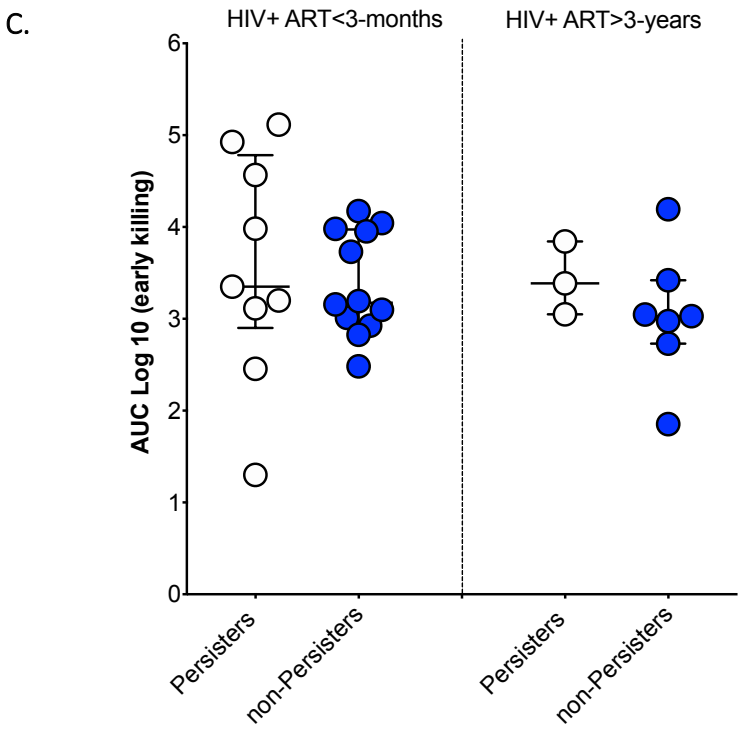
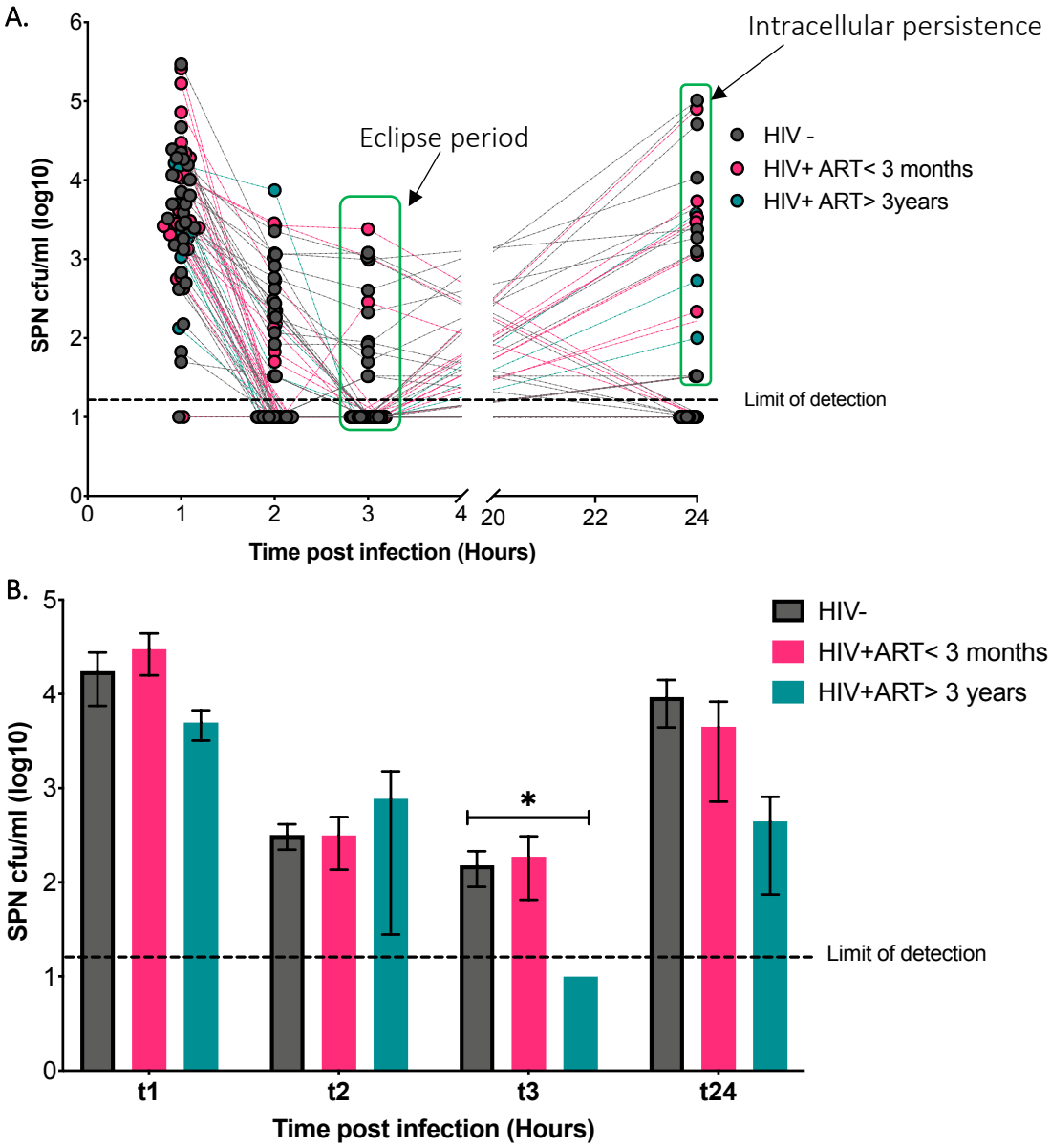


Figure 5.2. AMs *S. pneumoniae*-ST3 intracellular killing kinetics in HIV-infected individuals on short-term and long-term ART. **A**. Comparison of the reduction in AMs intracellular *S. pneumoniae-ST3* overtime between 1-hour and 24-hours post infection in HIV-infected adults on short-term ART; by Kruskal Wallis and Dunn multiple comparison test, ****p<0.0001, ***p=0.0002 (n=21). **B**. Comparison of the reduction in AMs intracellular *S. pneumoniae-ST3* overtime between 1-hour and 24-hours post infection in HIV-infected adults on long term ART; by Kruskal Wallis and Dunn multiple comparison test, **p=0.0030, **p=0.0050, *p=0.0214, *p=0.017 (n=10). **C**. Comparison of the early killing kinetics of persisters and non-persisters as demonstrated by the area under the curve (AUC) in HIV-infected adults on ART< 3-months and HIV-infected adults on ART>3-years, by Wilcoxon rank sum test, p>0.05. Abbreviations: AMs – alveolar macrophages, AUC – area under the curve, ART – antiretroviral therapy, SPN – *S.s pneumoniae*.

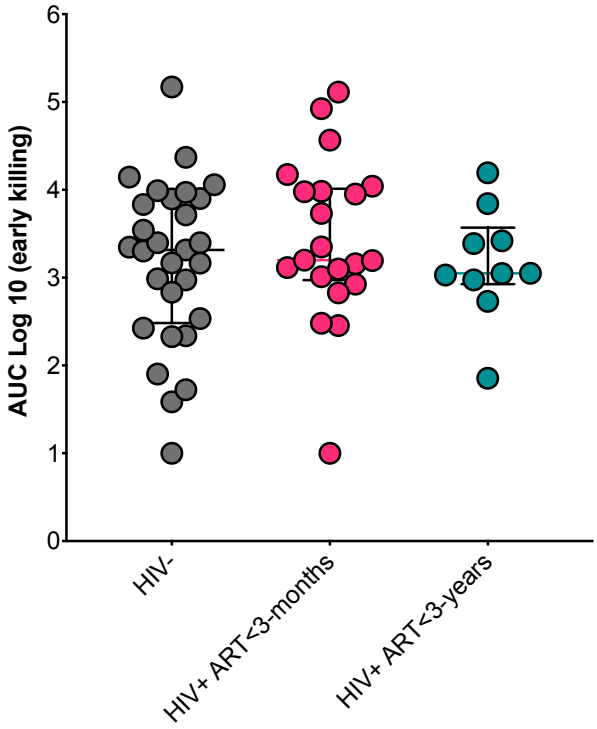
5.3.3. Early or long-term ART is not associated with impaired early intracellular AMs killing of *S. pneumoniae*.

To determine whether HIV-infected individuals on short-term or long-term ART exhibit altered killing of intracellular *S. pneumoniae* within AMs, I compared the microbiologically viable intracellular *S. pneumoniae* between the 3 groups (n=62) at each time point. Analyses of intracellular *S. pneumoniae* bacteria load at 1hr, 2hrs and 24hrs post infection showed no statistically significant difference among the three participant groups (all p>0.05). However, during the eclipse phase (Figure 5.3. A – B), the intracellular killing burden of *S. pneumoniae* was significantly different between the 3 groups (Kruskal-Wallis p=0.0332), with a marginal difference observed in the intracellular bacterial burden (3hrs post infection) between the HIV-uninfected adults and the HIV-infected individuals on long-term ART (10 [IQR, 10 – 83] vs 10 [10 – 10] cfu/ml; p=0.0530).

Next, I compared the early AM mediated killing kinetics amongst the three groups as measured by the AUC (1hr – 3hrs post infection). There was no statistically significant difference in the early killing kinetics amongst the three study groups (p>0.05). Even after pooling the groups of HIV-infected individuals on ART together, there was still no statistically significant differences in AMs-mediated early killing kinetics between HIV-uninfected adults and the ART group, (AUC: 1552 [IQR, 935 – 9536] vs 2058 [IQR, 294 – 7906]; p=0.6515, see figure 5.3 D). These findings show that AMs from individuals on short-term or long-term ART kill intracellular bacteria equally the same as those from HIV-uninfected during early pneumococcal infection.


A.
Intracellular persistence
Eclipse period
HIV -
HIV+ ART< 3 months
HIV+ ART> 3years
Limit of detection
SPN cfu/ml (log10)
Time post infection (Hours)
B.
HIV-
HIV+ART< 3 months
HIV+ART> 3 years
*
Limit of detection
SPN cfu/ml (log10)
t1
t2
t3
t24
Time post infection (Hours)

C.



D.

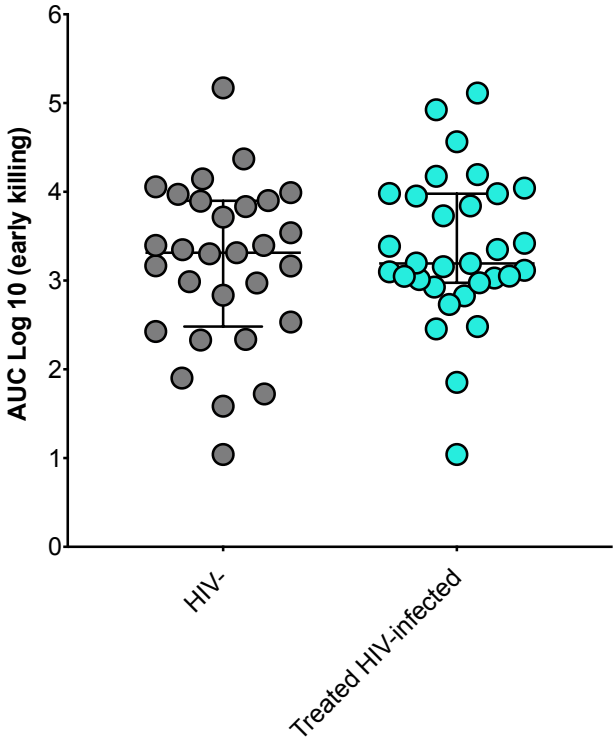


Figure 5.3. **Comparison of intracellular bacterial killing between HIV-uninfected adults and HIV-infected individuals on ART. A-B**. Comparison of intracellular bacterial load killing between HIV-negative, HIV-infected on ART<3-months and ART>3months (1hr, 2hrs, 3hrs, 24hrs post infection), HIV-uninfected (n=31), HIV-infected individuals ART<3-months (n=21), HIV-infected individuals ART>3years (n=10); analysis by Kruskal Wallis and Dunn multiple comparison test, *p<0.0332. **B**. A comparison of early intracellular bacterial killing kinetics represented by the area under the curve

between the 3 groups (HIV-uninfected (n=31), HIV-infected individuals ART<3-months (n=21), HIV-infected individuals ART>3years (n=10); analysis by Kruskal Wallis and Dunn multiple comparison test, p>0.05. **C**. A comparison of early intracellular bacterial killing kinetics represented by the area under the curve between the HIV-uninfected (n=31) and HIV-infected on ART individuals (n=31), (ART<3-months and ART>3-years, n=31); analysis by Kruskal Wallis and Dunn multiple comparison test, p>0.05. Abbreviations: AMs – alveolar macrophages, ART – antiretroviral therapy, AUC – area under the curve, cfu – colony forming units, GPA – gentamicin protection assay, HIV – Human immunodeficiency virus, hrs – hours, SPN – *S. pneumoniae*.

5.3.4. Treated HIV infection associated with a higher propensity of intracellular *S. pneumoniae* persistence within AMs *ex vivo*.

I next investigated the proportion of persisters within AMs between HIV-uninfected individuals and HIV-infected individuals on ART (n=60). When I compared the proportions of persisters (3hrs: 24hrs post infection), I observed higher proportion of persisters within the AMs from HIV-infected on short-term ART (2.60-fold increase, p=0.0107) and HIV-infected on long-term ART (22.42-fold increase, p<0.0001) when compared to the HIV-negative individuals (Figure 5.4 A). Furthermore, the HIV-infected on long-term ART also had higher proportion of persisters within the AMs compared to the HIV-infected on short-term ART (11.22-fold increase, p=0.0017). Lastly, I looked at the combined effect of treated HIV infection on the presence of persisters 24-hrs post infection. Treated HIV-infected individuals had higher proportions of intracellular *S. pneumoniae* persistence of 24-hours post infection (2.82-fold increase, p=0.0206) compared to their HIV-uninfected counterparts see Figure 5.4 B. These findings demonstrate that HIV-infected individuals on ART have reduced late intracellular *S. pneumoniae* killing capacity within AMs and an increased intracellular persistence of *S. pneumoniae* within AMs.

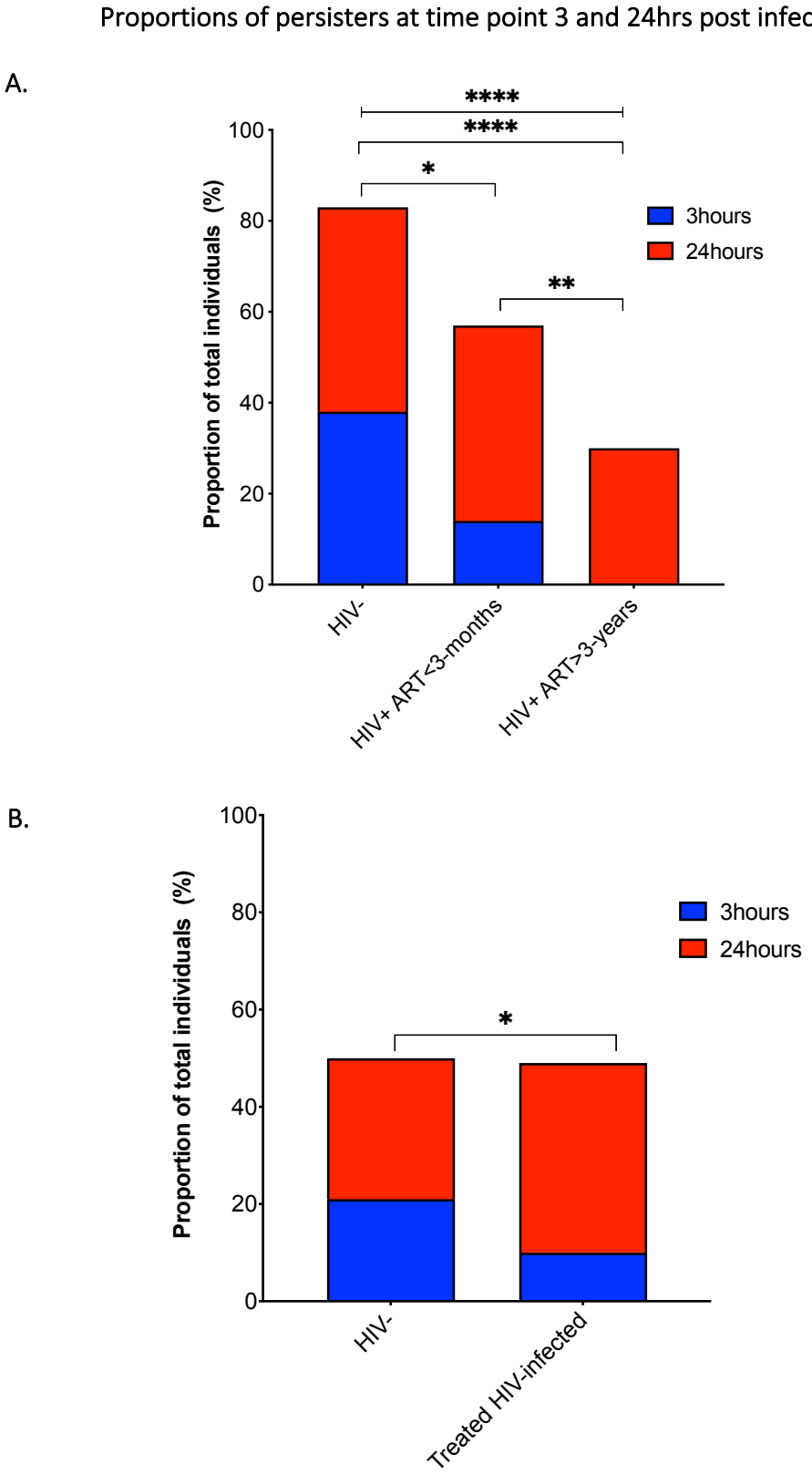


Figure 5.4. **HIV and ART is associated with a higher propensity of the persister phenotype during the late killing phase. A**. A comparison of the proportion of individuals with the persister phenotype stratified by HIV and ART status between the early killing phase (3-hrs post infection) and late killing phase (24-hrs post infection); HIV-uninfected (n=29), HIV-infected individuals

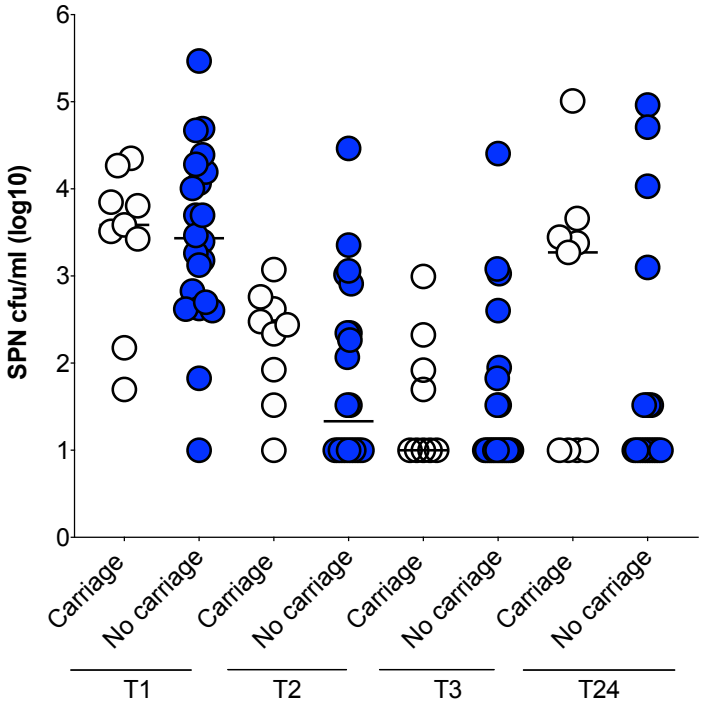
ART<3-months (n=21), HIV-infected individuals ART>3years (n=10); analyses by χ^2- test, ****p<0.0001. **B**. Impact of HIV on the proportion of persisters (HIV-infected on ART<3-months and HIV-infected on ART>3-years combined, n=31; HIV-uninfected n=24); analyses by χ^2- test, *p=0.0206. Abbreviations: ART – antiretroviral therapy, HIV – Human immunodeficiency virus, t – time point.

5.3.5. Pneumococcal nasal carriage is not associated with an enhanced AMs early intracellular killing propensity *ex vivo*.

It was recently shown in an experimental human pneumococcal challenge (EHPC) model that previous *S. pneumoniae* carriage (29 – 120 days post carriage) is associated with a heightened AMs uptake and responsiveness, attributed to trained immunity (Mitsi et al., 2019). With this in mind, I decided to investigate the impact of contemporaneous *S. pneumoniae* natural nasopharyngeal carriage on AMs mediated intracellular killing of *S. pneumoniae*-ST3. First, I explored the impact of *S. pneumoniae* natural nasopharyngeal carriage on time dependent killing in HIV uninfected, HIV-infected on ART<3-months and the HIV-infected on ART>3-years (Figure 5.5 A-C). I observed no difference between the *S. pneumoniae* carriage positive and carriage negative individuals in within intracellular killing capacity of AMs at all the time points (1hr, 2hrs, 3hrs and 24hrs post infection; all $p>0.05$). I further investigated whether *S. pneumoniae* carriage had an impact on the within early intracellular killing kinetics and we found no difference between the carriage positive and negative individuals ($p>0.05$, Figure 5.5 D). These findings report comparable AMs mediated intracellular killing kinetics between individuals with contemporaneous *S. pneumoniae* carriage compared to the carriage negative individual.

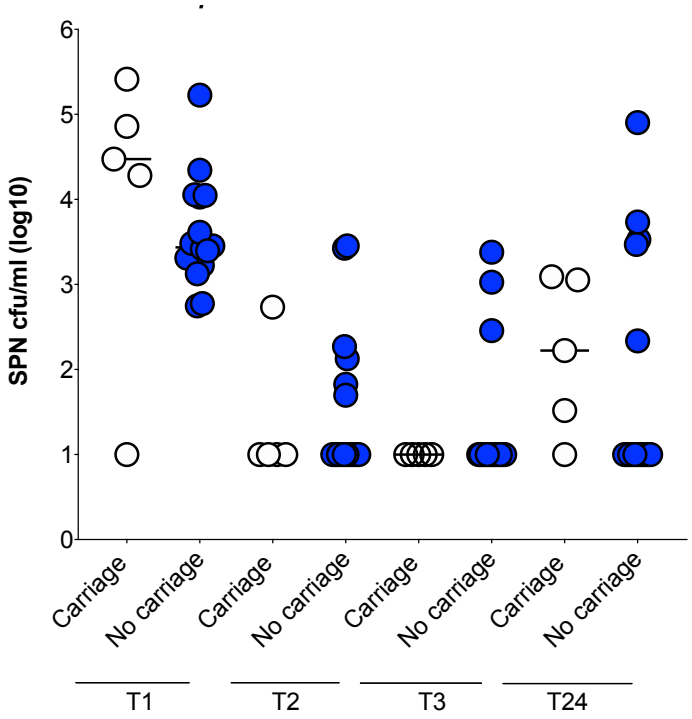
Impact of concurrent carriage on AMs mediated intracellular killing of *S. pneumoniae* in HIV-uninfected individuals

A.



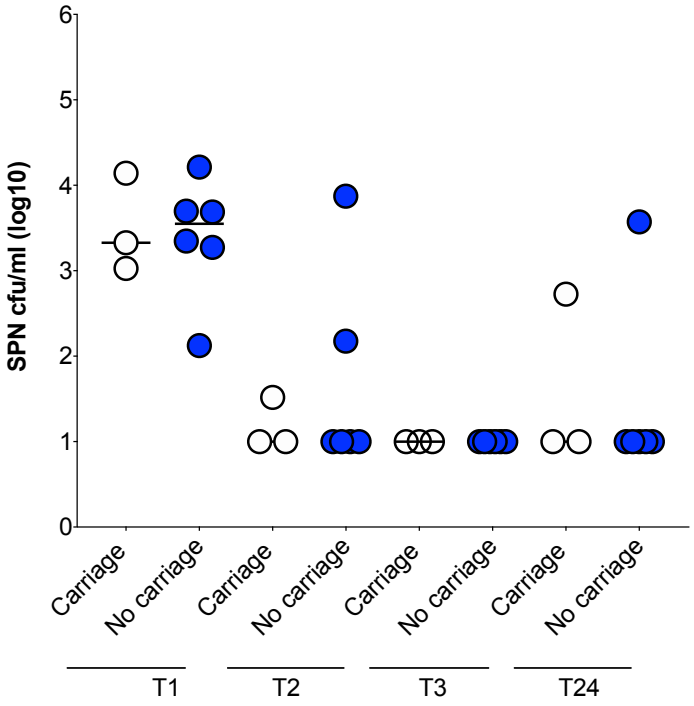
Impact of concurrent carriage on AMs mediated intracellular killing of *S. pneumoniae* in HIV-uninfected individuals

B.



Impact of concurrent carriage on AMs mediated intracellular killing of *S. pneumoniae* in HIV-uninfected individuals

C.



Impact of concurrent carriage on early killing kinetics (AUC)

D.

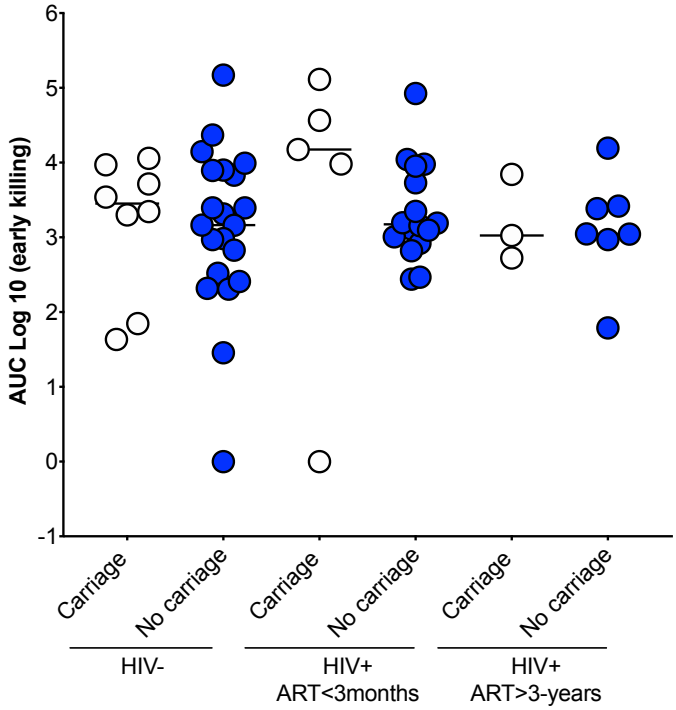


Figure 5.5. **Impact of pneumococcal carriage on AMs mediated intracellular killing activity. A.** A comparison of the impact of pneumococcal carriage on AMs mediated intracellular killing activity

in HIV-uninfected individuals (n=31) at time points 1-hour, 2-hours, 3-hours and 24-hours post infection; by Wilcoxon rank sum test, p>0.05. **B.** A comparison of the impact of carriage on AMs mediated intracellular killing activity in HIV-infected individuals on ART<3months (n=21) at time points 1-hour, 2-hours, 3-hours and 24-hours post infection; by Wilcoxon rank sum test, p>0.05. **C.** A comparison of the impact of carriage on AMs mediated intracellular killing activity in HIV-infected individuals on ART>3 years (n=10) at time points 1-hour, 2-hours, 3-hours and 24-hours post infection; by Wilcoxon rank sum test, p>0.05. **D.** A comparison of the impact of carriage on the early killing phase represented by the area under the curve (AUC) between HIV-uninfected individuals (n=31), HIV-infected individuals on ART<3-months (n=21) and HIV-infected individuals on ART>3 years (n=10); by Wilcoxon rank sum test, p>0.05. Abbreviations: AMs – alveolar macrophages, ART – antiretroviral therapy, AUC – area under the curve, HIV – HIV – Human immunodeficiency virus, SPN – *S. pneumoniae*.

5.3.6. The persister phenomenon and *S. pneumoniae*-ST3 survival can be detected using both microbiological and confocal microscopy analyses.

To further investigate the persister phenomenon and the presence of within AMs *S. pneumoniae* during the late killing phase using the gentamycin protection assay, AMs infected with opsonised *S. pneumoniae*-ST3, 3hrs and 24hrs post-infection were stained and imaged using confocal microscopy from a total of 14 individuals and data compared with microbiological analyses. As observed via microbiological culture, microscopy confirmed that not all AMs had cleared their intracellular *S. pneumoniae*-ST3 (Figure 5.6 A and B). Using confocal microscopy, *S. pneumoniae* was detected in 50% (n=7/14) of the individuals, with 35.7% (n=5/14) having intracellular or extracellular bacteria detected (Table 5.1). Only 1/14 individuals had *S. pneumoniae* on the surface and associated with AMs. Whereas, 57.1% (8/14) had viable culturable *S. pneumoniae* as detected by microbiological techniques, with 35.7% (n=5/14) having intracellular pneumococci and 50% (n=7/14) of the individuals having extracellular bacteria burden. These findings demonstrate that both confocal and microbiological analyses are able to demonstrate the presence of within AMs, extracellular bacteria and bacteria clearance of AMs at 3hrs and 24hrs post infection.

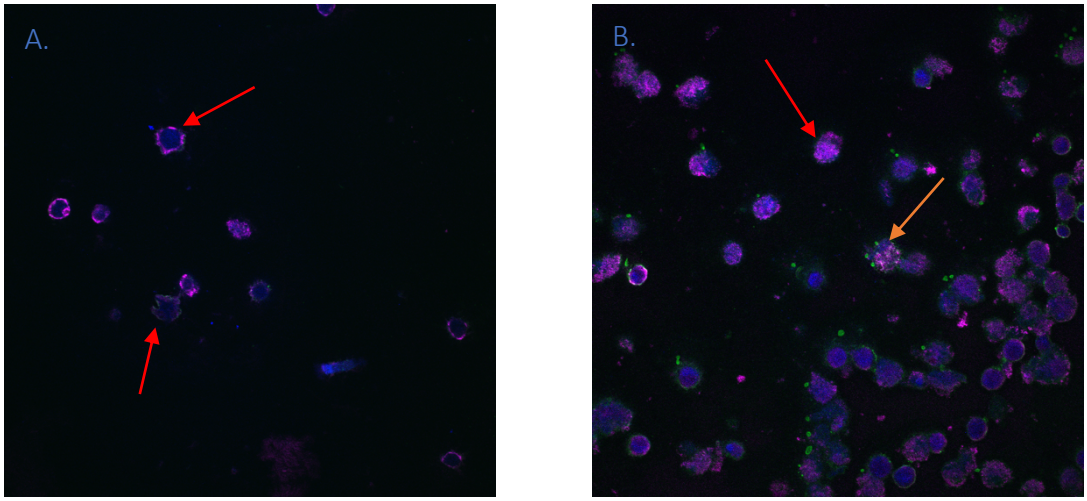


***Figure 5.6*. Evidence of *S. pneumoniae* internalisation and survival within alveolar macrophage *ex vivo*. A** Representative single fluorescent field image demonstrating the clearance of *S. pneumoniae* within AMs 2.5-hours post gentamicin application; AMs (WGA – purple; DAPI –

nucleus) with *S. pneumoniae* (bacteria capsule– green), arrow (red) indicates AMs that cleared *S. pneumoniae*. **B**. Representative single fluorescent field image demonstrating the presence, internalisation and survival of *S. pneumoniae*-ST3 within AMs 24-hours post gentamicin application; AMs (WGA – purple; DAPI – nucleus) with *S. pneumoniae* (bacteria – green), arrow (orange) *S. pneumoniae* within AMs, arrow (red) indicates AMs that cleared *S. pneumoniae*. Abbreviations: DAPI - 4′,6-diamidino-2-phenylindole, FITC - Fluorescein isothiocyanate, WGA-wheat germ agglutinin.

Table 5.1. *S. pneumoniae* association with AMs as detected by microbiological and culture confocal microscopy*.

ID	Time point post infection	Quantitative microbiology count		Average AMs counted	*S. pneumoniae* associated bacteria by confocal microscopy		
		Intracellular	Extracellular		Surface	Intracellular	Extracellular
PN1045S	T3	0	0	100	0	0	0
PN10990	T3	0	0	100	0	0	0
PN10990	T24	0	$8.53x10^3$	30	0	10	5
PN10958	T24	0	$2.34x10^5$	200	0	10	0
PN1077C	T24	33	$5.58x10^5$	100	1	0	0
PN10878	T24	$5.42x10^3$	$1.53x10^7$	100	0	8	4
PN1033X	T24	$1.02x10^5$	$4.92x10^6$	200	0	2	15
PN1044U	T24	217	$1.50x10^6$	200	0	4	6
PN1054Q	T24	0	0	200	0	0	2
PN1074L	T24	0	0	200	0	0	0
PN10470	T24	0	0	200	0	0	0
PN10305	T24	33	0	200	0	0	0
PN10894	T24	0	$1.93x10^5$	200	0	0	0
PN1072M	T24	0	0	200	0	0	0
PN10295	T24	0	0	200	0	0	0

*These data were derived from 14 volunteers with paired confocal microscopy and microbiological data.

5.3.7. The *S. pneumoniae*-ST3 persister phenomenon is associated with CD206^{+} alveolar macrophages 24-hours post infection.

I further used confocal microscopy to visualise the location of *S. pneumoniae*-ST3 within AMs, quantitate the bacteria and confirm the phenotype of AMs associated with the persister phenomenon *ex vivo*. Cytospin slides of AMs infected with *S. pneumoniae*-ST3 (24hrs post-infection) were stained with anti-CD206, DAPI, wheat germ hemagglutinin and FITC (bacteria) to identify AMs harbouring any *S. pneumoniae*. *S. pneumoniae* was observed within CD206^{+} AMs internalised is shown in Figure 5.7. This data confirms that CD206^{+} AM were the phagocytes harbouring intracellular *S. pneumoniae*-ST3 24hrs post infection.

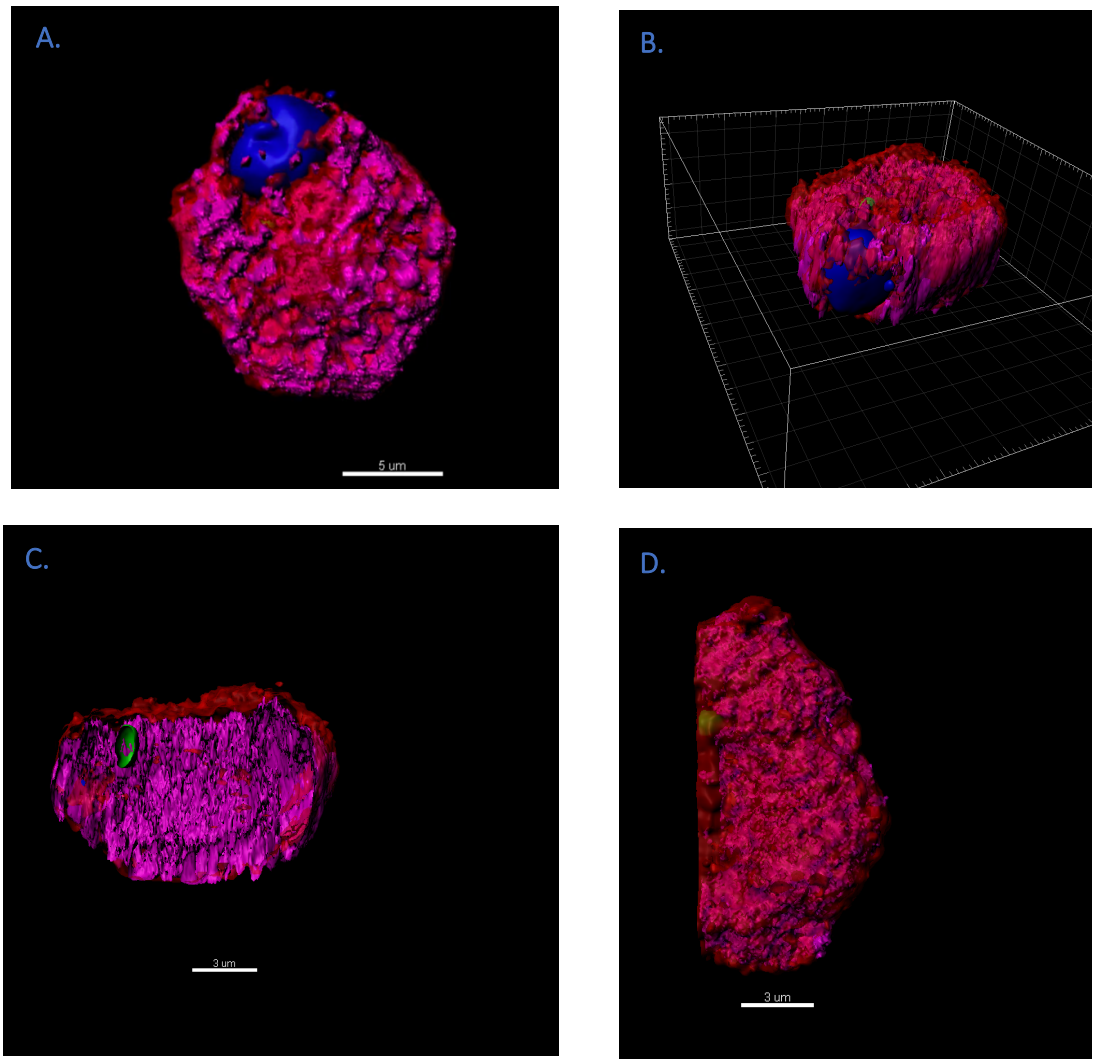


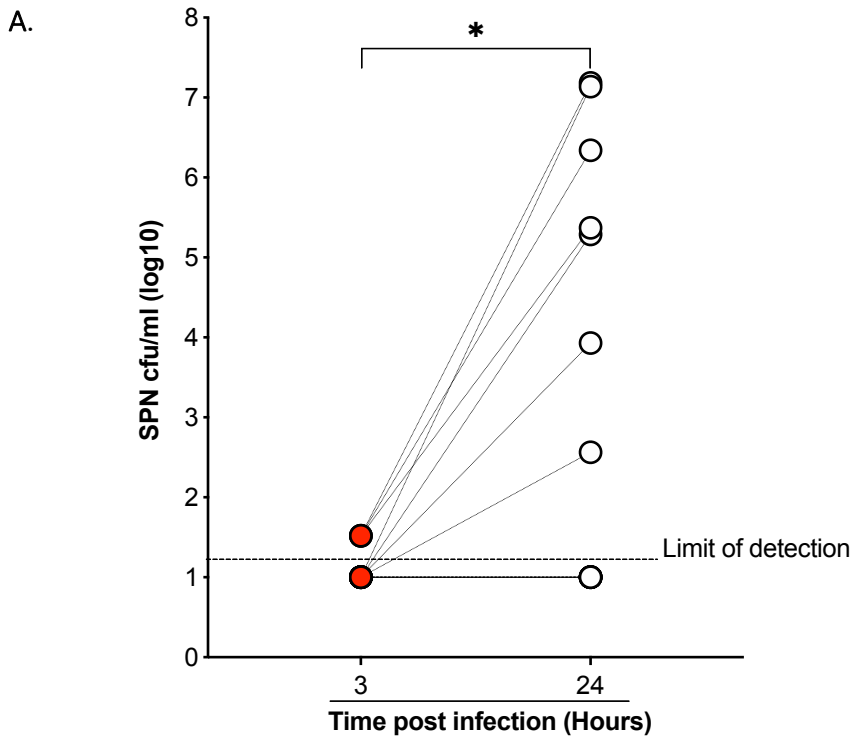
Figure 5.7. 3D reconstruction of deconvolved Z-stack images demonstrating evidence of the presence, internalisation and survival of *S. pneumoniae*-ST3 within CD206^{+} alveolar macrophages

24-hrs post infection *ex vivo* infection. A-D Representative 3D Z-stack images on different planes demonstrating the internalised *S. pneumoniae*-ST3 within $CD206^{+}$ alveolar macrophages (WGA; cell membranes – purple; DAPI – nucleus, red – monoclonal anti-CD206) with *S. pneumoniae*-ST3 (bacteria capsule, FITC– green). Abbreviations: CD – Cluster of differentiation, DAPI - 4′,6-diamidino-2-phenylindole, FITC - Fluorescein isothiocyanate, *S. pneumoniae*-ST3 – *S. pneumoniae* serotype 3, WGA- wheat germ agglutinin.

5.3.8. AMs serve as a reservoir for extracellular *S. pneumoniae* within the supernatants detected in some individuals during the late killing phase *ex vivo*.

Following the observation of *S. pneumoniae* persistence within AMs, I investigated whether AMs were acting as reservoirs for extracellular *S. pneumoniae* within the supernatants. I cultured the supernatants 3hrs and 24hrs post-infection for *S. pneumoniae*. Only individuals with paired samples were included in this analysis (n=14) (see Figure 5.8 A). I found higher extracellular burden of *S. pneumoniae* 24-hrs post infection 188.5 (IQR; 10 – 722 134) compared to 3-hrs post infection 10 (10 – 15.75; p=0.0156). Furthermore, I found a moderate strong positive correlation between presence of intracellular *S. pneumoniae* and extracellular *S. pneumoniae* during the late killing phase [r=0.6433 (95% confidence interval, 0.3850 – 0.8081); p<0.0001] (see Figure 5.8 B). These findings demonstrate that during the late killing phase (24hrs post-infection) survival of intracellular bacteria in AMs could serve as a reservoir to propagate pneumococcal infection.

Resurgence of extracellular *S. pneumoniae* 24hrs post infection



B.

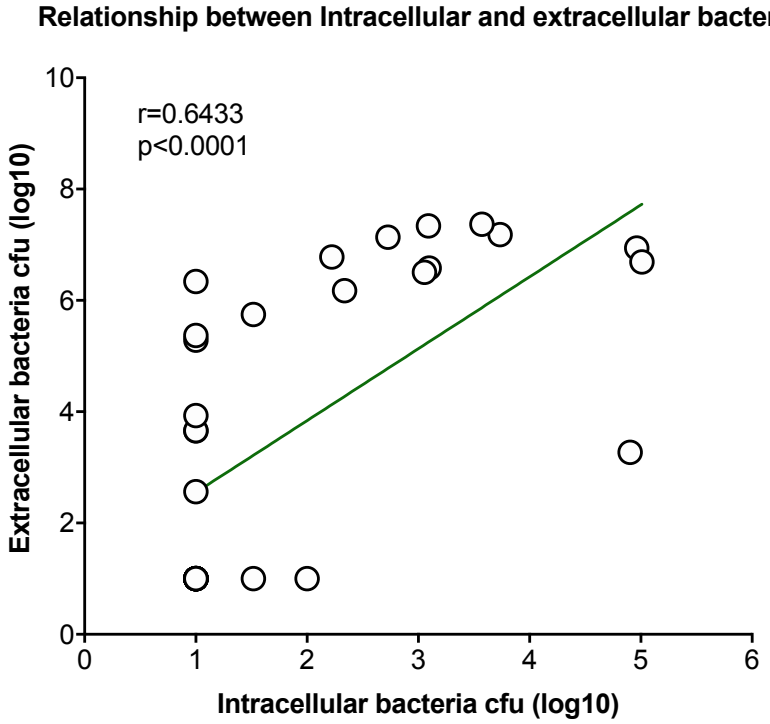


Figure 5.8. Relationship between within AMs pneumococci and extracellular pneumococci during the late killing phase *ex vivo*. A. Differential pneumococcal extracellular burden during the early killing (3hours post infection) and late killing phase (24hours post infection) n=14; by Wilcoxon signed rank test, *p=0.0156. B. A positive correlation between within AMs pneumococci and extracellular pneumococci (n=33) during the late killing phase (24hours post infection) *ex vivo*; by Pearson correlation test (r) and linear regression line shown; r=0.6433, p<0.0001. Abbreviations: cfu – colony forming units, GPA – gentamicin protection assay, SPN– *S. pneumoniae*.

5.4. Discussion

HIV-infected individuals are at an increased risk (at least 25-fold) of pneumococcal pneumonia compared to the general population (Cilloniz et al., 2014; Segal et al., 2011). The success of *S. pneumoniae* to cause disease in the lung depends on its ability to evade the lung chief resident phagocytic cell, the alveolar macrophages (AMs), consequently inducing inflammation whilst facilitating its spread and replication within the lung (Aberdein et al., 2013). In this study, I developed a gentamycin protection assay for understanding the early AMs-mediated intracellular control of pneumococcus. First, I demonstrated using microbiological methods and confocal microscopy that pneumococci persist in $CD206^{+}$AMs 24hrs post-infection. Second, slow early killing kinetics of intracellular bacteria within the first 3hrs post-infection in HIV-uninfected adults but not HIV-infected individuals on ART are associated with increased propensity for bacterial persistence in $CD206^{+}$AMs 24hrs post-infection. Third, treated HIV infection is associated with a reduced AMs-mediated intracellular killing capacity of *S. pneumoniae* during the late killing phase (24-hours post infection) leading to a higher propensity of intracellular *S. pneumoniae* persistence. Fourth, there was a direct correlation between presence of intracellular *S. pneumoniae* and extracellular *S. pneumoniae* during the late killing phase. Lastly, I did not find evidence to demonstrate that natural *S. pneumoniae* carriage augments AMs-mediated intracellular killing kinetics, as observed in the experimental human pneumococcal challenge model.

The early control mechanisms for pneumococcal infection within the lung depends on both the innate and adaptive immune responses (Wilson et al., 2015). Understanding the host-microbial interactions in humans is crucial in order to decipher the early events of pneumococcal control. It has recently been described in murine and porcine models that $CD169^{+}$ splenic macrophages can harbour *S. pneumoniae* and provide a site for intracellular replication with which after a *S. pneumoniae* eclipse phase in the blood, $CD169^{+}$ macrophages reseeds *S.*

pneumoniae into the blood to cause septicaemia (Ercoli et al., 2018). Consistent, with this observation, I found that that pneumococci exhibit an eclipse phase and persist within $CD206^+$ AMs 24hrs post-infection *ex vivo*, resulting in continued propagation of infection. This potentially serves as the *S. pneumoniae* invasion strategy into the lung parenchyma in susceptible individuals. Furthermore, recently it was discovered that pneumolysin (a cholesterol binding and pore forming toxin found on *S. pneumoniae*) dampens inflammatory cytokine responses by upregulating cytokine suppressor-1 (SOCS1), thereby facilitating lysosomal escape of *S. pneumoniae* in macrophages and dendritic cells by directly binding to the mannose receptor ($CD206^+$), thus promoting intracellular residency of *S. pneumoniae* (Subramanian et al., 2019). In another study it was shown that $CD206^+$ influences *S. pneumoniae* uptake and internalization in Schwann cells found in the peripheral nervous system (Macedo-Ramos et al., 2014). $CD206^+$ is a transmembrane glycoprotein and an endocytic receptor found abundantly expressed on AMs, responsible for binding mannose, fucose and acetylglucosamine including the present on pathogens (Byrne et al., 2016; Macedo-Ramos et al., 2014; Martinez-Pomares, 2012; Misharin et al., 2013; Varki, 2011). The binding of pneumolysin to the $CD206^+$ on the AM potentially gives insight to the persister phenomenon found in both HIV-uninfected and HIV-infected on ART individuals, however, this warrants further investigation.

Previously, it was shown that the efficient control of *S. pneumoniae* by AMs is dependent on IgG antibodies and HIV-infection does not impair the binding and internalisation of *S. pneumoniae* by AMs *ex vivo* (Gordon et al., 2000, 2001). However, recent evidence suggest that the HIV envelope glycoprotein (gp120) inhibits the mitochondrial-ROS (mROS) dependent killing in monocyte-derived-macrophages (MDM) and with a reduction in apoptosis-associated *S. pneumoniae* killing in ART-treated individuals (Collini et al., 2018). Consistent with previous findings (Collini et al., 2018), in this study I show that treated HIV infection is associated with a reduced AMs-mediated intracellular killing capacity of *S. pneumoniae* during the late killing phase (24-hours post infection), leading to a

higher propensity of intracellular *S. pneumoniae* persistence. In MDMs, HIV reduces caspase-dependent induction of mROS thereby facilitating survival of internalised *S. pneumoniae* (Collini et al., 2018). Furthermore, upregulation of the anti-apoptotic protein, the myeloid cell leukaemia 1 gene (mcl-1) a regulator for apoptosis, has been demonstrated to interfere with the late microbiocidal killing mechanism of AMs and is upregulated particularly in individuals with HIV or the chronic obstructive pulmonary disease (COPD) (Bewley et al., 2017; Collini et al., 2018; Preston et al., 2019). In mice models, lack of programmed death cell protein-1 (PD-1) expression is linked to significant protection against subsequent lethal pneumococcal challenge (McKay et al., 2015b). In a Simian immunodeficiency virus (SIV) rhesus macaque model, SIV infection is associated with increased (PD-1) expression in AMs (Hunegnaw et al., 2019). It is therefore plausible that HIV-mediated alterations of mROS and PD-1 expression could result in poor intracellular killing of pneumococci in human AMs. Persistence of pneumococci in AM is extremely important, as I further show that it results in AMs serving as reservoirs for extracellular *S. pneumoniae*. Survival of *S. pneumoniae* within the extracellular environment is propagated in an environment with high nutrients thus, bacteria can rapidly replicate to overcome AMs-mediated immune responses, resulting in neutrophil and inflammatory monocyte infiltration in the airway (Aberdein et al., 2013; Quinton et al., 2018). These processes could lead to an uncontrollable inflammation, which can cascade into pneumococcal pneumonia disease.

Previous nasal *S. pneumoniae* carriage was found to be associated with an heightened AMs responsiveness to bacterial pathogens including *S. pneumoniae* in the controlled human challenge model (Mitsi et al., 2019). Contrary to the earlier findings, contemporaneous *S. pneumoniae* natural carriage did not augment AMs-mediated intracellular killing kinetics and similar kinetics were observed in carriage negative individuals. This could be due to serotype differences; as I used serotype 3 which is an invasive strain whilst the EHPC used serotype 6B a carriage strain, hence their virulence and evasion capability might

be different. Furthermore, there was more serotype diversity amongst participants in my study as a result of natural carriage, whilst in the EHPC, the challenge serotype is the same as the laboratory experimental serotype (6B). Moreover, the differences observed could be due to the fact that this study investigated the effects of contemporaneous *S. pneumoniae* carriage as opposed to the effects of trained immunity as investigated in the EHPC model (Mitsi et al., 2019).The study setting is a high pneumococcal transmission setting, hence the impact of natural carriage on AMs-mediated immunity could be difficult to ascertain due to the lack of knowledge on the day of carriage acquisition and duration of carriage amongst the participants. These findings potentially point towards altered tolerance as a result of natural carriage and through repeated carriage exposure, though this needs to be further ascertained with a follow up longitudinal study.

The main limitation of this study, is the failure to include lysosomal associated membrane protein-1 (LAMP-1) or CD107a, a reading for lysosomal acidification (Gordon et al., 2001), to demonstrate whether the *S. pneumoniae* escaped the phagolysosomes or it was colocalized with the lysosomes. Furthermore, it was difficult to recruit antiretroviral therapy naïve individuals due to the Test and Treat strategy adopted by Malawi (Alhaj et al., 2019). Nevertheless, it is important to decipher effects of short- and long-term ART on early control of pneumococcus as the majority of HIV-infected adults will be on ART going forward.

5.5. Conclusion

This study demonstrates that although AMs readily internalize and kill *S. pneumoniae* within the first three hours of infection with intracellular killing being a rate-limiting step for bacterial clearance. The evasion of AM intracellular killing is exacerbated in HIV-infected adults on ART (short-term and long-term). This finding potentially uncovers why certain population groups are more susceptible to pneumococcal pneumonia. Nonetheless, intracellular killing is one of the mechanisms to control extracellular outgrowth of pneumococci, hence evasion of

intracellular killing is likely to have a direct impact on overall control of pneumococcal infection. In the next chapter, I will explore the control of pneumococcus outgrowth by alveolar cells and investigate the key mechanisms that drive this process.

CHAPTER 6

6.0. Airway cell control of *S. pneumoniae* extracellular outgrowth in asymptomatic adults residing in Malawi.

6.1. Introduction

The spread of *S. pneumoniae* to the lung is thought to be through microaspiration and this can lead to pneumonia if not cleared successfully by the host immunity (Mitsi et al., 2019; Ritchie and Evans, 2019). Airway phagocytic cells control extracellular bacteria like *S. pneumoniae* and *S. aureus* through bacterial internalisation, phagolysosomal killing, engagement of microbiocidal factors, cellular enzymes and metal intoxication (Ercoli et al., 2018; Flannagan et al., 2016; Johnson et al., 2015; Jubrail et al., 2016). In the previous chapter, I demonstrated that during the late killing phase AMs serve as a reservoir for extracellular pneumococci with HIV-infected individuals on ART having a higher propensity for intracellular persistence. Decreased late bacterial killing leads to neutrophil recruitment into the airway, which is the hallmark of pneumonia (Stout-Delgado, 2019).

It has been shown that intracellular bacterial killing is a finite process, as phagocytic cells (macrophages) have a bacteria internalisation threshold. This could result in some bacteria propagating in the extracellular space and persisting intracellularly as demonstrated with *S. aureus* (Aberdein et al., 2013; Jubrail et al., 2016; Preston et al., 2019; Stout-Delgado, 2019). This implies that intracellular killing alone will not suffice in controlling the bacteria burden, hence a secondary mechanism must be reengaged in controlling bacteria outgrowth. Moreover, the *S. pneumoniae* capsule and other virulence factors are known to profoundly inhibit bacterial phagocytosis, complement opsonisation as well as mucus entrapment (Andre et al., 2017; Hyams et al., 2010a, 2013a; Pathak et al., 2018). Phagocytic cells in the airway may employ other means like release of microbiocidal factors (reactive oxygen species (ROS), H_2O_2, hydroxyl radicals

(OH$^{\bullet}$), reactive nitrogen species (RNS), or regulate metal homeostasis within the extracellular space, to control the pneumococcal burden (Farshchi Andisi et al., 2012; Shenoy and Orihuela, 2016; Ullah et al., 2017; White et al., 2009).
With the growing antimicrobial resistance, pneumonia burden, pneumococcus serotype replacement, and low pneumococcal vaccine efficacy against pneumonia, understanding the contribution of extracellular killing is fundamental to the effective development of immunotherapies targeted enhancing the airway phagocytic killing activities. Furthermore, the role of extracellular control of *S. pneumoniae* outgrowth by human airway cells is still unclear and whether HIV infection perturbs this important function is equally unresolved. I hypothesised that airway cells from HIV-uninfected adults inhibit extracellular bacterial growth during early pneumococcal infection, but this process is impaired in HIV-infected individuals on short-term ART.

6.1.1. Research question

Do airway cells from HIV-infected individuals on short-term ART exhibit impaired control of *S. pneumoniae* extracellular outgrowth *ex vivo*?

6.1.2. Aims and Objectives

Aim

To investigate whether bronchoalveolar lavage airway cells from HIV-infected individuals on short-term ART show impaired control of *S. pneumoniae* extracellular outgrowth *ex vivo*.

Objectives

1. To determine the human bronchoalveolar lavage airway cell control of extracellular *S. pneumoniae* outgrowth in asymptomatic HIV-uninfected individuals.
2. To compare human bronchoalveolar lavage airway cell control of extracellular *S. pneumoniae* outgrowth in HIV-uninfected compared to HIV-infected adults on short-term or long-term ART.
3. To define the factors associated with human bronchoalveolar lavage airway cell control of extracellular *S. pneumoniae* outgrowth.

6.2. Methods

This section covers some of the methods used in chapter 4 as the same group of participants were used though the analysis done was different.

6.2.1. Study participants and design

This was a comparative cross-sectional study of asymptomatic HIV uninfected, HIV-infected on short-time ART (<3-months) and long-term ART (>3-years). Participants were recruited from the Malawi-Liverpool Wellcome-Trust's (MLW) Clinical investigation unit at the Queen Elizabeth Hospital in Blantyre, Malawi. All participants completed a specific questionnaire relating to their previous clinical history and social demographic characteristics that could potentially impact their alveolar cell functional response *ex vivo* before recruitment. The recruitment criteria used has been discussed elsewhere (see Chapter 2.4).

6.2.2. Ethical approval and study clearance

The study received ethical approval from both College of Medicine Research Ethics Committee (COMREC), Malawi and Liverpool School of Tropical Medicine Research Ethics Committee (LSTMREC), United Kingdom and this together with the recruitment criteria used have previously been described (see Chapter 2.3).

6.2.3. Sample collection and processing

Bronchoalveolar lavage and per nasal swab samples were collected from all participants and transported to the MLW laboratories within 30-minutes of collection. Sample collection and processing have been described (see Chapter 2).

6.2.4. Laboratory assays

The laboratory assays associated with this chapter have been described in Chapter 2. In particular for specific details *S. pneumoniae growth inhibition assay* (2.6); BAL processing and resting of cells (2.6.6); BAL *ex vivo* infections (2.6.9); per nasal swab processing and *S. pneumoniae* identification (2.6.15); and the identification of intracellular *S. pneumoniae* in uninfected BAL samples through

confocal microscopy (2.6). *S. pneumoniae* serotype 3 was used in all the experiments.

6.2.5. Statistical analysis

Descriptive statistics were used to for continuous variables by calculating medians and interquartile ranges. Groups were compared using non-parametric tests (Wilcoxon rank sum or Wilcoxon signed-rank test, Kruskal Wallis tests) depending on the distribution. For multiple comparison the Dunn test was used to adjust for *p*-values. Categorical data were summarised as proportions and compared using the $\chi 2$ tests. The relationship between the gentamicin protection (invasion) assay and outgrowth assay within the early time points (1 – 3 hours) was summarised using a simple linear regression model. The relationship between the outgrowth assay (persistence) and each variable including; age, sex, persistence within the gentamicin protection assay, carriage, AMs proportion, AMs binding index, HIV status, $CD4^+$ count and ART status were first summarised using univariate regression model. The relationship between the outgrowth assay (persistence) and all variable used in the univariate regression analysis namely; age, sex, persistence within the gentamicin protection assay, carriage, AMs proportion, AMs binding index, HIV status, $CD4^+$ count and ART status were summarised using multivariable regression model. All statistical tests were two-sided and were deemed statistically significant at an α value of 0.05 ($p<0.05$*, $p<0.01$**, $p<0.001$***, $p<0.0001$****) and all analyses were done in GraphPad Prism *v*9 software.

6.3. Results

6.3.1. Bronchoalveolar lavage airway cells from HIV-uninfected individuals control *S. pneumoniae* outgrowth during early infection.

S. pneumoniae is an extracellular pathogen with a polysaccharide capsule surrounding the bacterium that acts as a major virulence factor inhibiting phagocytosis, mucus entrapment and neutrophil extracellular traps (Hyams et al., 2010a; Moorthy et al., 2016). To determine the early *S. pneumoniae* outgrowth kinetics in the presence of airway cells, *S. pneumoniae* was grown in the presence and absence (control) of airway cells for 1-hr and 3-hrs. For these assays, airway cells from HIV-uninfected adults were used to establish baseline control in absence of HIV. I observed a 1.75-fold median reduction in extracellular *S. pneumoniae* burden in the presence of airway cells (1hr, median (interquartile range); 4.8 [4.5 – 5.5]x10^7 vs 1.7 [0.9 – 3.0]x 10^7 cfu/ml; p<0.0001) (Figure 6.1 A). I further compared the *S. pneumoniae* outgrowth kinetics at 3-hrs post incubation, and I observed a 2.38-fold median decrease in extracellular *S. pneumoniae* burden in the presence of airway cells (3hrs, median (interquartile range); 9.6 [7.2 – 13.8]x10^7 vs 2.8 [1.3 – 9.2]x 10^7 cfu/ml; p<0.0004) (see Figure 6.1 B). Taken together, these findings demonstrate that bronchoalveolar lavage airway cells from HIV-uninfected individuals control the *S. pneumoniae* extracellular outgrowth kinetics during early infection.

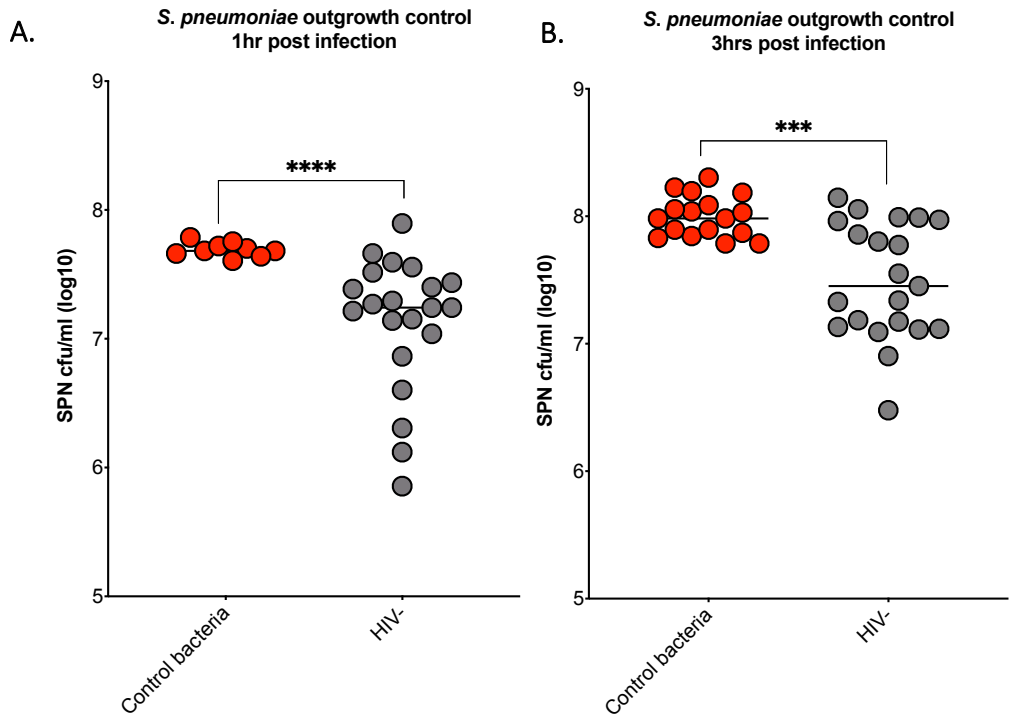


Figure 6.3.1. S. pneumoniae outgrowth kinetics in the presence and absence of alveolar cells. **A.** Comparison of *S. pneumoniae* outgrowth kinetics in infection media in the presence and absence of airway cells 1-hour post incubation; by Wilcoxon rank sum test, ****p<0.0001; SPN (red circles – control bacteria; n=9); HIV-uninfected (grey circles, n=21). **B.** Comparison of *S. pneumoniae* outgrowth kinetics in infection media in the presence and absence of airway cells 3-hours post incubation; by Wilcoxon rank sum test, ***p=0.0004; SPN (red circles – control bacteria; n=17); HIV-uninfected (grey circles, n=21). Abbreviations: cfu – colony forming units, HIV- – HIV-uninfected individuals, SPN – *S. pneumoniae*.

6.3.2. HIV-infection and ART does not impair bronchoalveolar lavage airway cells early control of *S. pneumoniae* extracellular outgrowth *ex vivo*.

Having established that bronchoalveolar lavage airway cells from HIV-uninfected individuals control the *S. pneumoniae* extracellular outgrowth during early infection. I next sought to investigate the control of extracellular *S. pneumoniae* outgrowth by airway cells in asymptomatic HIV-infected adults on short-term ART *ex vivo* (n=19). I observed no statistically significant difference in the *S. pneumoniae* extracellular bacteria burden between the HIV-uninfected adults and HIV-infected adults on short-term ART at 1-hr post infection (median, IQR; 1.7 [0.9 – 3.0]x 10^7 vs 2.2 [0.9 – 3.6]x10^7 cfu/ml; p=0.7226) (Figure 6.2 A). At 3-hrs post infection, there was also no statistically significant differences (median, IQR; 2.8 [1.3 – 9.2]x 10^7 vs 5.2 [0.9 – 9.8]x10^7 cfu/ml; p=0.9207) between the HIV-uninfected adults and HIV-infected adults on short-term ART (Figure 6.2 B). I also compared the airway cell control of *S. pneumoniae* extracellular outgrowth in individuals on long-term ART. I observed no significant differences in the median between *S. pneumoniae* extracellular bacteria burden from HIV-uninfected individuals and HIV-infected adults on long-term ART at 1-hr post infection (median, IQR; 1.7 [0.9 – 3.0]x 10^7 vs 1.0 [0.3 – 2.1]x10^7 cfu/ml; p=0.3016) (see Figure 6.2 C). At 3-hrs post infection, no statistically significant differences (median, IQR; 2.8 [1.3 – 9.2]x 10^7 vs 3.9 [7.0 – 8.8]x10^7 cfu/ml; p=0.9503) were observed between the extracellular *S. pneumoniae* burden from HIV-uninfected individuals and HIV-infected adults on long-term ART (Figure 6.2 D). Collectively, this data show that short-term and long-term ART is not associated with impaired early control of extracellular *S. pneumoniae* outgrowth.

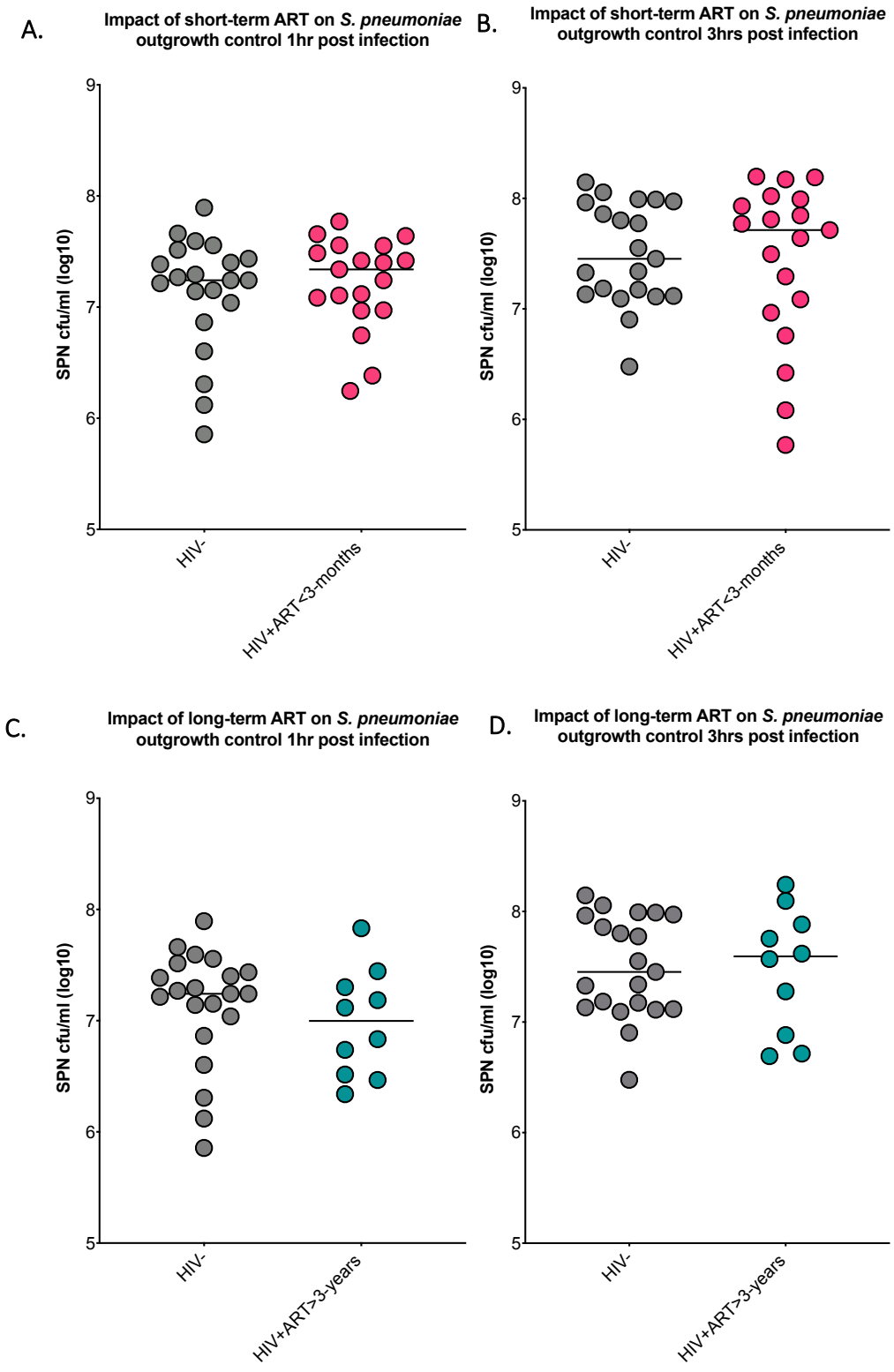


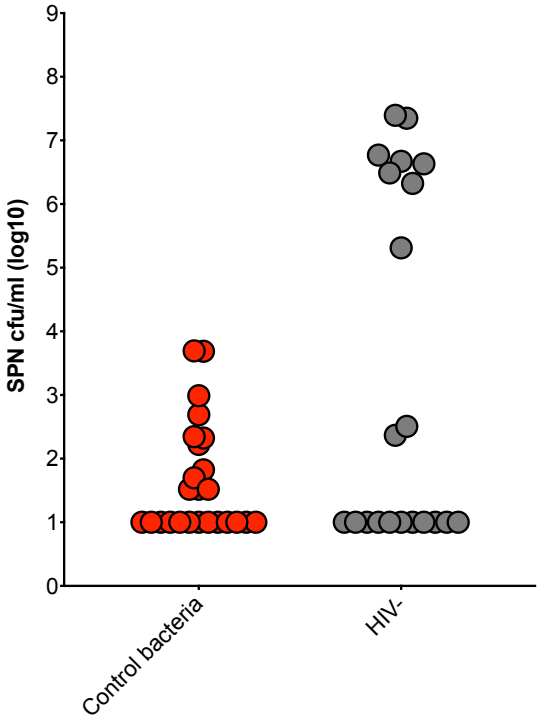
Figure 6.2. Impact of HIV and ART on bronchoalveolar lavage airway control of extracellular *S. pneumoniae*. A. Comparison of the extracellular *S. pneumoniae* burden between HIV-uninfected (grey circles; n=21) and HIV-infected on ART<3-months (cyan circles; n=19) at 1-hour post infection. B. Comparison of the extracellular *S. pneumoniae* burden between HIV-uninfected (grey circles; n=21) and HIV-infected on ART<3-months (cyan circles; n=19) at 1-hour post infection. C. Comparison of the extracellular *S. pneumoniae* burden between HIV-uninfected (grey circles;

n=21) and HIV-infected on ART>3-years (cyan circles; n=10) at 1-hour post infection. D. Comparison of the extracellular *S. pneumoniae* burden between HIV-uninfected (grey circles, n=21) and HIV-infected on ART>3-years (cyan circles; n=10) at 3-hour post infection. All analysis by Wilcoxon rank sum test, p>0.05. Abbreviations: ART – antiretroviral therapy, cfu – colony forming units, HIV- –HIV-uninfected individuals, SPN – *S. pneumoniae*.

6.3.3. The presence of human bronchoalveolar lavage airway cells augments *S. pneumoniae* outgrowth *ex vivo* 24-hrs post infection.

Following previous observations of extracellular *S. pneumoniae* control by bronchoalveolar lavage airway cells within the early phase of *ex vivo* infection (1-3 hours), I investigated whether airway cells are capable of controlling the extracellular *S. pneumoniae* outgrowth during the late infection phase infection (24-hrs post infection). Firstly, I observed no statistically significant difference in the proportions between cell free conditions and airway cells from HIV-uninfected individuals (48% vs 47.6%; p>0.9999). I also observed no difference in *S. pneumoniae* extracellular bacteria burden (median, IQR; 10[10 – 189] vs 10[10 – 255.3] cfu/ml; p=0.4366) between cell free conditions and airway cells from HIV-uninfected individuals (Figure 6.3 A). Furthermore, when I looked at the impact of treated HIV infection on extracellular pneumococcal persistence 24hrs post infection, airway cells from HIV-uninfected individuals had higher proportions of persisters compared to airway cells from HIV-infected adults on short-term ART (48% vs 15.8%, p=0.0455) or HIV-infected adults on long-term ART (48% vs 10%, p=0.0550) see (Figure 6.3 B). Furthermore, I compared viable culturable pneumococci in the conditions with and without airway cells at 24hrs post infection. The airway cells had higher bacterial burden than those without (median, IQR; 3.7[0.2 – 230] $x10^6$ vs 2[0.5 – 9.8]$x10^2$ cfu/ml; p=0.0007) (Figure 6.3 C). These findings show that during the late infection phase (24hrs post-infection), airway cells potentially promote pneumococcal survival, with HIV-infected individuals on ART killing extracellular pneumococcus faster than HIV-uninfected individuals.

A.


SPN cfu/ml (log10)
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2
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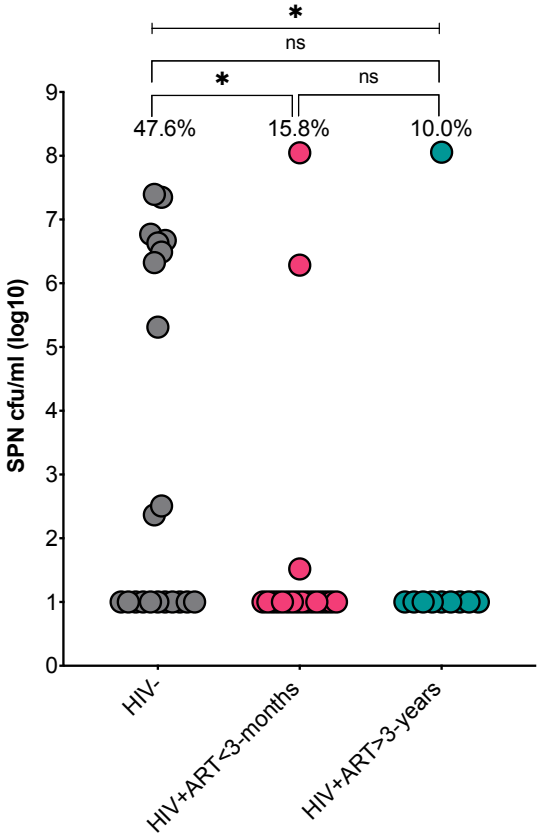
Control bacteria
HIV-

B.


*
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SPN cfu/ml (log10)
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HIV+ART>3-years

C.

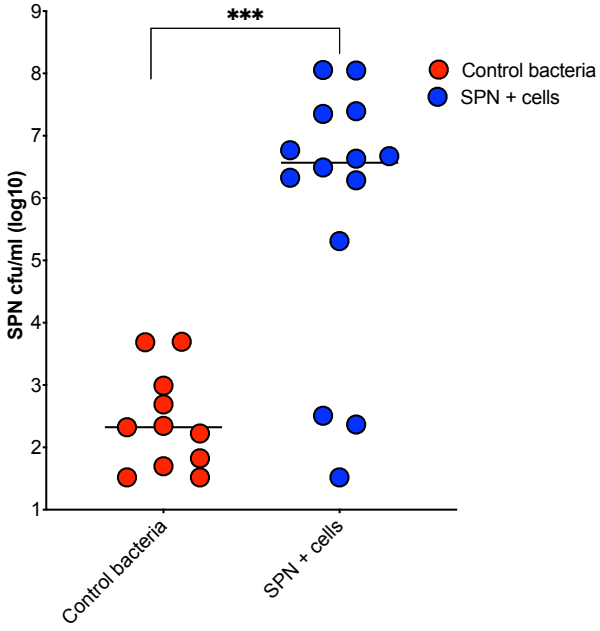


Figure 6.3. S. pneumoniae* growth enhancement 24-hrs post infection *ex vivo. **A**. Comparison of *S. pneumoniae* outgrowth bacterial load in the presence and absence of airway cells 24-hours post incubation; by Wilcoxon rank sum test, p>0.05; SPN (red circles, control bacteria, n=25); HIV-uninfected (grey circles, n=21).**B.** Comparison of the proportions of outgrowth bacterial load between HIV-uninfected (grey circles, n=10/21), HIV-infected on ART<3-months (purple circles, n=3/19) and HIV-infected individuals ART>3-years (cyan circles, n=1/10) 24-hr post infection; by Kruskal-Wallis (*p=0.0446) and Dunn multiple comparison test (*p=0.0455). **C**. Comparison of the extracellular *S. pneumoniae* burden in persisters [(individuals pooled from HIV-uninfected (n=10), HIV infected individuals ART<3-months (n=3), HIV-infected individuals ART>3-years (n=1), blue circles)] 24hrs post infection, and compared with SPN (red circles, n=11) in airway cell free condition; analysis by Wilcoxon rank-sum test, ***p=0.0007.Abbreviations: ART – antiretroviral therapy, cfu – colony forming units, SPN – *S. pneumoniae*.

6.3.4. The relationship between early intracellular killing and bacterial outgrowth assay of *S. pneumoniae ex vivo*.

Having established that airway cells could support the growth of *S. pneumoniae* during late infection, and that early intracellular killing kinetics (1 – 3 hours) in HIV-uninfected individuals predict *S. pneumoniae* intracellular persistence in the late killing phase (Chapter 5). I investigated whether there was a direct relationship between intracellular killing kinetics and extracellular bacterial outgrowth control during early infection (1-3hrs post infection), as this could dictate the outcome of *S. pneumoniae* clearance. I found a moderate positive correlation between the early intracellular killing kinetics and extracellular bacterial outgrowth control [r=0.5389 (95% confidence interval, 0.3037 – 0.7122); p<0.0001] (see figure 6.4). Taken together, this shows that a relationship exists between the intracellular and extracellular killing kinetics of *S. pneumoniae* within the first 3-hours of infection.

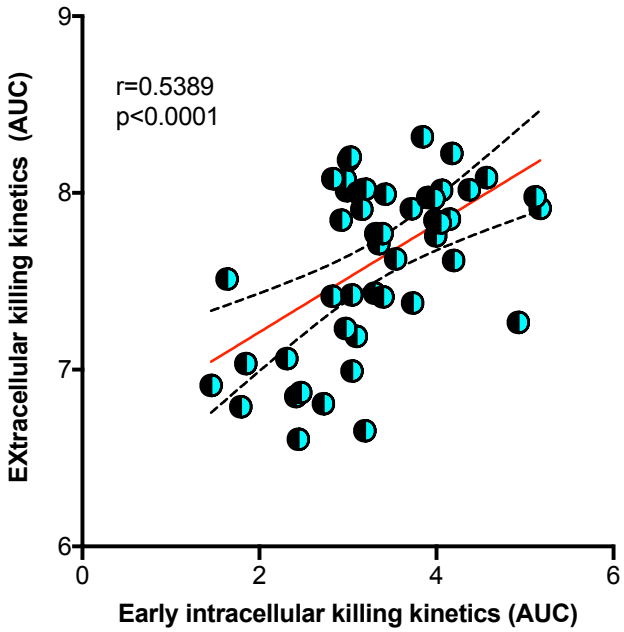
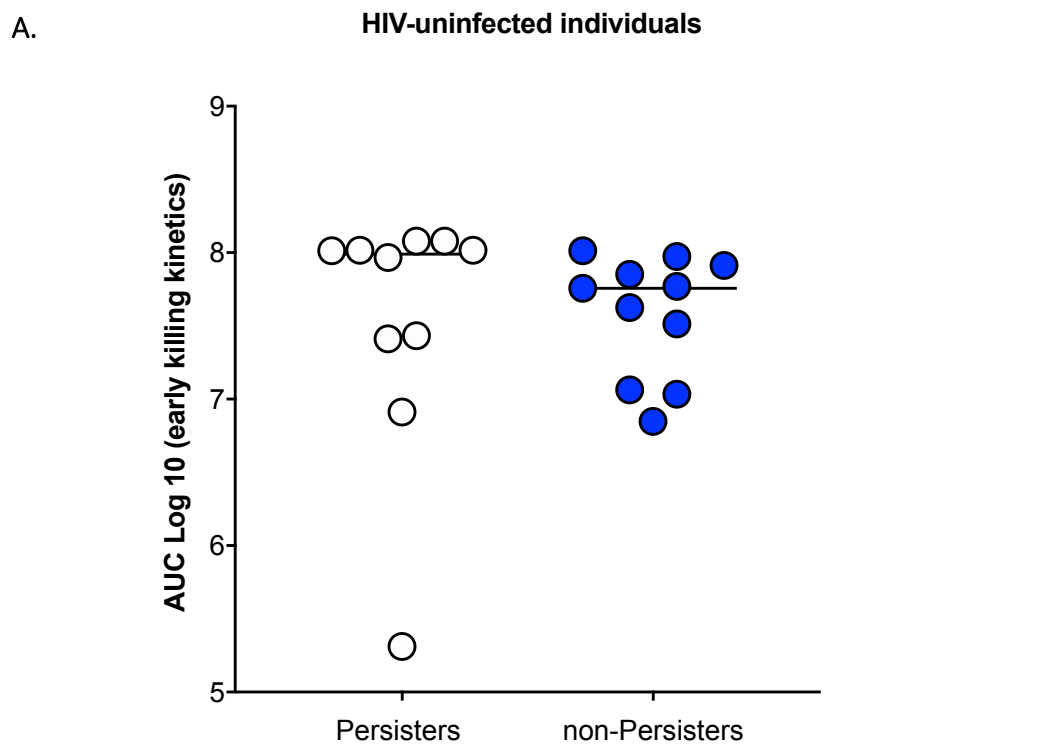


Figure 6.4. **Relationship between early intracellular killing and early bacterial outgrowth assay of *S. pneumoniae ex vivo*.** A moderate positive correlation was observed between early intracellular within AMs pneumococci (gentamicin protection assay) and extracellular pneumococci (bacterial outgrowth assay) during the early killing phase (1-3hours post infection, n=49) *ex vivo*; by Pearson correlation test (r), linear regression line with 95% confidence interval are shown; r=0.5389, 95%CI 0.3037 – 0.7122, p<0.0001. Abbreviations: AUC – area under the curve, GPA – gentamicin protection assay, EI – extracellular inhibition.

6.3.5. Early *S. pneumoniae* extracellular killing kinetics do not predict bacteria persistence *ex vivo*.

I then investigated whether early extracellular outgrowth control kinetics represented by the area under the curve (AUC) for time points (1 – 3hrs) could distinguish between extracellular persistence and non-persistence of pneumococcus. Within the HIV-uninfected individuals, I found no statistically significant difference in the extracellular outgrowth control kinetics between persisters and non-persisters during the early time points (AUC, 9.3 [IQR; 2.1 – 10.8]x10^7 vs. 5.7 [1.2 – 8.2]x10^7, p=0.2877) (Figure 6.5 A). I found no statistically significant differences between persisters and non-persisters from the combined treated HIV-infected individuals (AUC, 11.1 [IQR; 3.8 – 15.0]x10^7 vs. 5.1 [IQR; 1.3 – 9.7]x10^8, p=0.5593) (Figure 6.5 B). I was not able to do any analysis in HIV infected individuals on long-term ART as there were not enough data points (Figure 6.5. C). Collectively, these findings demonstrate that persistence of *S. pneumoniae* 24hrs post infection is not due to poor early extracellular outgrowth control.



B.

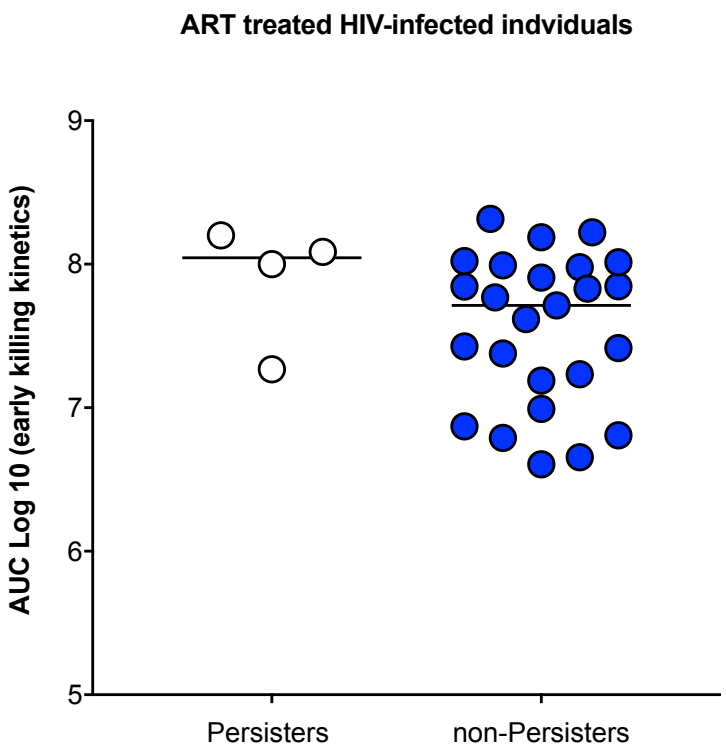


Figure 6.5. A comparison of early killing kinetics between persister and non-persisters. A. Comparison of the early outgrowth bacterial load between persisters and non-persisters within the HIV-uninfected individuals as demonstrated by the area under the curve (AUC), by Wilcoxon rank sum test, p>0.05. B. Comparison of the early outgrowth bacterial load between persisters and non-persisters within the combined ART treated HIV infected individuals as demonstrated by the area under the curve (AUC), by Wilcoxon rank sum test, p>0.05. Abbreviations: AUC – area under the curve, ART – antiretroviral therapy, HIV – Human immune deficiency virus.

6.3.6. Intracellular bacterial persistence predicts survival of extracellular *S. pneumoniae* 24hrs post infection

Next, I used univariate and multivariate analysis to determine key factors that could explain the extracellular persistence of *S. pneumoniae* 24hrs post infection, using demographic data and experimental parameters describe in Chapter 4, 5 and 6. I found a positive association between intracellular *S. pneumoniae* persistence and extracellular *S. pneumoniae* persistence on univariate analysis (coefficient estimate, 95% CI: 0.354, 95%CI 0.096 to 0.61; p =0.008) (Table 6.1.). This association between intracellular *S. pneumoniae* persistence and extracellular *S. pneumoniae* persistence remained marginally significant following multivariate analyses (0.304, 95%CI 0.001 to 0.606; p=0.049), after adjusting for AMs proportion, carriage, age, sex, proportion of alveolar macrophages associated with *S. pneumoniae*, binding index, HIV status, $CD4^+$ count and ART status. However, the other variables like sex (coefficient estimate, 95% CI: -0.330, 95%CI -0.600 to -0.061; p=0.017), HIV status (coefficient estimate, 95% CI: -0.418, 95%CI -0.672 to -0.165; p=0.002), ART duration (short-term ART; coefficient estimate, 95% CI: -0.418, 95%CI -0.760 to -0.077, p=0.018; Long-term ART, (coefficient estimate, 95% CI: -0.418, 95%CI -0.699 to -0.138, p=0.004) were only positively associated with extracellular pneumococcal persistence on univariate analysis but the association was lost in multivariable analysis. Collectively, the survival of extracellular *S. pneumoniae* 24hrs post infection is likely driven by intracellular bacterial persistence.

Table 6.1. Factors associated with the presence of extracellular *S. pneumoniae* following *ex vivo* infection of airways cells.

Characteristic	Univariate analysis		Multivariable analysis	
	Coefficient estimate (95% CI)	p-value	Coefficient estimate (95% CI)	p-value
T24 GPA- P	0.354 (0.096 – 0.61)	**0.008**	0.304 (0.001 – 0.606)	**0.049**
AM prop	0.009 (-0.009 – 0.026)	0.338	0.003 (-0.014 – 0.020)	0.737
Carriage	0.019 (-0.279 – 0.317)	0.898	0.033 (-0.277 – 0.344)	0.828
Age	-0.009 (-0.025 – 0.007)	0.253	-0.006 (-0.022 – 0.009)	0.413
Sex	-0.330 (-0.600 – -0.061)	**0.017**	-0.115 (-0.426 – 0.197)	0.456
AM ST3 Prop	0.002 (-0.003 – 0.007)	0.432	0.004 (-0.003 – 0.012)	0.257
AM binding index	-0.002 (-0.069 – 0.064)	0.07	-0.028 (-0.137 – 0.080)	0.597
HIV status	-0.418 (-0.672 – -0.165)	**0.002**	-0.272 (-0.635 – 0.091)	0.137
$CD4^+$	0.0003 (-0.0002 – 0.0009)	0.243	-9.92^{e-06} (-0.0007 – 0.0007)	0.976
ART status (1) **(2)**	-0.418 (-0.760 – -0.077) -0.418 (-0.699 – -0.138)	**0.018** **0.004**	0.012 (-0.361 – 0.386) – θ	0.946

Definition of abbreviation

GPA-P – gentamicin protection assay (intracellular persistence 24-hours post infection)
AM prop – alveolar macrophage proportion
AM ST3 prop – proportion of alveolar macrophages associated with *S. pneumoniae* (serotype 3) 1-hr post infection
AM binding index – Proportion of *S. pneumoniae* bound to each alveolar macrophage 1hr post infection
$CD4^+$ - cluster of differentiation 4
ART – antiretroviral therapy

(1) These are HIV infected individuals on ART<3-months
(2) These are HIV infected individuals on ART>3-years

θ - omitted from the multivariable analysis because of collinearity with HIV status
In bold are the p-values that were significant following univariable and multivariable analysis.

6.4. Discussion

The success of *S. pneumoniae* to cause infection in the airway depends on its ability to evade airway cell mediated killing mechanisms. Previous studies have shown that peripheral CD4+ T-cells depletion, predisposes individuals to higher carriage densities and bacterial pneumonia amongst the HIV-infected individuals (Glennie et al., 2013; Hirschtick et al., 1995). In this study, I developed a primary airway cell model for understanding the early kinetics of airway cell-mediated control of *S. pneumoniae* extracellular outgrowth *ex vivo*. Using this approach, first, I demonstrated that airway cells from HIV-uninfected adults control *S. pneumoniae* extracellular outgrowth during early infection. Secondly, HIV infection does not impair the airway cell-mediated control of extracellular *S. pneumoniae* outgrowth during early infection. Thirdly, during the late killing phase (24hrs post-infection), airway cells promote *S. pneumoniae* survival. Lastly, presence of extracellular *S. pneumoniae* 24hrs post infection is propagated by intracellular bacterial persistence.

Airway cells play a key role in lung immune homeostasis including early control of bacterial pathogens in the airspaces (Boehme et al., 2017; Byrne et al., 2015; Collini et al., 2018; Wilson et al., 2015). In this study, airway cells from HIV-uninfected individuals were able to control the extracellular *S. pneumoniae* outgrowth during early infection. Previous studies have primarily focused on single populations particularly AMs, shorter infection studies, as well as intracellular killing (cell invasion) probably due to their increased proportions of AMs in the airway (Gordon et al., 2000, 2013; Mitsi et al., 2019). However, this study provides a more holistic insight using unfractionated airway cells to understand early control of extracellular outgrowth of *S. pneumoniae,* as this process is likely critical for development of pneumonia. In line with the increased susceptibility to pneumococcal pneumonia in HIV-infected individuals, we expected to observe poor early control of pneumococcal outgrowth during HIV infection. However, short-term or long-term ART-treated HIV infection was not associated with poor control of pneumococcal extracellular outgrowth during

early infection *ex vivo*. This is consistent with previous findings focusing on AM, that demonstrated no impairment in the binding, internalisation and intracellular killing of opsonised pneumococci (Gordon et al., 2000, 2001, 2013), as well as results in Chapter 4 and 5.. This suggests that HIV infection may not significantly impact early control of pneumococcal infection.

Intriguingly, the presence of airway cells enhanced *S. pneumoniae* outgrowth 24-hrs post-infection, leading to a higher propensity of extracellular *S. pneumoniae* burden in conditions with cells compared to those without cells. This observation could be serotype dependent as the isolate used in this study was isolated as a meningitis causing isolate (serotype 3, sequence type 700), therefore it could have developed mechanism to evade host extracellular killing. The meningitis strain was only used in this study to model invasive disease as we did not have access to a strain known to cause pneumococcal pneumonia. On the other hand, the observed phenomenon could be a mechanism *S. pneumoniae* has evolved to exploit host immunity by obtaining micronutrients such as zinc, iron and manganese that are necessary for its propagation and maintenance (Cao et al., 2018; Eijkelkamp et al., 2019; Martin, 2020; Miao et al., 2018; Ong et al., 2013; Plumptre et al., 2014).

This study demonstrates that the presence of extracellular *S. pneumoniae* 24hrs post infection is associated with persistence of intracellular bacteria. The strength of this observation is confirmed through a multivariate analysis, as well as the fact the parameters were generated from two separate assays (Chapter 5 and 6). Furthermore, this study demonstrates that pneumococcal infection in the airway is a dynamic process as evidenced by the association between extracellular and intracellular pneumococcal survival, which could potentially influence the outcome of the infection. This dynamic survival mechanism through evasion of extra-intracellular killing has been shown to promote survival of other pathogens such *Cryptococcus* and *S. aureus* in macrophages (Flannagan et al., 2016; Goldman et al., 2000; Jubrail et al., 2016; Rollin et al., 2017; Shourian and Qureshi,

2019). Intracellular persistence of *S. pneumoniae* in alveolar macrophages could be a major driver of pneumonia due to its potential to propagate extracellular bacterial outgrowth. Therefore, understanding the mechanisms of intracellular pneumococcal survival in airway cells is critical and warrants further investigation.

Phagocytes are able to control extracellular burden of bacteria through production of H_2O_2 (which can be toxic to epithelia cells), reactive oxygen and nitrogen species. However, pneumococcus has developed mechanisms to survive in high oxygen tension environments, ROS and H_2O_2 detoxifying enzymes such as NADH oxidase, superoxide dismutase, thiol peroxidase and alkyl hydroperoxidase and metal scavengers such as manganese (Mn^{2+}) (Erttmann and Gekara, 2019; Hajaj et al., 2012; Rai et al., 2015; Yesilkaya et al., 2013). The host is also able to sequestrate metal cofactors such as Fe, Mn, Zn, Ni which are critical for the function pneumococcal enzymes used in ROSN detoxification, like superoxide dismutase (Cao et al., 2018; Eijkelkamp et al., 2019; Miao et al., 2018). The host can also control extracellular growth of pneumococci by producing excess Zn which prevents the uptake of manganese by binding irreversibly to the high affinity manganese solute binding protein PsaA, resulting in it becoming hypersensitive to oxidative stress (Ong et al., 2015; Yesilkaya et al., 2013). The host can also increase intracellular Zn as shown with murine phagocytic cells from zinc restricted mice, which demonstrated poor pneumococcal control, increased bacterial burden and reduced survival times (Eijkelkamp et al., 2019).

The main limitations of this study are that I did not measure any microbiocidal factors like reactive oxygen and nitrogen species (RONS), transitional metals, cytokine milieu that could be key in the clearance of extracellular pneumococci and further explain my findings. However, the strength of this study is that it provides a more holistic insight using unfractionated airway cells critical to the control of extracellular control pneumococcus. This model acts as proxy and helps explain how human airway cells could control pneumococcal infection.

6.5. Conclusion

Collectively, this study elucidates how *S. pneumoniae* exploits host airway cells for its extracellular survival *ex vivo* and this could be a critical step in pneumococcal pathogenesis. This work highlights the importance of both intracellular (cell invasion) and extracellular killing (outgrowth) assays in understanding the pathogenesis of *S. pneumoniae* infection in the airway.

CHAPTER 7

7.0. Overall Discussion

7.1. Introduction

S. pneumoniae is the leading cause of community-acquired pneumonia in all ages, with the greatest incidence occurring in children, the elderly and HIV-infected individuals (Aston et al., 2019; Azzari et al., 2016; Troeger et al., 2017). The airway is the major port of entry for respiratory pathogens, with the lung serving as the main site of pulmonary defence. Pneumococcal pneumonia is preceded by asymptomatic colonisation and both pneumonia and colonisation are highest amongst children and the HIV infected individuals (Bogaert et al., 2004; Heinsbroek et al., 2015, 2016). Alveolar macrophages (AMs) are the major effector cells found in the airway, responsible for the early clearance and control of respiratory pathogens. To date, few studies exist in humans on the lower airway mediated immunity against *S. pneumoniae*, although most of the knowledge gathered is derived from studies done in animal models.

This thesis tested the **primary hypothesis** that **HIV infection is associated with impaired airway alveolar phagocyte killing function, leading to survival and propagation of pneumococci**. The secondary hypotheses tested that: **(i)** HIV-infected adults on short-term antiretroviral therapy (ART) possess differentially impaired lower airway phagocyte pneumococcal binding and internalisation capacity to *S. pneumoniae*, **(ii)** HIV is associated with impairment of AMs killing of *S. pneumoniae* leading to its intracellular survival, and **(iii)** HIV-infected adults on short-term ART show impairment of airway control of extracellular outgrowth of *S. pneumoniae ex vivo*.

To answer the research question, three groups of individuals aged between 18 – 60 years were recruited consisting of asymptomatic HIV-uninfected adults, HIV-infected adults on short-term and long-term ART residing in Blantyre, Malawi. Lower airway samples (bronchoalveolar lavage fluid) were collected from the

participants and used to investigate airway mediated defence immunity against *S. pneumoniae* in an *ex vivo* model. The study was a comparative cross-sectional design, which allowed a direct comparison of airway pulmonary immunity amongst the three groups and also shedding light on the possible mechanisms involved that render HIV-infected individuals more susceptible to pneumococcal pneumonia. This chapter focuses the discussion on the major findings in this thesis, their implications, and ideas for future work.

7.2. Summary of research findings in context

This thesis has generated three major conclusions arising from the airway *ex vivo* model and were centred on 1) the binding and internalisation of pneumococcus by alveolar macrophages (AMs) and neutrophils 2) the AMs intracellular killing kinetics of pneumococcus and 3) airway cell extracellular killing kinetics of pneumococcus. The findings generated in this thesis are summarised in Figure 7.1.

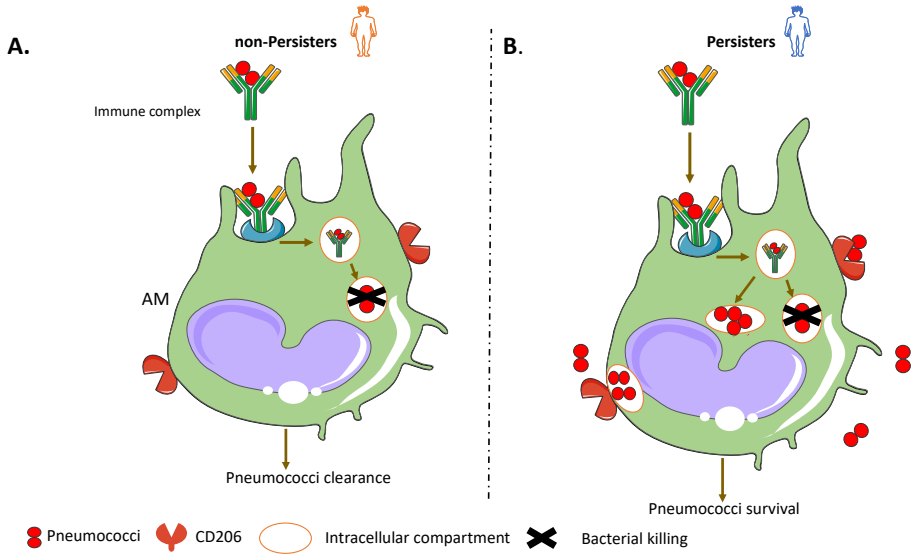


***Figure 7.1*. The model summarises the thesis findings. A.** Individuals who are able to control pneumococci (non-persisters), their alveolar macrophages internalise opsonised pneumococci through their Fc portion resulting in pneumococcal killing and clearance from the intracellular and extracellular compartments. **B.** Individuals who are able to unable clear pneumococci (persisters), their alveolar macrophages internalise opsonised pneumococci through their Fc portion resulting in the failure to kill internalised pneumococci from the intracellular compartments. Internalised pneumococci propagate the survival of extracellular pneumococci. Abbreviations: AMs – alveolar macrophage. This figure contains some artwork produced by Servier Medical Art (http://smart.servier.com), under creative commons license 3.0.

Firstly, the thesis investigated whether HIV-infected adults on short-term ART possess differentially impaired airway phagocyte pneumococcal binding and internalisation capacity. Using an *ex vivo* infection model of primary airway cells, it was observed that AMs are the principal phagocytic cell in the airway and the major cell associated with IgG-opsonised pneumococcal-ST3 following *ex vivo* infection, irrespective of HIV status. It was also observed that HIV-infected adults on short-term or long-term ART do not exhibit impaired association of IgG-opsonised pneumococcal-ST3 with AM. Furthermore, it was demonstrated that HIV-infected adults on short-term ART exhibited enhanced binding of IgG-opsonised pneumococcal-ST3 to neutrophils, but not to AMs.

Secondly, this thesis investigated whether HIV-infection was associated with an impaired AMs killing of *S. pneumoniae* resulting in its intracellular survival. Using the gentamycin protection (invasion) assay which I developed for investigating early AMs-mediated intracellular control of pneumococcus. Using confocal microscopy, it was observed that pneumococci persist in $CD206^{+}$AMs 24hrs post-infection. It was also observed that slow early killing kinetics of intracellular pneumococci within the first 3hrs post-infection in HIV-uninfected adults but not HIV-infected individuals on ART was associated with increased propensity for bacterial persistence in $CD206^{+}$AMs 24hrs post-infection. Furthermore, HIV-infection and ART were associated with a reduced AMs-mediated intracellular killing capacity of *S. pneumoniae* during the late killing phase (24-hours post infection) leading to a higher propensity of intracellular *S. pneumoniae* persistence. It also was demonstrated that natural *S. pneumoniae* carriage did not augment AMs-mediated intracellular killing kinetics.

Lastly, this thesis investigated whether airway cells from HIV-infected individuals on short-term ART show impaired control of *S. pneumoniae* extracellular outgrowth. This thesis demonstrated that airway cells from HIV-uninfected adults control *S. pneumoniae* extracellular outgrowth during early infection. It was also shown that HIV-infection did not impair the airway cell-mediated control of

extracellular *S. pneumoniae* outgrowth during early infection. It was further demonstrated that the presence of extracellular *S. pneumoniae* 24hrs post infection is propagated by intracellular bacterial persistence.

7.3. Implications of findings

The data presented in this thesis have plausible implications on future studies particularly on airway mucosal immunity (human and animal), disease pathogenesis and vaccine development. Implications of findings from this thesis are discussed in sections below.

7.3.1. Airway mucosal immunity

The thesis demonstrated a reduced AMs-mediated intracellular killing capacity of *S. pneumoniae* during the late killing phase (24-hours post infection) leading to a higher propensity of intracellular *S. pneumoniae* persistence HIV-infected on ART. These findings concur with previous work from Dockrell's group; where HIV gp120 was found to be associated with an impairment in apoptosis intracellular pneumococci killing in MDM (Collini et al., 2018). These findings have also been shown in other human lung respiratory disease states such COPD (Bewley et al., 2017). The same group has also shown that CD68.hMcl-1 transgenic mouse model with macrophage-specific overexpression of the human anti-apoptotic Mcl-1 protein resulted in a blunted microbiocidal killing response of internalised pneumococci (Preston et al., 2019). Taken together these findings suggest that chronic disease conditions impair AMs response to pneumococci and possibly render these individuals more susceptible to pneumococcal pneumonia. Furthermore, we also uncovered that the use of single immune cell type and global airway immune cells both aids in deciphering deficits in the broad pneumococcal immune responses as shown in the findings.

However, since it is challenging to obtain airway samples, previous studies have shown that using monocyte derived macrophages (MDMs) or monocyte derived dendritic cells (MoDCs), can be used as a surrogate immune cells to decipher AMs

and DCs pneumococcal intracellular immune kinetics (Collini et al., 2018; Subramanian et al., 2019). What remains to be shown though is to demonstrate whether MDMs and MoDCs can be used as a substitute for primary airway cells (AMs and DCs) in studying pneumococcal immunity in a high carriage setting. Many other studies use epithelial cell lines but not airway epithelial cells due to the challenge associated with their limited numbers during collection (Weight et al., 2019). The use of animal studies to investigate airway pneumococcal immunity could offer an alternative model. However, in our setting it maybe more challenging to use animal studies due to the costs associated with the programme. In Malawi, an experimental human pneumococcal challenge has been established (Gordon et al., 2017) and it offers a better alternative of studying mucosal immunity against *S. pneumoniae*.

7.3.2. Pneumococcal pathogenesis within the airway

This thesis has also demonstrated that the presence of extracellular *S. pneumoniae* 24hrs post infection is propagated by intracellular bacterial persistence. This suggests that though, *S. pneumoniae* is believed to be an extracellular pathogen, its fate in the airway depends on the ability of phagocytes to kill the internalised bacteria irrespective of HIV status. These observations also suggest that it is plausible that the *S. pneumoniae* might have an intracellular propagation phase in airway phagocytes, though this merits further experimental investigations to elucidate this postulation. No human studies exist so far with which to compare our results. These findings have an implication on our understanding of the pneumococcal pathogenesis within the airway and the establishment of infection. Previously, in a murine model of sepsis it was shown that sepsis is established by a single pneumococci bacterium (Ercoli et al., 2018). This aligns well with our invasion *ex vivo* experiment, whereby during the eclipse phase the bacteria were virtually cleared from the AMs but re-emerged during the late infection phase. Macrophages may represent an immune-privileged sanctuary and a reservoir for the reseeding of bacteria into the extracellular compartment to cause pneumococcal infection in the airway (Ercoli et al., 2018).

AMs have been shown to serve as a reservoir for other pathogens such as HIV, *S. aureus*, *Mycobacterium tuberculosis* and *C. neoformans* (Boliar et al., 2019; Flannagan et al., 2016; Honeycutt et al., 2017; Shourian and Qureshi, 2019).

I have also demonstrated that the pneumococci establishes persistence within $CD206^{+}$AMs and this concurs with the recent findings showing that pneumolysin and CD206 interactions mediate pneumococcal internalisation into non-lysosomal compartments thus promoting its survival (Subramanian et al., 2019). Notably, these findings are not exclusively based on primary airway cell model, but have previously been shown in different models such as an MoDc, murine AMs and AMs from EHPC model (Mitsi et al., 2019; Subramanian et al., 2019). These findings are a departure from the commonly accepted dogma of *S*. *pneumoniae* being an obligate extracellular pathogen, but are in alignment with previous findings which demonstrated that pneumococcal septicaemia is initiated by a single bacterial cell within murine splenic macrophages (Ercoli et al., 2018). This is despite the belief that AMs are efficient in the clearance and control of pneumococcus. As a field we may need to shift our focus of pneumococci only being a classic obligate extracellular pathogen but also consider that the bacteria might be able to establish intracellular residency before being reseeded extracellularly to cause serious infections. Furthermore, these findings are unique and novel in our setting of both high HIV burden and pneumococcal carriage. This opens a new avenue for further investigations into the insights of human immune responses elicited in the pulmonary mucosa. At the same time, our findings affirm previous findings which demonstrated that HIV infection or being on ART does not impair the binding and internalisation of opsonised pneumococci by AMs (Gordon et al., 2001, 2013). This shifts the focus of the field to understanding events occurring within the AMs between the early and late killing phase and virulence factors involved during early events of pneumococcal infection.

7.3.3. Vaccine development

This thesis sheds some insights into why the current PCVs and PPSV-23 are not effective pneumococcus serotype 3 strains. This might be debatable because we used an invasive serotype-3 strain and it could behave differently from carriage serotypes. However, evidence from literature shows that the current vaccines are not effective against pneumococcus serotype 3 due to its clonal diversity (Azarian et al., 2018; Groves et al., 2019; Swarthout et al., 2020). More research needs to be carried out in understanding the differences in global pneumococcal serotype strains and their preponderance for vaccine escape.

AMs are the most abundant cell type within the airway and its proportions are less affected by HIV and antiretroviral therapy. Previously, it was shown that AMs from carriage positive individuals had a heightened opsonophagocytic capacity, and also displayed non-specific response to pneumococcal stimulus (Mitsi et al., 2019). This demonstrates that AMs are excellently placed to initiate an immune response in the lower airway should they encounter pneumococcus. Thus, they provide a critical target for pneumococcal vaccine research and could be boosted to elicit non-specific responses against common respiratory pathogens thus successfully preventing pneumonia in susceptible individuals. The administration of a live- attenuated intranasal pneumococcal vaccine could augment the pulmonary immune-responses and confer serotype-independent protection (Mitsi et al., 2019).

7.4. Limitations

Though this thesis produced intriguing findings, it also has limitations which affect the generalisability of some of the findings. In this section, I will focus on the limitations that were not discussed in the previous chapters.

The main limitation was the lack of a sufficient sample size, we recruited fewer individuals than expected especially in the HIV-infected individuals on ART>3-years (12 vs 30). This was due to study time constraints, we lost 5-months of

recruitment as our two research bronchoscopes malfunctioned around the same time, and we could not continue with recruitment till a replacement bronchoscope had been purchased and shipped to Malawi. In, addition not all the experiments that were done (invasion assay, bacteria outgrowth, uptake assay), had the expected number of individuals per group (n=30), because of the limited number of airways cells returned during the bronchoscope procedure. In such circumstance, airway cells were first prioritised for the binding and internalisation and invasion assays, rather than the outgrowth assay. To resolve the limited number of cells for the experiments, the use of MDMs and peripheral lymphocytes in optimised ratios could have been adopted for the invasion assay.

In addition, I was not able to decipher the mechanism that differentiates individuals found to be persisters and non-persisters in this thesis. This unusual phenomenon could possibly be strain related, or a natural phenomenon associated with all clinically relevant pneumococcus. To solve this, we could have included other strains of pneumococcus (carriage and invasive) or mutant strains as comparators to observe if we could replicate similar findings before trying to understand deficits associated with the intracellular killing pathways. Furthermore, I could have also increased the earlier killing time points to six hours and the late killing phase to include time point 20, 22- and 24-hours post infection for us to observe the exact time points when AMs reached the limit of intracellular detection of pneumococcus by microbiological methods.

Lastly, another possible methodological criticism could be that all participants recruited were asymptomatic middle-aged adults. It is unknown if the findings described in this thesis would be reflective of results in children, the elderly, or the immunocompromised. However, our results are hypothesis generating, pointing to deficits within the intracellular killing of phagocytic pathways, which could have a bearing on all the susceptible populations on how their phagocytic cells kills and clear pneumococcus, but this remains to be clarified.

7.5. Future studies

The findings in this thesis have addressed several hypotheses and some intriguing points came up that warrant further investigation and if addressed would better the understanding of the early events leading to pneumococcal infection in the airways.

7.5.1. Deciphering the mechanism of survival for serotype 3 pneumococcus in human macrophages

It will be desirable to understand the biology of pneumococcus serotype 3 and virulence factors used for its evasion of the airway immunity. Pneumococcus serotype 3 has been shown previously to be clonally diverse and this is one of the possible reasons why the current vaccines are not effective against it (Azarian et al., 2018; Binsker et al., 2017; Swarthout et al., 2020). In Malawi, majority of the serotype 3 found are sequence type 700 and are different from those found in other African and European countries (Azarian et al., 2018; Everett et al., 2012; Groves et al., 2019). It will be interesting to research the difference between carriage and invasive pneumococcus serotype 3 found in Malawi and whether have the same invasive and evasion potential.

Since, airway samples are difficult to obtain than peripheral samples; it will also be worth verifying the use of MoDCs or the MDM as alternative models of airway for studying the control of pneumococcus by DCs and AMs in a high carriage setting. This knowledge for the alternate model will be crucial for us to further demonstrate the main biological differences between persisters and non-persister individuals at gene (transcriptomics) and metabolic level (seahorse). To date, we still don't know which cellular pathways are differentially upregulated and downregulated between persisters and non-persister individuals. Previous studies have used MDMs and MoDCs, but this was in regions of low pneumococcal carriage (Collini et al., 2018; Subramanian et al., 2019). So, it remains to be investigated whether the two models perform equally the same in a high carriage setting.

Recently, it has been shown that pneumolysin (haemolytic region) is able to interact with CD206 found on DCs and AMs and mediate pneumococcal internalisation into non-lysosomal compartments (Subramanian et al., 2019). We were not able to show in this study how the pneumococcus was able to evade the phagocytic killing and survive intracellularly as this needed more time to develop fully. Further work is required to investigate whether pneumolysin or other virulence factors are key in the evasion of the killing machinery and by which mechanism it exerts these effects. Creation of *S. pneumoniae* ST3 pneumolysin mutants will be required to demonstrate whether the pneumolysin virulence factor is used by the bacteria in the evasion of intracellular killing machinery. In addition, we suggest doing time-lapse experiments, to prove this hypothesis and at the same time demonstrate which cell structures pneumococcus localises as it evades the phagolysosome. Single cell analysis of the AMs might also be critical to investigate molecular signatures associated with the intracellular survival of pneumococcus and the AMs subpopulations associated with this phenomenon.

Lastly, it will be critical to demonstrate the pro-and anti-inflammatory cytokine profile from the supernatants of *ex vivo* infections between the persisters and non-persister individuals at earlier and late time-points during the infection. Preliminary data generated exist from few individuals recruited earlier into the study, but this is not substantial to support our hypothesis. Furthermore, it will also be important to show whether the pneumolysin is associated with pro-inflammatory cytokine repression in persister individuals.

7.6. Concluding remarks

In summary, I have observed that pneumococcal binding and internalisation by alveolar macrophages is not impaired in treated HIV infection, but that deficits exists in the intracellular killing pathways leading to reduced bacterial killing. The data shows that the survival of extracellular *S. pneumoniae* during the late killing phase of infection is largely driven by intracellular bacterial persistence. Importantly, deficits in intracellular killing were observed in alveolar macrophages amongst all the three groups recruited comprising of asymptomatic adults namely; HIV-uninfected, HIV-infected on short-term and those on long-term antiretroviral therapy. A failure of AMs to kill internalised pneumococci could explain an important role underlying the increased risk pneumococcal pneumonia in susceptible populations. There is a need for further research on pneumococcal serotype 3 and evasion of airway cellular immunity and the role of natural carriage on the early events of pneumococcal infection in the lower airways. The work conducted for this thesis adds to current understanding of the early cellular events leading to pneumococcal clearance and establishment of infection in the lower airways.

8.0. REFERENCES

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APPENDICES

Appendix 1: Study institutional support

THE COLLEGE OF MEDICINE
Malawi-Liverpool-Wellcome Trust
Clinical Research Programme
www.mlw.medcol.mw

Faculty of Basic Sciences and Health Professions
Department of Medical Laboratory Sciences
www.medcol.mw

11th December 2017

Chair,
College of Medicine Research and Ethics Committee
College of Medicine
PO Box 160
Blantyre

Dear Dr. Yohannie Mlombe,

Characterisation of viral-associated immune environment that favours growth of *Streptococcus pneumoniae* in the alveoli: A pilot study

I am delighted to write this letter confirming our support to Tinashe Nyazika as PI on the proposal entitled 'Characterisation of viral-associated immune environment that favours growth of *Streptococcus pneumoniae* in the alveoli: A pilot study'. Tinashe is a PhD student in Viral Immunology group at MLW. He is co-supervised by Drs. Kondwani Jambo and Henry Mwandumba.

This project is part of Tinashe's PhD programme. It is a pilot study to be nested within PERSIST (P.03/16/1907; Dr Jambo) and HITUB (P.05/15/1728; Dr Mwandumba) studies. It fits within the scope of our ongoing work investigating the immunological basis for the increased risk to pneumococcal pneumonia in HIV-infected Malawian adults. Specifically, it builds on our recent observation that pneumococcal-specific alveolar $CD4^+$ T cell responses are preserved in HIV-infected adults (Peno, C. *et al.* J Infect. 2017), suggesting that there are other alternative mechanisms rendering HIV-infected adults more susceptible to pneumococcal pneumonia. Therefore, in this pilot study, we hypothesise that "the presence of HIV in alveoli suppresses alveolar phagocyte anti-pneumococcal immunity leading to poor clearance of the pneumococci"

MLW has the necessary infrastructure and expertise to successfully execute this research study within the MLW, College of Medicine, Queen Elizabeth Central Hospital and Blantyre Health office framework. Specifically, we will grant access to this study for use of our laboratories, data management service, grant's management service and clinical facilities.

As MLW, we will provide all the required support to the study.

Yours sincerely,

Professor Stephen Gordon
MA MD FRCP FRCPE DTM & H
Director, MLW

www.mlw.medcol.mw

Malawi-Liverpool-Wellcome Clinical Research Programme
PO Box 30096, Chichiri, Blantyre 3, Malawi Tel: +265 187 6444 Fax: +265 187 5774

Appendix 2: Hospital Support

Telephone: (265) 1 874 333 / 677 333

Facsimile: (265) 1 876 928

Email: queenshosp@globemw.net

All Communications should be addressed to:

The Hospital Director

In reply please quote No

QUEEN ELIZABETH CENTRAL HOSPITAL

PO BOX 95

BLANTYRE

MALAWI

04/05/2018

The Hospital Director
Queen Elizabeth Central Hospital
P. o. Box 95
Blantyre 3

The Chairman
COMREC
Blantyre

Dear Sir

RE: A CHARACTERISATION HIV ASSOCIATED CHANGES IN ALVEOLAR IMMUNE ENVIRONMENT THAT FAVOUR THE GROWTH OF STREPTOCOCCUS PNEUMONIAE: A PILOT STUDY.

The above project has our departmental support. The details of the projects and due process will be taken to fulfill all hospital set requirements. We will not hesitate to inform you sir should there be changes to this or non-adherence to stipulated agreements.

Kindly provide your institutional letter of support for the same. I wish Dr Mwandumba the best of luck in execution of this important project.

Yours truly

DR L. MASAMBA

HOD MEDICINE DEPARTMENT.

Appendix 3: COMREC Certificate of ethics approval

CERTIFICATE OF ETHICS APPROVAL

This is to certify that the College of Medicine Research and Ethics Committee (COMREC) has reviewed and approved a study entitled:

P.01/18/2335 - Characterisation of HIV-associated changes in alveolar immune environment that favour the growth of Streptococcus pneumoniae version 1.1 dated 16 February 18 by Mr Tinashe Kenny Nyazika

On 26-Mar-18

As you proceed with the implementation of your study, we would like you to adhere to international ethical guidelines, national guidelines and other requirements by COMREC as indicated on the next page

Dr. YB. Mlombe - Chairperson (COMREC)

Approved by College of Medicine
26 MAR 2018
(COMREC)
Research and Ethics Committee

26-Mar-18
Date

Appendix 4: LSTM Sponsor letter

Mr Tinashe Kenny Nyazika
Malawi-Liverpool-Wellcome-Trust
Clinical Research Programme
P.O Box 30096
Chichiri
Blantyre 3
Malawi

LSTM
LIVERPOOL SCHOOL OF TROPICAL MEDICINE
Pembroke Place,
Liverpool, L3 5QA, UK
Tel: +44(0)151 705 3100
Fax: +44(0)151 705 3370
www.lstmed.ac.uk

Tuesday, 03 April 2018

Dear Mr Nyazika,

Re. Research Protocol (18-007) Characterisation of HIV-associated changes in alveolar immune environment that favour the growth of Streptococcus pneumoniae: A Pilot study

I am pleased to confirm that LSTM has agreed to act as Sponsor for the above mentioned clinical research study.

Please note that LSTM approval to allow your study to proceed is conditional upon compliance with the relevant regulatory requirements.

All study staff should be given the appropriate training in Protocol, GCP, Consent and Data Protection, relevant to their responsibilities as defined within the study protocol.

LSTM Research Governance and Ethics Office should receive annual study progress and final close out reports via lstmgov@lstmed.ac.uk

Yours Sincerely,

pp. L. Taylor

Carl Henry
Research Governance Manager
Research Governance and Ethics Office

Appendix 5: Material transfer agreement

UNIVERSITY OF MALAWI
COLLEGE OF MEDICINE

COLLEGE OF MEDICINE RESEARCH and ETHICS COMMITTEE (COMREC)

MATERIAL TRANSFER AGREEMENT FORM ON SHIPPING OF SAMPLES

Samples collected from Malawi for research purposes is the property of the Government of Malawi represented by the College of Medicine (CoM), under the authority of COMREC and such samples can be accessed or recalled by the Government of Malawi and CoM at any time without let or hindrance.

Shipment of samples outside the country without proper justified reasons is not allowed.

Investigators are encouraged to develop capacity to do all tests required in the country. In special cases where this may not be possible the investigators must justify in the proposal the reason for importation and exportation of samples.

In review process the following have to be considered:-

- There must be a justification for importation and exportation of samples.
- COMREC shall make sure that there is material transfer agreement between relevant institution in the context of exportation and importation of samples. The material transfer agreement shall include the following:-

1. The intention of the importation and exportation
2. The duration of storage
3. Location of storage
4. The appropriate informed consent authorizing the exportation and importation
5. To whom it will be accessible
6. Who will be the controlling officer of the samples.
7. Ownership of samples
8. Capacity Building

- For studies that requires shipping of samples should fill the Material Transfer Agreement (MTA) form at COMREC. In case there are issues of Intellectual Property Rights (IPR) the committee shall advise the concerned parties to have prior agreement of IPR which has to be signed by all stakeholders before COMREC approval.
- Samples collected in Malawi will not be sold.
- Initial authorization to store samples can last up to 5 years, if one wishes to use sample beyond 5 years, one must seek authorization from COMREC. This Authorization will last another 5 years before it is due for renewal.

Approved by
College of Medicine
08 SEP 2018
(COMREC)
Research and Ethics Committee

COMREC MATERIAL TRANSFER AGREEMENT FORM
Protocol Number: P.01/18/2335
Title of protocol: Characterisation of HIV-associated changes in alveolar immune environment that favour the growth of Streptococcus pneumoniae: A Pilot study
(a) **Intention and Justification of transfer:** We would like to transfer fixed brochoalveolar lavage (BAL) cells, pneumococcal isolates and cytospins (slides) to Prof Marco Oggioni's laboratory at University of Leicester in the United Kingdom, for electron/confocal microscopy analysis and bacterial sequencing, to determine whether pneumococcus survive intracellularly in human alveolar macrophages. While MLW and the College of Medicine are expanding the range of their immunological techniques, they do not have the expertise and capacity for electron and confocal microscopy, as well as, bacterial sequencing. However, this facility is available at our co-investigator's (Marco Oggioni) institution. With input from the Oggioni's laboratory, we plan to analyse intracellular bacteria in relevant cells and pneumococcal isolates from the samples from Malawi. As part of our local capacity development and technology transfer initiative, we will develop SOPs for fluorescence microscopy at MLW together with researchers from the University of Leicester. In addition, this work forms part of a PhD project for Tinashe Nyazika who is jointly registered between the University of Malawi-College of Medicine and the Liverpool School of Tropical Medicine.
(b) Duration of storage: Indicate date, month or years
We will store the shipped sample for a maximum of 5 years in case we need to repeat some of the analysis. If we need to store the samples for more than 5 years, we will apply to COMREC to extend the storage period. Samples leftover at the end of the approved storage period (including extension period) will be destroyed by incineration in accordance with local health and safety guidelines for the disposal of bio-hazardous human materials. We will inform COMREC when the samples are destroyed.
(b) Responsible Party:
At the University of Leicester, Dr Kondwani Jambo and Dr. Marco Oggioni will have overall responsibility for the samples, although their ownership will remain with the government of Malawi through the University of Malawi's College of Medicine.

Approved by
College of Medicine
08 SEP 2018
(COMREC)
Research and Ethics Committee

(c) Location of stored samples:
Shipped samples will be stored safely in a secure designated freezer in the Oggioni laboratory at the University of Leicester, United Kingdom
(d) Transportation of samples:
World Courier will ship samples. This company has a long service history with the MLW Clinical Research Programme and has reliably shipped ambient, cold and frozen human samples to various collaborating institutions and laboratories around the world.
(e) Ownership of samples:
Although Dr Jambo and Dr Oggioni will have custodianship and responsibility for exported samples, their ownership will be with the government of Malawi through the University of Malawi's College of Medicine
(e) After all laboratory testing has been completed: Describe what will happen to the samples
Samples leftover after all analyses have been completed will be destroyed by incineration in accordance with local health and safety guidelines for the disposal of bio-hazardous human materials.
(g) Appropriate informed consent authorizing the exportation and importation of samples
Consent to ship samples to collaborating institutions abroad will be sought when obtaining consent from study participants at recruitment. This information has been included in both the English and Chichewa versions of the participant information sheets. Personal identifiers of study participants will not accompany shipped samples and no sample will be shipped without the written consent of the study participant from whom it was obtained.
(h) To whom will the samples be accessible
To the PI (Tinashe K. Nyazika), co-investigators (Dr Kondwani Jambo) and Collaborators (Prof Marco R. Oggioni and Dr Megan De Ste Croix)
(i) Who will be the controlling officers of the samples
Dr Jambo and Dr Oggioni

Approved by
College of Medicine
08 SEP 2018
(COMREC)
Research and Ethics Committee

- **Capacity building plan:** This has been outlined in part (a)
- **Please note exportation of samples should be considered as a last resort. Effort to build the local capacity and expertise should be a priority.**

Signed by

Name of the PI: Tinashe Nyazika

Name of Institution: MLW Clinical research programme

Signature:

Date Signed: 16th July 2018

Name of Co-PI: Dr Kondwani Jambo

Name of Institution: MLW Clinical research programme

Signature:

Date Signed: 16th July 2018

COMREC APPROVAL

Name of the Chairperson:

Signature:

Date Signed:

Name of IRB Administrator:

Signature:

Date Signed:

COMREC STAMP OF APPROVAL:

Approved by
College of Medicine
08 SEP 2018
(COMREC)
Research and Ethics Committee

Appendix 6: LSTM Support for the MTA amendment

LSTM
LIVERPOOL SCHOOL OF TROPICAL MEDICINE
Pembroke Place,
Liverpool, L3 5QA, UK
Tel: +44(0)151 705 3100
Fax: +44(0)151 705 3370
www.lstmed.ac.uk

Mr Tinashe Kenny Nyazika
PO Box 30096
Blantyre 3
Malawi

Monday, 15 October 2018

Dear Mr Nyazika,

Re. Research Protocol (18-007) Characterisation of HIV-associated changes in alveolar immune environment that favour the growth of Streptococcus pneumoniae: A Pilot study.'

Thank you for your correspondence of 14 September 2018 providing the LSTM Research Ethics Committee and LSTM Research Governance Manager with details to include the use of laboratories at the University of Leicester, broader recruitment criteria and inclusion of new collaborators.

This amendment has now been reviewed, noted and accepted on the behalf of the Committee and the LSTM, as study Sponsor. Please continue to adhere to the conditions of approval and to update us of any further changes to the study that may arise.

Please continue to adhere to the original conditions of approval following notification of study conclusion with specific regard to confidentiality and data management.

Yours sincerely,

Dr Angela Obasi
Chair
LSTM Research Ethics Committee

LSTM Research Governance and Ethics Office
Mr Carl Henry
LSTM Research Governance Manager

Yours sincerely,

Appendix 7: English consent form

PneumoVI

Consent Form

Version 1.1 (2018/02/16)

CONFIDENTIAL

Title of Project	**Characterisation of HIV-associated changes in alveolar immune environment that favour the growth of *Streptococcus pneumoniae*: A Pilot study.**
Principal investigator	**Mr. Tinashe K. Nyazika**
Organisation	**Malawi Liverpool Wellcome Trust**
Sponsor	**Liverpool School of Tropical Medicine**
Contact details for Co-investigator	**01 876 444** **Dr Henry Mwandumba (+265) 881 07 38 22**

Study number

Approved by College of Medicine 26 MAR 2018 (COMREC) Research and Ethics Committee

Study Number

Informed consent to take part in the study

	If you agree with each statement, please INITIAL the box provided	
1	I confirm that I have read (or it has been read to me) and understand the Participants Information Documents. I understand what taking part in the study involves and have been given sufficient time to decide whether or not to take part.	
2	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason and without my medical care or legal rights being affected.	
3	I was given the opportunity to ask questions about the study.	
4	My questions have been answered clearly and I am satisfied that I have been given enough information about the study.	
5	I consent to give information about my medical history, to be examined by the study clinicians, and to give nasal, bronchoalveolar lavage and blood samples as required by the study.	
6	I consent to undergo phlebotomy, nasal and bronchoalveolar lavage collection. I understand what these procedures involve and I am aware of their potential risks and complications.	
7	I give consent for some experiments to be performed in United Kingdom on my samples since these experiments cannot be performed in Malawi.	
8	I consent to having my samples stored for a period of 5-years and that they can be used for other tests related to this project	
9	**I voluntarily agree to participate in this study**	

Print name of participant	
Signature/Thumb print of participant	
Date	

Approved by
College of Medicine
26 MAR 2018
(COMREC)
Research and Ethics Committee

Study Number	

Statement by the researcher/person taking consent

I confirm that the participant was given the opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

Print name of witness	
Signature	
Date	

Print name of researcher or staff taking consent	
Signature	
Date	

When complete: 1 copy for participant; 1 copy (original) for research

Appendix 8: Chichewa consent form

PneumoVI

Fomu ya chilorezo

Gawo lolumikizidwa 1.1 (2018/02/16)

ZA CHINSINSI

Mutu wa kafukufuku	**Characterisation of HIV-associated changes in alveolar immune environment that favour the growth of *Streptococcus pneumoniae*: A Pilot study.**
Mkulu wa kafukufuku	**Mr. Tinashe K. Nyazika**
Bungwe	**Malawi Liverpool Wellcome Trust**
Wopereka ndalama zoyendetsera Kafukufukuyu	**Liverpool School of Tropical Medicine**
Tsatane-tsatane wa uthenga wa m'mene mungalumikizirane ndi mkulu wa kafukufukuyu	**01 876 444** **Dr Henry Mwandumba (+265) 881 07 38 22**

Nambala ya kafukufuku

Approved by College of Medicine 26 MAR 2018 (COMREC) Research and Ethics Committee

Study Number

Mau olankhulidwa momveka bwino potenga nawo mbali mukafukufukuyu

1	Ndikutsimikizira kuti ndawerenga (kapena lawerengedwa kwa ine) ndipo ndikumvesetsa tsamba la chilorezo cha wotenga nawo mbali. Ndikumvesetsa zimene zizichitika mukafukufukuyu potenga nawo mbali, ndipo ndapatsidwa nthawi yokwanira yopangira chiganizo choti ndikatenge nawo mbali kapena ayi.	
2	Ndikumvesetsa kuti kutenga nawo mbali kwanga ndikosakakamizidwa, ndipo ndili ndi ufulu wotuluka mu kafukufukuyu nthawi ina iliyonse, popanda kupereka chifukwa, komanso popanda kukhudza chisamaliro chimene ndingalandire kuchipatala kapena kukhudza ufulu wanga wovomerezeka.	
3	Ndinapatsidwa mwayi wofunsa mafunso okhudzana ndi kafukufuku.	
4	Mafunso anga ayankhidwa momveka bwino ndipo ndine okhutitsidwa kuti ndapatsidwa uthenga wokwanira wokhudzana ndi kafukufukuyu.	
5	Ndikupereka chilorezo choti ndikapereke uthenga wokhudzana ndi mbiri yanga yokhudzana ndi za chipatala, kuti ikaunikidwe ndi madotolo a kafukufuku, ndikupereka totokosela m'phuno ndi kukhosi komanso masampulo a magazi, mongak uli kofunikira kutero.	
6	Ndikupereka chilorezo choti ndikatengedwe magazi komanso totokosela m'phuno. Ndikumvesetsa zimene zizchitika, komanso ndikudziwa za chiopyezo komanso mavuto amene angakhalepo.	
7	Ndikupereka chilorezo kuti ntchito ina yoyeza masampulo anga ikathe kuchitikira ku maiko a ku United Kingdom, popeza ntchito yoyezayi siingathe kuchitika ku Malawi kuno.	
8	Ndikuvomeleza kuti masampulo anga akhoza kusungidwa kwa zaka zisanu ndipo atha kuzayezedwa mwapadela mogwilizana ndi kafukufukuyi.	
9	**Ndikuvomeleza mosakakamizidwa kutenga nawo mbali mukafukufukuyu**	

Lembani dzina la wotenga nawo mbali	
Siginichala /chidindo cha chala cha wotenga nawo mbali	
Deti	

Approved by College of Medicine 26 MAR 2018 (COMREC) Research and Ethics Committee

Study Number

Mawu olanhkulidwa ndi wopangitsa kafukufuku/munthu amene akupempha chilorezo

Ndikutsimikizira kuti wotenga nawo mbali anapatsidwa mwayi wofunsa mafunso okhudzana ndi kafukufuku, ndipo mafunso onse amene afunsidwa ndi wotenga nawo mbali, ayankhidwa moyenelera komanso mwakuyesetsa kwanga. Ndikutsimikizira kuti munthuyu sanakakamizidwe kuti akapereke chilorezo,ndipo chilorezo chaperekedwa mwa ufulu komanso mosakakamizidwa.

Lembani dzina la mboni	
Siginichala	
Deti	

Lembani dzina la wopangitsa kafukufuku kapena munthu amene akupempha chilorezo	
Siginichala	
Deti	

Mukamaliza, kopi imodzi ya wotenga nao mbali, kopi imodzi (yoyambirira) kwa kafukufuku

Approved by
College of Medicine
26 MAR 2018
(COMREC)
Research and Ethics Committee

Appendix 9: English participant information sheet (HIV on short-term ART)

Malawi-Liverpool-Wellcome Trust
Clinical Research Programme
P.O Box 30096, Chichiri, Blantyre 3,
Malawi.Tel. +265 1 876444 Fax +265 1 875774

INFORMATION DOCUMENT FOR AN ASYMPTOMATIC, HIV-INFECTED INDIVIDUAL ON ANTIRETROVIRAL THERAPY FOR A PERIOD OF LESS THAN 90 DAYS

STUDY TITLE: CHARACTERISATION OF HIV-ASSOCIATED CHANGES IN ALVEOLAR IMMUNE ENVIRONMENT THAT FAVOUR THE GROWTH OF *STREPTOCOCCUS PNEUMONIAE*: A PILOT STUDY

INTRODUCTION

Pulmonary infections are still a major cause of morbidity and mortality among those living with the human immunodeficiency virus. The bacteria pneumococcus is estimated to be a cause of disease in approximately 15-million individuals and mortality in 1.5-million annually. In Malawi, pneumococcal disease peaks during the colder and drier months and is common in the HIV infected adults and younger children. The exact mechanism through which HIV and co-viral infections renders individuals susceptible to pneumococcus pneumonia remains unclear.

WHAT IS THIS RESEARCH FOR?

We would like to know how infection with HIV affects the way in which lungs fight and control infections such as pneumococcus pneumonia. To do this, we will compare the function of the lung cells obtained from people without HIV infection from those who have HIV infection. This study also serves as a fulfilment of the joint Doctor of Philosophy Degree with the Liverpool School of Tropical Medicine (UK) and College of Medicine (Malawi).

WHY AM I BEING ASKED TO TAKE PART?

You are being asked to participate in the research study named above because you have been on antiretroviral therapy (ART) for not more than 90 days.

WHAT WILL HAPPEN TO ME?

The study involves the examination of the large and small airways in the lungs with a flexible tube passed through the nose and the mouth. The tube has a camera attached to it and this test is called ***bronchoscopy***.

You should not eat or drink anything for at least 4 hours before the bronchoscopy. During the test, you will be given medicine to numb your nose and the back of the mouth and the airways to minimise discomfort. You will be given a sedative or oxygen if necessary. Once the tube has reached the right place in the lungs, a small part of the lung will be washed with warm salt water, the fluid

Malawi-Liverpool-Wellcome Trust
Clinical Research Programme
P.O Box 30096, Chichiri, Blantyre 3,
Malawi.Tel. +265 1 876444 Fax +265 1 875774

will be collected and tested for infections and will be used to assess the ability of the lungs to control infections. This test will be done by our trained staff guided by Dr Henry Mwandumba (co-investigator).

What other tests will you do.

Before the bronchoscopy, we will examine you and arrange for you to have a chest x-ray. Bronchoscopy will only be performed if these are normal. You will also be screened for the presence of influenza virus which causes flu. This will be done by inserting a nasal swab into one of nostrils to collect mucus. Additionally, you will be tested You will be offered HIV counselling and testing if you consent to this. A blood sample will be required for this test.

You will not take part in the study if you decline this test. If, however, you agree to have it but do not wish to know the results, we will ask for your permission to perform the test anonymously. We encourage you to consider having the test and knowing its result even if you choose not to take part in the study as such knowledge can help you plan your life better, whatever the result of the test.

In total 20-mls (4 teaspoons) of blood will also be collected from you. The blood samples will be used to measure your blood count, liver and kidney function, and the number of cells that protect you against infection (CD4+ T cells), which are particularly destroyed by HIV. To ensure confidentiality, the results of your tests will be known only by the members of the study team. We will not share them with anybody unless you have given us the permission to do so.

WHAT WILL HAPPEN TO MY SAMPLES?

Samples collected will be transported to the MLW laboratories immediately after collection on ice in biohazard cooler boxes for laboratory testing and analysis. Bronchoalveolar lavage and whole blood collected will be used to identify immune cells and factors that are help to control growth of pneumococcus in your lungs. For tests that cannot be performed in Malawi, samples will be shipped abroad to the United Kingdom (UK) for analysis. Left over samples will be stored for a period maximum 5 years and will be used for study associated tests.

WHAT IMPACT WILL THE STUDY HAVE ON ME?

RISKS: Bronchoscopy is generally a safe test. However, some people find it uncomfortable mainly due to coughing. Every effort will be made to minimise this by giving you medicine to stop or reduce coughing. Occasionally breathing problems can occur during the test; you will be monitored constantly and should this happen, you will be given oxygen or the test maybe stopped if necessary. Some people develop fever following bronchoscopy; this is quite common and usually short lived, often settling with simple ant-fever medicines. We will give you the necessary treatment should this happen. Other rare complications include bleeding and lung infections. We will take special care to minimise the risks, and treat you appropriately should they happen. After bronchoscopy you will be followed up in clinic 2 days later to ensure that no problem had arisen from the test.

Malawi-Liverpool-Wellcome Trust
Clinical Research Programme
P.O Box 30096, Chichiri, Blantyre 3,
Malawi.Tel. +265 1 876444 Fax +265 1 875774

BENEFITS: No, you will benefit directly from this study but your involvement will help advance the current understanding of how lungs control pneumococcus in adults and how this is affected by HIV/AIDS. This knowledge could help facilitate new treatments for pneumococcus in HIV infected people.

Will I be paid by participating in the study?

Yes, you will be compensated for your involvement in the study. This compensation will be MK7,200 (US$10) to cover your transport and meals since you will not be allowed to eat or drink anything before bronchoscopy.

WHO WILL HAVE ACCESS TO THE INFORMATION YOU COLLECT ABOUT ME?

The investigators of in this study and the doctors managing you will have access to your information.

WILL I FIND OUT ABOUT THE STUDY RESULTS?

Results for the rapid influenza screening test, information regarding to the presence or absence of an acute respiratory disease, will be revealed to you.

WHAT HAPPENS IF I DON'T WANT TO TAKE PART?

You may choose not to enter the study without loss of benefits entitled to you. **Your decision will not affect your treatment in any way.** You do not have to explain your reasons for declining to participate.

WHO CAN I GO TO FOR MORE INFORMATION ABOUT THIS?

Please feel free to contact the following researcher if you have any questions, concerns or issues about your involvement in this research

Dr Henry Mwandumba, Malawi-Liverpool-Wellcome Trust, Clinical Research Programme
P.O Box 30096, Chichiri, Blantyre 3, Malawi, Telephone: +265 1 876 444 Mobile: +265 881 07 38 22

If you have any questions regarding your rights as a research participant, or concerns on how you have been treated in the study, please feel free to contact COMREC Secretariat, College of Medicine, Private Bag 360, Chichiri, Blantyre 3 or call on 01877 245 or 01 877 291 extn 334.

Appendix 10: Chichewa participant information sheet (HIV on short-term ART)

Malawi-Liverpool-Wellcome Trust
Clinical Research Programme
P.O Box 30096, Chichiri, Blantyre 3,
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CHIKALATA CHA UTHENGA CHA ANTHU AMENE ALI NDI HIV-AMENE SAKUONETSA ZIZINDIKIRO AMENE AKHALA KULANDIRA THANDIZO LA MANKHWALA A ANTIRETROVIRAL KWA MASIKU OSAPITILIRA MAKUMI ASANU NDI ANAYI

MUTU WA KAFUKUFUKU: CHARACTERISATION OF HIV-ASSOCIATED CHANGES IN ALVEOLAR IMMUNE ENVIRONMENT THAT FAVOUR THE GROWTH OF *STREPTOCOCCUS PNEUMONIAE*: A PILOT STUDY.

CHIYAMBI

Matenda a m'mapapo ndi vuto lalikulube limene limayambitsa matenda komanso limabweretsa imfa pakati pa anthu amene ali ndi kachirombo ka HIV. Kachirombo ka bacteria ka pneumococcus kamatengedwa kukhala koyambitsa matenda kwa anthu pafupi-fupi 15-million kubweretsa imfa kwa anthu 1.5-million pa chaka. Ku Malawi, matenda a ***pneumococcal*** amakwera mu nyengo ya miyezi yozizira komanso yotentha ndipo amapezeka kwambiri kwa anthu aakulu komanso ana amene ali ndi HIV. Njira imene anthu amene ali ndi kachirombo ka HIV komanso matenda ena okhala ndi tizirombo ta mitundu iwiri co-viral amakhalira pachiopsyezo cha matenda a chibayo (***pneumococcus pneumonia***) siikudziwika bwino.

KODI KAFUKUFUKUYU NDI WACHANI?

Tikufuna kuti tidziwe momwe HIV imakhudzira chitetezo cha m'mpapo chimene chimalimbana ndimatenda monga ***pneumococcus pneumonia***. Tidzachita izi posiyanitsa kagwiridwe ntchito ka ma ***cells*** kuchokera kwa gulu la anthu amene alibe kachilombo ka HIV ndi amene ali ndi HIV. Kafukufukuyu akugwira ntchito yolumikiza maphunziro a ukachenjede a Philosophy Degree ndi masukulu a ukachenjede a Liverpool School of Tropical Medicine (UK) ndi College of Medicine (Malawi).

NDICHIFUKWA CHANI NDIKUFUNSIDWA KUTI NDITENGE NAWO MBALI?

Mukufunsidwa kutenga nawo mbali mukafukufukuyu amene walembedwa m'mwambamu chifukwa mwakhala mukulandira mankhwala otalikitsa moyo a ART kwa masiku osapitilira makumi asanu ndi anayi.

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CHITACHITIKE NDI CHANI KWA INE?

Mu kafukufukuyu timaunika m'mpapo ndi kapaipa kakang'ono kudzera m'mphuno kapena m'kamwa. Kapaipiko kali ndi makina otolera zithunzi. Kuunika kumeneku kumachedwa **bulonkosikope**. Mudzachitidwa bulonkosikope kamodzi kokha pa nthawi ya kafukufuyu.

Tisanachite bulonkosikope, tidzakupemphani kuti musadye kapena kumwa chiri chonse kwa maola anayi kufikira nthawi yokupimani. Pokonzekera kupimaku, tidzakupatsani mankhwala oziziritsa m'mphuno, m'kamwa ndi m'mapapo kuti musamve ululu uli wonse pamene mukupimidwa. Nthawi zina timapereka nso mankhwala kuti mufewe ndikukhala ndi tulo pang'ono. Pamene tikuchita bulonkosikope, tidzapitiriza kukupatsani mankhwala kuti musamve ululu kapena kukhosomola kwambiri. Tidzatsuka mbali yaying'ono ya phapo lanu ndi madzi ofunda okhala ndi mchere pang'ono, ndipo pomaliza kutsukaku madziwo tidzawatenga kuti tikayese ku labotale momwe chitetezo cha m'mapapo chilimbana ndi HIV komanso pneumococcus. Izi zizachidwa ndi ogwila ntchito omwe anaphunzitsidwa bwino zantchitoyi ndi adokotala akulu amukafukufuku Dr Henry Mwandumba.

Kodi palinso zoyesa zina zomwe mudzachite?

Tisanachite bulonkosikope tidzakufunsani mafunso okhudza umoyo wanu. Tidzayesa kuthamanga kwamagazi anu (buladi pulesha) ndi kukupimani thupi lonse kuti titsimikize kuti palibe vuto lonse. Mudzajambulidwa chinthunzi cha mu chifuwa (x-ray). Tidzatengaso nao zoyesa mu mphuno pokwetsa ka thonje nkutenga mamina zoyesazi tikaunika ngati muli tizilombo toyambitsa chimfine. Tidzakufunsani ngati tingakulongosoleleni za matenda a HIV, ndikukupemphani kuti tiyese magazi anu kufufuza matendawa. **Simungatenge nawo mbali mu kafukufukuyu ngati simuvomera kuyezedwa HIV. Ngati muvomera kuyesedwa koma simukufuna kudziwa zotsatira zake tidzakufunsani ngati mungatipatse chililezo kuti tichite zoyesazo mwa chinsinsi kotero kuti zotsatila zake sizingalimukizidwe ndi dzina kapena nambala yanu**. Tikukulimbikitsani kuti muganize mofasa zoyesedwa HIV komaso kudziwa zotsatira za kuyesaku ngakhale musatenge nawo mbali kafukufukuyu kudziwa ngati muli ndi HIV kapena ayi kukhoza kukuthandizani pamene mukukhonza tsogolo lanu.

Magazi omwe titenga tikawayesanso kuti tidziwe kuchuluka kwa magazi nthupi mwanu, kuti tidziwe momwe chiwindi ndi impfyo zanu zikugwilila ntchito komanso kuti tidziwe kuchuluka kwachitetezo cha nthupi chomwe chimaononged wa ndi HIV. Ngati pokumimani tapeza kuti mwatentha nthupi, magaziwo tikawayesanso kuti tidziwe ngati muli ndi malungo kapena tizilombo tina tomwe timayambisa matenda am'magazi. Onse pamodzi magazi omwe titenga adzakhala ochuka pafupifupi kudzadza masipuni akulu anayi (4 tablespoons/20mls). Omwe akuchitisa kafukufukuyu ndi okhao namene azadziwe zotsatira zakuyesa kwa magazi anu. Sitizauza wina aliyense zotsatira pokapeda chilolezo chanu.

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Pali zoyesa zina sitingakhe kuchita kuno kumalawi ndipo padzafunika kuti titumize ena mwa magazi anu ndi zotsuka za mmapapu ku maiko akunja (United Kingdom) kuti zikayesedwe choncho tikupempha chilolezo chanu kuti tizatumize zoyesazi . Mukavomela kulowa nawo mukafukufukuyu ndiye kuti mwavomelanso kuti zoyesa zanu zikhoza kutumizidwa kunja kwadziko lino.

CHITACHITIKE NDI CHANI PA ZOYESA ZANGA?

Zoyesa zimene zingatengedwe zidzaikidwa mu ma bokosi osungiramo zozizira m'mene muli ***ice*** ndikutumizidwa ku malo oyezerako zinthu a MLW zikazangotengedwa kuti zikayezedwe komanso kufufuzidwa. ***Bronchoalveolar lavage*** komanso magazi amene atengedwa adzagwiritsidwa ntchito kuzindikira timagawo ting'onoting'ono ta chinthu cha moyo ta chitetezo cha mthupi komanso zinthu zimene zimathandizira kumera kwa ***pneumococcus*** m'mapapo mwanu. Pa ntchito yoyeza imene siingathe kuchitikira ku Malawi, zoyesa zidzatumizidwa kunja kwa dziko ku United Kingdom (UK) kuti zikafufuzidwe. Zotsala za zoyesazi zidzasungidwa kwa zaka zosachepera zisanu(5), zisanu(5) ndikuzagwiritsidwa ntchito mtsogolo.

KODI KAFUKUFUKUYU ADZABWERETSA CHANI PA INE?

ZIOPSWEZO: Bulonkosikope siyoospya ayi. Koma anthu ena amalepera kukazikika bwino pamene akupimidwa makamaka chifukwa chakukhosomola. Tidzayesetsa kuchepetsa zimenezi pokupatsani mankhwala oletsa kapena kuchepetsa kukhosomola. Nthawi zina kuvutika kupuma bwino pamene mukupimidwa kungathe kuoneka; tidzakuyang'anirani bwino ndipo vuto limeneli litatilaoneka tizakupatsani mpweya wothandiza kupuma (okosijeni) kapena kuimitsa kupima kuli kofunikila kutero. Anthu ena amatentha mthupi akapimidwa mmapapo. Izi zimaoneka kawirikawiri koma kwa kanthawi kochepa ndimankhwala oletsa kutentha mthupi amathandiza kuchepetsa vuto limeneli. Zovuta zina zomwe sizioneka kawirikawiri ndi kutaya magazi komanso chibayo. Tidzakuyanganilani mwachifase kuti mavutowa asaoneke, koma atati anoneka, mudzalandila chithandizo choyenela mwansanga.

Ndipofunika kuti tidzakuoneninso kuno kuchipatala patatha masiku awiri chichitikileni bulonkosikope kuti tidzatsimikize kuti palibe vuto lililonse.Ngati palibe vuto lililonse sitidzakuonaninso mukafukufukuyu pokhapokha ngati mwadwala ndipo mukufuna chithandizo cha anthu omwe akuchita kafukufukuyu.

PHINDU: Palibe, koma kutenda nao mbali mukafukufuku kuthandiza kumvetsetsa mwa tsopano momwe mapapo amatetezera pneumococcus mwa akulu amene ali ndi matenda a HIV/AIDS. Chidziwitso ichi chidzathandiza kupeza thandizo lina la momwe tingachizire pneumococcus mwa anthu amene alindi kachilombo ka HIV.

Kodi ndizalipilidwa ndikatenga nawo mbali?

Eya muzabwezeledwa ndalama yokwana MK7200 (US$10), imene mudzayendele ndikugulila chakudya chifukwa muzakhala kuti mwasalitsidwa kudya.

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NDI NDANI ANGAGWIRITSE NTHCITO UTHENGA WOKHUDZANA NDI INE UMENE MUNGATOLERE?

Akulu-akulu mu kafukufukuyu komanso ma dotolo amene akukuyang'anirani adzakhala ndi kuthekera kogwiritsa ntchito uthenga wanu.

KODI NDIZADZIWA ZA ZOTSATIRA ZA KAFUKUFUKUYU?

Mudzauzidwa zotsatira zochokera mu ntchito yoyeza mwachangu chimfine, uthenga wokhudzana ndi kupezeka kapena kusapezeka kwa matenda oyamba kumene a munjira yopumira.

CHITACHITIKE NDI CHANI NDIKAPANDA KUFUNA KUTENGA NAWO MBALI?

Mukhoza kusankha kusalowa nawo mukafukufukuyu popanda kutaya phindu limene mukuyenera kulandira. **Chisankho chanu sichidzakhudza thandizo lanu la mankhwala amene mukulandira mu njira ina iliyonse.** Simukuyenera kufotokoza chifukwa chimene mwakanira kutenga nawo mbali m'kafukufukuyu.

KODI NDINGAPITE KWANDANI KUTI NDIMVE ZAMBIRI ZOKHUDZANA NDI ZIMENEZI?

Chonde khalani omasuka kulumikizana ndi opangitsa kafukufuku otsatirawa ngati muli ndi mafunso,madandaulo kapena mavuto okhudzana ndi kutenga nawo mbali kwanu mukafukufukuyu

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Ngati muli ndi mafunso ena aliwonse okhudzana ndi ufulu wanu ngati wotenga nawo mbali mukafukufuku, kapena madandaulo pa m'mene mwathandizidwira mukafukufukuyu, chonde khalani omasuka polumikizana ndi mlembi wa ***COMREC***, College of Medicine, Private Bag 360, Chichiri, Blantyre 3 kapena imbani foni pa 01877 245 kapena 01 877 291 extn 334.

Approved by
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