

**The modulation of macrophage apoptosis by
HIV-1 during *Streptococcus pneumoniae*
infection**



A thesis for the degree of Doctor of Philosophy

Paul John Collini

April 2014

Department of Infection and Immunity
Faculty of Medicine, Dentistry and Health
University of Sheffield

Contents

Acknowledgements.....	9
Declaration.....	13
Abstract.....	15
Abbreviations.....	17
List of Figures and Tables.....	23
1 Chapter 1. Introduction	27
1.1 Organisation of the immune system.....	28
1.1.1 Innate Immunity.....	28
1.1.2 Adaptive Immunity.....	33
1.1.3 Apoptosis	37
1.2 The spectrum of pneumococcal disease.....	40
1.2.1 Vaccination against <i>Streptococcus pneumoniae</i>	41
1.3 The pathogenesis of <i>Streptococcus pneumoniae</i> infection	42
1.3.1 Colonisation	43
1.3.2 From colonisation to disease	44
1.3.3 Alveolar macrophage control of <i>Streptococcus pneumoniae</i> infection.....	45
1.3.4 Pneumococcal bacteraemia and meningitis	48
1.3.5 Adaptive immune responses to <i>Streptococcus pneumoniae</i> in the lung.....	50
1.4 HIV-1 infection	53
1.4.1 The natural history of HIV-1 infection.....	53
1.4.2 HIV-1 structure.....	55
1.4.3 HIV-1 replication	56
1.4.4 The immune response to HIV-1 infection	58
1.4.5 Consequences of HIV-1 for the immune system	60
1.5 Macrophages in HIV-1.....	63
1.5.1 Macrophage apoptosis and its circumvention by HIV-1.....	65
1.6 Invasive pneumococcal disease and pneumococcal pneumonia in HIV-1	69
1.6.1 The epidemiology of <i>Streptococcus pneumoniae</i> infection in HIV-1.....	69
1.6.2 HIV-1 and <i>Streptococcus pneumoniae</i> pathogenesis.....	73
1.7 Aims.....	83
2 Chapter 2. Materials and Methods.....	85
2.1 Cell culture and differentiation.....	85

2.1.1	Cell lines	85
2.1.2	Human peripheral blood cells	86
2.1.3	Human alveolar cells	88
2.1.4	Counting cell numbers	91
2.2	HIV-1 propagation	92
2.2.1	HIV-1 propagation in lymphoblasts	92
2.2.2	HIV-1 propagation in MDM	92
2.2.3	HIV-1 titration	93
2.3	<i>Streptococcus pneumoniae</i> culture and cell challenge	97
2.3.1	<i>Streptococcus pneumoniae</i> culture	97
2.3.2	Miles and Misra viable bacterial count	97
2.3.3	<i>Streptococcus pneumoniae</i> opsonisation with human serum	97
2.3.4	Cell challenge with <i>Streptococcus pneumoniae</i>	99
2.3.5	Gentamicin protection assays	100
2.4	MDM incubation with HIV-1 gp120	100
2.5	MDM infection with HIV-1	100
2.6	Flow cytometry	101
2.6.1	Flow Cytometry	101
2.6.2	Antibodies	101
2.6.3	Cell viability staining	102
2.6.4	Measurement of alveolar macrophage polarisation	103
2.6.5	Measurement of lymphocyte subsets and activation	103
2.7	Measuring cell death	107
2.7.1	Nuclear Morphology	107
2.7.2	TUNEL assay	109
2.7.3	Measurement of Caspase 3/7 activity	111
2.7.4	Measuring hypodiploid DNA	114
2.8	Measurement of protein expression by Western Blot	114
2.8.1	Protein extraction	114
2.8.2	Protein quantification assays	115
2.8.3	SDS PAGE	115
2.8.4	Western blot semi dry transfer	115
2.8.5	Protein detection by chemiluminescence	116
2.8.6	Densitometry	116

2.8.7	Measurement of mitochondrial superoxide	117
2.8.8	Measurement of mitochondrial density	117
2.9	Statistical Methods	118
3	Chapter 3. HIV-1 infection is associated with attenuation of macrophage apoptosis in response to <i>Streptococcus pneumoniae</i>	119
3.1	Introduction	119
3.2	Results	122
3.2.1	U937 and U1 promonocytic cell lines can be differentiated similarly towards a macrophage-like phenotype	122
3.2.2	The induction of apoptosis by <i>Streptococcus pneumoniae</i> is reduced in U1 compared with U937 cells after a brief differentiation period.....	125
3.2.3	Characterisation of U937 and U1 cell lines following a more extended PMA differentiation period.....	128
3.2.4	The induction of apoptosis by <i>Streptococcus pneumoniae</i> is reduced in U1 compared with U937 cells after an extended differentiation period.....	130
3.2.5	There is reduced induction of caspase 3/7 following challenge with <i>Streptococcus pneumoniae</i> in U1 compared with U937 cells after an extended differentiation period.....	133
3.2.6	The induction of apoptosis by <i>Streptococcus pneumoniae</i> is reduced in U1 compared with U937 cells after a prolonged, eight day differentiation period.....	135
3.2.7	Infection of monocyte-derived macrophages with HIV-1.....	135
3.2.8	HIV-1 infection of MDM is not associated with altered phagocytosis of <i>Streptococcus pneumoniae</i>	136
3.2.9	HIV-1 infection of MDM is associated with altered induction of apoptosis	141
3.2.10	HIV-1 infection is associated with reduced induction of caspase 3/7 activity following <i>Streptococcus pneumoniae</i> infection.....	144
3.2.11	HIV-1 infection of MDM is associated with reductions in late bacterial killing	144
3.3	Discussion.....	147
4	Chapter 4. HIV-1 infection is associated with persistent alterations in bronchoalveolar fluid T lymphocytes and alveolar macrophage phenotype despite antiretroviral therapy	151
4.1	Introduction	151
4.2	Results.....	154
4.2.1	Patient demographics	154
4.2.2	Alveolar macrophages from a minority HIV-1-seropositive volunteers on fully suppressive ART are infected with HIV-1	155
4.2.3	HIV-1-seropositive individuals have a greater proportion of lymphocytes in their BAL fluid than HIV-seronegative controls.	158

4.2.4	The HIV-1 associated BAL lymphocytosis is predominantly from CD8 ⁺ T cells.	160
4.2.5	Alterations in T lymphocyte subset proportions in the lung are distinct from those in the peripheral blood of HIV-1-seropositive individuals	165
4.2.6	Alveolar macrophage cell surface expression of polarisation markers is similar in HIV-1-seropositive individuals and controls	165
4.2.7	Alveolar macrophages from HIV-1-seropositive and seronegative individuals exhibit similar rates of <i>Streptococcus pneumoniae</i> phagocytosis.....	168
4.2.8	Alveolar macrophages from HIV-1-seropositive individuals on ART undergo less apoptosis than those from control individuals following challenge with <i>Streptococcus pneumoniae</i>	170
4.2.9	The induction of caspase 3/7 activity by <i>Streptococcus pneumoniae</i> infection is reduced in AM from HIV-1-seropositive volunteers on fully suppressive ART compared to controls	173
4.2.10	Killing of internalised <i>Streptococcus pneumoniae</i> may be reduced in AM from HIV-1-seropositive volunteers on fully suppressive ART at 20 hours post challenge.....	173
4.3	Discussion.....	176
5	Chapter 5. Investigating the mechanism of HIV-1 associated changes in the apoptotic response of macrophages to <i>Streptococcus pneumoniae</i>	183
5.1	Introduction	183
5.2	Results.....	185
5.2.1	There is no association between the level of MDM HIV-1 infection and <i>Streptococcus pneumoniae</i> associated apoptosis	185
5.2.2	Exposure to HIV-1 gp120 is not associated with any altered early phagocytosis of <i>Streptococcus pneumoniae</i> by MDM.....	185
5.2.3	Exposure to HIV-1 gp120 reduces the degree of apoptosis in MDM following <i>Streptococcus pneumoniae</i> challenge.....	188
5.2.4	Exposure to HIV-1 gp120 reduces the degree of <i>Streptococcus pneumoniae</i> associated caspase 3/7 activation in MDM.	190
5.2.5	Exposure to gp120 is associated with increased bacterial survival in MDM at 20 hours post <i>Streptococcus pneumoniae</i> challenge, despite similar levels of early killing .	192
5.2.6	HIV-1 infection of MDM is associated with persistent expression of Mcl-1 during <i>Streptococcus pneumoniae</i> challenge.....	194
5.2.7	Mitochondrial superoxide is increased by HIV-1 and by <i>Streptococcus pneumoniae</i> challenge	196
5.2.8	The level of induction of AM apoptosis following <i>Streptococcus pneumoniae</i> challenge shows a relationship to the proportion of CD4 ⁺ and CD8 ⁺ T cells in the bronchoalveolar lavage fluid.....	198

5.2.9	Receipt of a non nucleoside reverse transcriptase inhibitor or protease inhibitor as a third agent in an ART regimen is not associated with any difference in the apoptosis response of AM to <i>Streptococcus pneumoniae</i> challenge.....	200
5.3	Discussion.....	203
6	Chapter 6. Discussion.....	209
6.1	HIV-1 infection results in decreased macrophage apoptosis following challenge with <i>Streptococcus pneumoniae</i> which is linked to impaired bacterial killing.	209
6.2	HIV-1 is associated with persistence of Mcl-1 expression and failure to augment mitochondrial superoxide by macrophages following <i>Streptococcus pneumoniae</i> challenge 211	
6.3	HIV-1-seropositive individuals on fully suppressive ART continue to have a CD8 ⁺ T lymphocytosis with a reversed CD4:CD8 ratio in the BAL	213
6.4	Future work.....	217
6.5	Conclusion.....	219
	Chapter 7.References.....	221
	Appendix 1.Buffers.....	245
	Appendix 2. Publications & Awards	249
	Peer reviewed articles.....	249
	Presented abstracts	249
	Awards	250

Acknowledgements

During my PhD and writing this thesis I received invaluable support and advice from many people.

My PhD supervisor, Professor David Dockrell has been a strong source of guidance from the inception of this work right through to submission. David helped me enormously to develop my early research questions into a successful MRC clinical training fellowship application which enabled the research presented here to happen. Only with his constant drive and encouragement did each of the grant application, interviews, research applications and ethics committee forms and interview get completed with success. David has been thorough in his supervision of my experimental work, generous in introducing me to collaborators and has given up significant amounts of his evenings and weekends to reviewing abstracts and manuscripts that I have submitted, not least this thesis. Most of all David has been a strong advocate for my training in the often conflicting world of clinical academic training and continues to be a great career mentor.

I must also acknowledge the support of the Dockrell research group; Dr Helen Marriott, Dr Martin Bewley, Dr Julie Preston and Dr Marc Daigneault for teaching me so many core techniques as well as Jamil Jubrail, my fellow PhD student in the group, for help with microscopy. I have had fantastic technical and moral support from Katie Cooke, Ian Geary, Vanessa Singleton and Jon Kilby and, in the early days, Margaret Lee and Anne Cook. Kevin Oxley and Katie Cooke have been particularly helpful in keeping the CL3 facility up and running for me to work in.

As mentioned, David introduced me to some very generous collaborators; Dr Maddy Noursadeghi and his team Dr Jhen Tsang and Dr Gillian Tomlinson at University College London who took me under their wing and taught me the core techniques for HIV propagation and

purification and kindly donated the NP2 cell line for my use in Sheffield. Professor Steve Gordon and his team at the Liverpool School of Tropical Medicine, Dr Sarah Glennie and Dr Adam Wright, taught me and advised on alveolar macrophage harvest by bronchoscopy. Dr Charlie Elliot, Dr Robin Condliffe, Dr David Kiely, Professor Moira Whyte and the staff in the Royal Hallamshire Hospital endoscopy suite and lung MDT office were fantastically accommodating arranging and performing bronchoscopies.

Professor Ian Sabroe has been my PhD mentor and tutor and given freely of his time both in my initial MRC application and interview preparation and subsequently during my PhD. He also gave me invaluable help when designing the bronchoscopy protocols and preparing my ethics applications. Professor Robert Read helped guide my fellowship application and initial work. Others in the department of Infection and immunity who gave me very helpful advice during my fellowship application and during lab meetings were Professor Moira Whyte and Professor Steve Renshaw. Sarah Moll in the STH research office was a great help steering my applications through all the research governance bodies. Thank you to Dr Colin Bingle and Dr Mark Thomas who carefully read and discussed my transfer report at the end of my first year. I'd also like to thank the postgraduate research team, led by Martina Daly at the Medical School for keeping my PhD on track.

I would like to thank my funders, the MRC, who awarded me my clinical training fellowship and made this research possible.

Key to the success of this research were, of course, the patients and healthy volunteers who both gave blood and agreed to undergo bronchoscopy and lavage. I am in their debt. Specialist HIV nurses Karen Sherry and Charlie Hughes and the research nurses Lynne Smart in communicable diseases and Charlotte Morgan and her team in the CRF were a fantastic help during patient recruitment and enrolment.

Finally I would like to thank my wonderful family; Magda, Joseph and Matthew for their support and understanding, particularly every time I disappeared to the lab in the evening or for the weekend and my parents without whose care and guidance I would never have been in the position I am.

Declaration

All the work presented in this thesis is my own. Ian Geary, Katie Cooke and Jonathan Kilby helped with peripheral blood mononuclear cell isolation.

Abstract

Invasive pneumococcal disease (IPD) causes significant global morbidity and mortality. IPD is also more common in HIV-1-seropositive individuals. Although anti-retroviral therapy (ART) has transformed the outlook of HIV-1 infection, IPD remains up to 30 times more common. The currently available strategies against IPD, vaccination and antimicrobial therapy, have had only modest impact and are less effective in HIV-1. New approaches based on a better understanding of the underlying immunology of these two diseases are needed.

A programme of host-mediated macrophage apoptosis ensures killing of pneumococci when canonical phagolysosomal killing capacity is exhausted. HIV-1 is associated with resistance of macrophages to apoptosis. I hypothesised that HIV-1 mediated resistance to *S. pneumoniae* associated macrophage apoptosis resulting in reduced bacterial killing.

I measured the effect of HIV-1 and the HIV-1 antigen gp120 on rates of macrophage apoptosis and bacterial survival *in vitro* following challenge with *S. pneumoniae*. Macrophages were derived from monocytic cell lines U937 and U1 (latently infected with HIV-1) or primary human monocytes or alveolar macrophages (AM) were harvested from the lungs of HIV-1-seropositive individuals naïve to, or receiving ART and controls by bronchoalveolar lavage (BAL). I also measured the levels of anti-apoptotic Mcl-1 and mitochondrial superoxide following pneumococcal challenge. Additionally, I characterized and compared the T lymphocyte subsets and AM phenotype from the BAL fluid of each group.

I found that HIV-1 infection is associated with reduced macrophage apoptosis, a persistence of Mcl-1 expression, altered mitochondrial superoxide generation and decreased bacterial killing following pneumococcal challenge. Altered apoptosis was observed even with low rates of *in vitro* HIV-1 infection and in AM from virally suppressed, HIV-1 ART treated individuals. gp120 alone was sufficient to mediate these effects. There was a CD8 T cell predominant

lymphocytosis in the BAL of HIV-1-seropositive individuals and, in some cases, evidence of ongoing HIV-1 replication in AM, despite ART.

These observations demonstrate that apoptosis-associated killing of *S. pneumoniae* is impaired in HIV-1 infection, potentially through altered Mcl-1 expression and ROS generation.

Importantly this defect appears to persist in the alveolar macrophages of virally suppressed HIV-1-seropositive individuals on ART and is associated with an altered T lymphocyte environment in the lung. It is likely that these immune defects contribute to the increased risk of IPD in the HIV-1-seropositive population.

Abbreviations

ACID	Activation-induced cell death
AIDS	Acquired immunodeficiency syndrome
AM	Alveolar macrophage
AMC	7-amino-4-methylcoumarin
ANOVA	Analysis of variance
APAF-1	Apoptotic protease activating factor 1
APC	Allophycocyanin
ART	Antiretroviral therapy
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
BAL	Bronchoalveolar lavage
Bax	Bcl-2-associated x protein
Bcl-2	B cell lymphoma-2
Bcl-XL	B-cell lymphoma-extra large
BHI	Brain-heart infusion
Bid	BH3 interacting-domain death agonist
BSA	Bovine serum albumin
CBA	Columbia blood agar
CCR	Chemokine receptor
CD	Cluster of differentiation
c-FLIP	FADD-like IL-1 β -converting enzyme-inhibitory protein
CFU	Colony forming units
CL2	Containment level 2 laboratory
CL3	Containment level 3 laboratory

COPD	Chronic obstructive pulmonary disease
cpm	Copies per mL
CRF	Clinical research facility
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CTLA-4	Cytotoxic t-lymphocyte-associated protein-4
DAPI	4',6-diamidino-2-phenylindole
dATP	Deoxyadenosine triphosphate
DEVD	Asp-glu-val-asp
DISC	Death inducing signalling complex
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dUTP	2' –deoxyuridine, 5' –triphosphate
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetracetic acid
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FADD	Fas-associated via death domain
FasL	Fas ligand
FEV1	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate
FLICE	FADD-like IL-1 β -converting enzyme
FSC	Forward scatter

G418	Geneticin
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMR	Geometric mean ratio
gp	Glycoprotein
H ₂ O ₂	Hydrogen peroxide
HAART	Highly active antiretroviral therapy
HBSS	Hank's balanced salt solution
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIFCS	Heat inactivated fetal calf serum
HIV-1	Human Immunodeficiency Virus - 1
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPD	Invasive pneumococcal disease
IQR	Inter-quartile range
IRIS	Immune reconstitution inflammatory syndrome
ITAM	Immunoreceptor tyrosine-based activation motifs
JPEG	Joint photographic experts group
LN	Liquid nitrogen
LPS	Lipopolysaccharide
LTNP	Long term non progression/progressor

LTR	Long terminal repeat
Mcl-1	Myeloid cell leukemia protein-1
M-CSF	Macrophage colony-stimulating factor
MDA	Malondialdehyde
MDM	Monocyte-derived macrophages
MFI	Geometric mean fluorescence intensity
MHC	Major histocompatibility complex
MI	Mock-infected
MIP-1 α	Macrophage inflammatory protein 1 α (CCL3)
MIP-1 β	Macrophage inflammatory protein 1 β (CCL4)
MOI	Multiplicity of infection
Nab	Neutralizing antibody
NF- κ B	Nuclear factor kappa B
NLR	Nod like receptor
NNRTI	Nucleotide reverse transcriptase inhibitor
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NP2	astrocytoma cell line
O ₂ ⁻	Superoxide
OD	Optical density
PAF	Platelet activating factor
PAMP	Pathogen associated molecular pattern
Pav	Pneumococcal adhesion and virulence
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
PCV	Pneumococcal conjugate vaccine
PE	Phycoerythrin
PFA	Paraformaldehyde
PHA	Phytohemagglutinin
PI	Protease inhibitor
PI	Propidium iodide
pIgR	Polymeric immunoglobulin
PKC	Protein kinase C
Ply	Pneumolysin
PMA	Phorbol 12-myristate 13-acetate
PPV23	23 valent pneumococcal polysaccharide vaccine
PRR	Pattern recognition receptor
Psp	Pneumococcal surface proteins
PUMA	P53 upregulated modulator of apoptosis
RANTES	Regulated on activation, normal T cell expressed and secreted (CCL5)
RFU	Relative fluorescent units
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute
RT	Reverse transcriptase
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulphate poly acrylamide gel electrophoresis
SIV	Simian Immunodeficiency Virus

SOD2	Superoxide dismutase 2, manganese superoxide dismutase
SSC	Side scatter
STH	Sheffield teaching hospitals NHS foundation trust
STS	Staurosporine
TAR	Trans-activating response element
Tat	Trans-activator of transcription
TB	Tuberculosis
TBS	Tris buffered saline
TCA	Tri-chloroacetic acid
TCL	Tissue culture laboratories
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
THP-1	Human acute monocytic leukaemia cell line
TLR	Toll like receptor
TNF α	Tumour necrosis factor alpha
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase dutp nick end labelling
U1	HIV-1 infected clone of U937 cell line
U937	Histiocytic lymphoma cell line
UC	Ultracentrifuge
UV	Ultra-violet
ViD	Viability dye
VL	Plasma viral load
X4	CXCR4 - C-X-C chemokine receptor type 4
Xgal	5-bromo-4-chloro-3-indolyl—galactopyranoside

List of Figures and Tables

Figure 2-1 Photomicrographs of four wells of NP2 cells stained for HIV-1 p24	94
Figure 2-2 Photomicrographs and histograms of GFP expression in ghost cells indicating HIV-1 infection	96
Figure 2-3 Human serum from PCV-23 vaccinated subjects opsonises D39 <i>Streptococcus pneumoniae</i>	99
Figure 2-4 Gating strategy for measuring macrophage surface markers	105
Figure 2-5 Gating strategy for measurement of BAL fluid lymphocyte subsets	106
Figure 2-6 Chromatin fragmentation and nuclear condensation in DAPI stained U937 nuclei following <i>Streptococcus pneumoniae</i> challenge.....	108
Figure 2-7 Photomicrograph of DAPI and TUNEL staining nuclei	110
Figure 2-8 Representative AMC standard curve as used to derive relative caspase activity from RFU measurements with fluorimetric caspase 3/7 assay.....	113
Figure 3-1 Characterisation of morphological changes in U937 and U1 following treatments with PMA.....	124
Figure 3-2 After a brief differentiation protocol there is loss of adherent cells following challenge with <i>Streptococcus pneumoniae</i> , which is less marked for U1 than U937	126
Figure 3-3 After a brief differentiation protocol U1 demonstrate less induction of apoptosis than U937 during infection with <i>Streptococcus pneumoniae</i>	127
Figure 3-4 Characterisation of morphological changes in U937 and U1 following extended PMA differentiation protocols.....	129
Figure 3-5 Representative flow cytometry dot plots and histograms for U937 and U1 hypodiploid DNA measurement following <i>Streptococcus pneumoniae</i> challenge or mock-infection.	131
Figure 3-6 After an extended differentiation period U1 demonstrate less induction of apoptosis than U937 following challenge with <i>Streptococcus pneumoniae</i>	132

Figure 3-7 Following an extended differentiation period U1 demonstrate less induction of caspase 3/7 activity than U937 cells following <i>Streptococcus pneumoniae</i> challenge.	134
Figure 3-8 After a prolonged, 8 day differentiation protocol U1 demonstrate less induction of apoptosis than U937 following exposure to <i>Streptococcus pneumoniae</i>	137
Figure 3-9 Photomicrographs of sham and HIV-1 treated MDM.....	138
Figure 3-10 HIV-1 infection of monocyte-derived macrophages is variable and donor dependent.....	139
Figure 3-11 HIV-1 infection is not associated with any alteration in internalisation of <i>Streptococcus pneumoniae</i> at 4 hours.....	140
Figure 3-12 HIV-1 infection is associated with reduced induction of apoptosis by MDM after <i>Streptococcus pneumoniae</i> challenge.....	142
Figure 3-13 HIV-1 infection is associated with reduced induction of MDM apoptosis between 16 and 20 hours following <i>Streptococcus pneumoniae</i> challenge.....	143
Figure 3-14 HIV-1 infection is associated with reduced induction of caspase 3/7 activity by MDM following <i>Streptococcus pneumoniae</i> challenge.	145
Figure 3-15 HIV-1 infection is associated with reduced killing of internalised <i>Streptococcus pneumoniae</i> in MDM at 20 hours post infection.....	146
Figure 4-1 Alveolar macrophage yields from BAL.....	157
Figure 4-2 Representative photomicrograph showing alveolar macrophages from an HIV-1 seropositive donor on fully suppressive ART staining positive for p24	157
Figure 4-3 Bronchoalveolar lavage fluid lymphocyte proportions are elevated in HIV-1-seropositive volunteers.....	159
Figure 4-4 There are fewer CD4 ⁺ and more CD8 ⁺ T-cells in the bronchoalveolar lavage fluid of HIV-1-seropositive individuals on fully suppressive antiretroviral therapy.....	162
Figure 4-5 The BAL T lymphocyte CD4:CD8 ratio is reduced in HIV-1-seropositive individuals on fully suppressive antiretroviral therapy	163

Figure 4-6 There is similar low level expression of CD38 on CD8 T lymphocyte from BAL of HIV-1-seropositive individuals on fully suppressive antiretroviral therapy and controls.....	164
Figure 4-7 T lymphocyte subsets and ratios in the blood and bronchoalveolar lavage fluid of HIV-1-seropositive individuals on fully suppressive antiretroviral therapy show no association	166
Figure 4-8 Cell surface expression of polarisation markers on AM from HIV-1-seropositive individuals on fully suppressive antiretroviral therapy is similar to controls	167
Figure 4-9 Rates of <i>Streptococcus pneumoniae</i> phagocytosis by alveolar macrophages from HIV-1-seropositive on fully suppressive antiretroviral therapy and control volunteers are similar 4 hours after bacterial challenge.	169
Figure 4-10 There is significant loss of AM from control donors but not HIV-1-seropositive volunteers on fully suppressive ART 20 hours following challenge with <i>Streptococcus pneumoniae</i>	171
Figure 4-11 AM from HIV-1-seropositive volunteers on fully suppressive ART show reduced levels of apoptosis following <i>Streptococcus pneumoniae</i> challenge when compared with AM from controls.	172
Figure 4-12 AM from HIV-1-seropositive volunteers on fully suppressive ART demonstrate less induction of caspase 3/7 activity following <i>Streptococcus pneumoniae</i> challenge.....	174
Figure 4-13 There was a non significant trend for greater bacterial survival in AM from HIV-1-seropositive volunteers on fully suppressive ART compared with controls after 20 hours of <i>Streptococcus pneumoniae</i> challenge.....	175
Figure 5-1 The level of direct MDM infection with HIV-1 does not correlate with the difference in level of induction of apoptosis following <i>Streptococcus pneumoniae</i> challenge.	186
Figure 5-2 Rates of viable internalised <i>Streptococcus pneumoniae</i> in MDM four hours after infection are similar irrespective of gp120 exposure	187

Figure 5-3 gp120 exposure reduces the induction of MDM apoptosis following <i>Streptococcus pneumoniae</i> challenge.	189
Figure 5-4 gp120 treatment is associated with reductions in MDM caspase 3/7 activity with and without <i>Streptococcus pneumoniae</i> challenge.	191
Figure 5-5 Exposure to gp120 is associated with reduction in late phase killing of <i>Streptococcus pneumoniae</i> by MDM.	193
Figure 5-6 HIV-1 infection is associated with persisting expression of Mcl-1 in MDM at 20 hours following <i>Streptococcus pneumoniae</i> challenge.	195
Figure 5-7 In MDM HIV-1 infection and <i>Streptococcus pneumoniae</i> infection are associated with elevated mitochondrial superoxide levels without any alteration in mitochondrial density.	197
Figure 5-8 Induction of AM apoptosis following <i>Streptococcus pneumoniae</i> challenge correlates with the bronchoalveolar lavage CD4:CD8 ratio and CD4%.	199
Figure 5-9 Receipt of protease inhibitor or non-nucleoside reverse transcriptase inhibitor therapy is not associated with any difference in <i>Streptococcus pneumoniae</i> associated AM apoptosis.	201
Figure 5-10 Receipt of protease inhibitor or non-nucleoside reverse transcriptase inhibitor therapy is not associated with any difference in the proportions of CD4 ⁺ and CD8 ⁺ lymphocytes in the BAL.	202
Table 2-1 Inclusion and Exclusion criteria for study enrolment	89
Table 2-2 Fluorophore labelled antibodies.	102
Table 4-1 Volunteers undergoing bronchoalveolar lavage.	156

Chapter 1. Introduction

Human Immunodeficiency Virus (HIV) -1 infection and disease caused by *Streptococcus pneumoniae* (the pneumococcus) have been members of the top five on the World Health Organisation's (WHO) list of global infectious causes of mortality for two decades (WHO 2013). Furthermore they are epidemiologically linked; HIV-1-seropositive individuals are also many times more likely to suffer illness from pneumococcal infection, especially invasive pneumococcal disease (IPD). As made clear by its name, untreated HIV-1 infection results in a deficiency of the immune response and is characterised by increased susceptibility to opportunistic infections and cancer. Fortunately, the vast majority of HIV-associated diseases are significantly less frequent since the advent of highly active antiretroviral therapy (HAART). Daily use of this combination drug treatment effectively suppresses the production of HIV-1 virions enabling reconstitution of the immune system. However, despite HAART being widely available for more than a decade, many epidemiological studies have observed that a substantially increased risk of IPD and pneumococcal pneumonia remains in HIV-1-seropositive individuals. This raises questions about the extent to which the immune system is reconstituted in HAART treated individuals living with HIV-1 infection and the degree to which subtle deficits remain.

Of further concern is that vaccination against pneumococcal disease is not effective in all populations, particularly those with HIV. Moreover, even with antimicrobial therapy IPD carries a substantial mortality risk in both HIV-1-seropositive and -seronegative individuals. New approaches to understanding and strengthening the immune response to the pneumococcus are now a growing area of research activity. The role of innate immunity has received particular interest and, independently, groups have focused on innate immune responses to the pneumococcus and the effects of HIV-1 infection on innate immunity. This work has brought new insights into the importance of the macrophage both as a target of HIV-

1 infection and also a critical element of the body's immune response against *S.pneumoniae* and HIV-1. Macrophage apoptotic responses are relevant to both diseases; apoptosis is a key, host initiated, part of the macrophage immune response to the pneumococcus. By contrast, HIV-1 infection is associated with a macrophage phenotype of prolonged survival and resistance to apoptosis.

In this thesis the role of the macrophage during pneumococcal infection in the context of HIV-1 infection will be considered. In this chapter the fundamental aspects of the innate and adaptive immune system will be summarised then the epidemiology, clinical history and pathogenesis of pneumococcal disease and then HIV-1 infection will each be described, with a specific focus on the importance of macrophage apoptosis. Next the epidemiological relationship between HIV-1 and pneumococcal disease will be explored and the current state of research into the underlying immunopathogenesis of pneumococcal infection during HIV-1 will be examined.

1.1 Organisation of the immune system

With respect to infection, the role of the immune system is to protect the host from any harmful effects of microorganisms. The complexity and time scale of the immune response differs with the nature and pathogenic potential of the microorganism; a wide range of microorganisms are encountered daily and rapidly dealt with by an immediate and non-specific innate response. A more specific response is individually tailored to the pathogen, that develops over days and results in immunological memory.

1.1.1 Innate Immunity

Innate immunity draws on physical barriers (e.g. skin, mucosa), soluble molecules (e.g. salivary enzymes, antibacterial peptides) and phagocytic leukocytes (macrophages, neutrophils and natural killer cells) which can recognise and then become activated to limit microorganisms' replication and reduce the potential of these to invade host tissues. The principal sites where

S.pneumoniae encounters the innate immune system are the upper and lower airways, thus the respiratory system provides an appropriate paradigm in which to describe this arm of the immune system.

1.1.1.1 Soluble factors of the respiratory immune system

Mucus secreted from cells of the respiratory mucosa contains mucin which coats microorganisms and inhibits their adherence to epithelial surfaces. Antimicrobial peptides include β -defensins which are cationic peptides made by epithelial cells, that destroy bacterial cell membranes. Also abundant in the alveolar fluid are surfactant proteins A (SP-A) and D (SP-D) and mannose binding lectin (MBL), members of the collectin family (named by virtue of their collagen and lectin domains). By binding and coating pathogens they aggregate and immobilise them to limit their spread, activate complement and enhance their phagocytosis (reviewed in (Kadioglu and Andrew 2004)).

The complement family of proteins is another group of the soluble factors of the innate immune system. Complement proteins interact with one another to generate cascades of activated intermediates that opsonise pathogens to promote phagocytosis, directly damage the membranes of microorganisms and form chemoattractants for neutrophils aiding the induction of an inflammatory response (reviewed in (Paterson and Orihuela 2010)). The complement system can be activated by direct interaction with foreign surfaces; carbohydrates on microbial surfaces trigger the lectin pathway of complement activation and in the absence of inhibitors of complement, present normally on host cells but not microbial surfaces, the alternative pathway is stimulated. Complement can also be activated in association with signals from the adaptive immune system, in this instance by antibody which has bound to an antigen, to set off a third cascade known as the classical pathway (Paterson and Orihuela 2010).

1.1.1.2 Macrophages in innate immunity

Should a pathogen breach epithelial and other physical barriers and enter tissue it will usually be recognised, ingested and killed by a phagocyte. Macrophages are specialised, long lived phagocytes of the myeloid lineage that reside in the tissues and can be replenished from both the pool of circulating monocytes or by local proliferation (Jenkins, Ruckerl et al. 2011). In the lung, most macrophages are differentiated alveolar macrophages (AM) residing in the alveoli (van oud Alblas and van Furth 1979). AM express pathogen recognition receptors (PRR) including the mannose receptor (CD206), MARCO and Toll like receptors (TLRs) (Wileman, Lennartz et al. 1986; Palecanda, Paulauskis et al. 1999; Droemann, Goldmann et al. 2003). PRRs recognise both environmental particles and components of microorganisms, such as the lipopolysaccharide component of a bacterial cell wall. These microbial ligands are referred to as pathogen associated molecular patterns (PAMPs), and allow the macrophage to identify a broad range of pathogens rapidly and without immunologic memory. Ligation of the cell surface PRR activates the macrophage and upregulates receptors that stimulate phagocytosis of the pathogen and generation of microbicidal molecules such as nitric oxide (NO) which kill the pathogen in an intracellular vesicle called the phagolysosome. Macrophage killing of *S. pneumoniae* will be considered in more detail in the next section. PRRs also trigger complex intracellular signalling pathways which result in activation of nuclear factor kappa B (NF- κ B) and interferon regulatory factor (IRF), key transcription factors involved in immune signalling (reviewed in (Calbo and Garau 2010)). NF- κ B is essential for a second response of the macrophage to pathogens, namely the production and release of specialised molecules called cytokines and chemokines which signal inflammation and attract neutrophils and plasma proteins to the site of the pathogen, thus helping the macrophage co-ordinate the inflammatory response more broadly, in addition to helping the macrophage ingest and kill pathogens (reviewed in (Janeway and Medzhitov 2002)).

1.1.1.3 Macrophage polarisation

Crucially, and particularly important in the alveolus, the AM has adapted to become an efficient phagocyte yet limit cytokine production in the face of constant exposure to microorganisms in order to constrain an otherwise damaging inflammatory response (Cassol, Cassetta et al.). It also has a prolonged life span and estimates suggest it lives for 1-2 years or longer (Kjellstrom, Ichimura et al. 2000; Murphy, Summer et al. 2008). These features distinguish the resting alveolar macrophage from macrophages seen at the site of acute inflammation which are short lived and produce stronger cytokine responses (Maus, Janzen et al. 2006; Jenkins, Ruckerl et al. 2011). Plasticity is a hallmark of macrophages and their differing phenotypes are recognised to fall along polarised pathways of activation; the inflammatory or M1 macrophage is driven by inflammatory signals provided by IFN- γ and bacterial LPS and at the other extreme the alternatively activated M2 macrophage is induced by a range of different stimuli. M2 polarisation can be subdivided according to the phenotype of the resulting cell with specific stimuli associated with each phenotype; M2a (IL-4 and IL-13), M2b (immune complexes and TLR ligands) and M2c (IL-10 and glucocorticoids)(Cassol, Cassetta et al. ; Ambarus, Krausz et al. 2012) (and reviewed in (Dockrell, Collini et al. 2012)). As a consequence of polarisation macrophages differ in expression of surface receptors, cytokine production and generation of NO and reactive oxygen species (ROS) with consequences for their function in host defence, wound healing or immune regulation (reviewed in (Mosser and Edwards 2008)). Thus, the alveolar macrophage has the capacity to alter its phenotype in response to the microbiological and immunological environment it encounters.

1.1.1.4 Macrophage signalling

The macrophage communicates with other arms of the innate and adaptive immune response by releasing cytokines and chemokines, including interleukin (IL) -1 β , tumour necrosis factor (TNF) - α , IL-6, IL-8 and IL-12. They serve the ultimate purpose of containing the spread of infection when the macrophage is overwhelmed. Their actions may be autocrine (further activating the macrophage and enhancing phagocytosis), paracrine (local) or endocrine

(inducing responses from remote locations, e.g. leukocyte release from the bone marrow, acute phase response protein production by the liver and release of prostaglandin E2 which acts on the hypothalamus to alter the body's temperature)(reviewed in (Janeway 2001))

1.1.1.5 Neutrophils, dendritic cells and natural killer cells

One action of the cytokine response of the macrophage is to upregulate adhesion molecules on endothelial cells in both local blood vessels and circulating neutrophils, resulting in neutrophil extravasation into the tissue. Here they migrate in huge number along chemokine gradients to the epicentre of the infection. Neutrophils, abundant in the blood but not normally in healthy tissues, are short lived phagocytes. By devoting the major part of their cellular energy to the respiratory burst required to generate toxic oxygen species they have a greater capacity than macrophages for intracellular killing but at the cost of their own survival: they die soon after completing a round of phagocytosis. Thus the rapid accumulation of neutrophils at the site of infection serves to exponentially increase phagocytic capacity while also contributing to a dramatic transformation of the local tissue. Dead neutrophils, which are the main component of pus, must be removed from the tissue during the resolution of infection, another phagocytic role served by the macrophage known as efferocytosis (reviewed in (Janeway 2001)).

Related to macrophages, dendritic cells also have the ability to respond to micro-organisms. They are similarly activated through the binding of pathogen constituents to PRRs. Instead of being phagocytes they are best adapted to process the microorganisms and migrate to the local lymph glands, where after they present pathogen specific molecules, termed antigens, to the antigen-specific naive T lymphocytes (T cells, discussed further below). In turn the T cells become activated to mature into effector cells and migrate to the original site of the infection. Another cell activated in response to macrophage signalling is the natural killer (NK) cell. Derived from a common lymphoid progenitor in the bone marrow it is similar to B and T

lymphocytes but lacks antigen specific receptors. It recognises host cells that have become infected with intracellular pathogens, and by releasing cytotoxic granules onto the surface, induces apoptotic death of their target. Related is the invariant natural killer T (iNKT) cell, which shares the properties of T cells and NK cells but possesses only invariant receptors against non specific lipids and glycolipids displayed by CD1d molecules on infected cells' surfaces. They thus act as an innate cell rather than a component of the adaptive immune response but can produce considerable quantities of immunoregulatory cytokines including IFN- γ , IL-4, IL-17 (reviewed in (Janeway 2001) and (Wu, Gabriel et al. 2009)). Another T cell, called the $\gamma\delta$ T cell also acts as an innate immune cell and has the capacity to phagocytose. It possesses an alternative pair of $\gamma\delta$ glycoprotein chains in the T cell receptor (TCR) rather than the $\alpha\beta$ type of CD4⁺ and CD8⁺ T cells (see below) which is more restricted and acts like an innate cell PRR (Born, Reardon et al. 2006).

1.1.2 Adaptive Immunity

The innate immune response is rapid but lacks immunological memory. To develop a more long-lasting response the adaptive immune response is required (Janeway 2001). The adaptive immune response also has the potential to be harmful to host tissue and so it is characterised by an enhanced capacity to distinguish foreign material from self. This antigen specific adaptive immune response is provided by B and T lymphocytes. These cells can act in most areas of the body through the secretion into the circulation and body fluids of specialised soluble proteins called immunoglobulins, manufactured by B cells, or by migrating to remote sites where they have local and direct effects on other cells, a function exemplified by T cells. A key feature is the development of long lived immunological memory of this primary response to a specific pathogen such that a secondary immune response can be more rapidly induced in the event of a subsequent encounter.

1.1.2.1 B lymphocytes

After their generation in the bone marrow, B lymphocytes develop into a diverse number of distinct clones. Domain variability, somatic mutation and hypermutation as well as class switching all contribute to the diversity of antibody responses using a restricted number of genes. Thus B cells have the capacity to produce thousands of subtly distinct immunoglobulins, each specific to different antigens on the surfaces of pathogens. A microorganism specific B-cell response is generated when a B cell encounters its antigen on an antigen presenting cell in a lymph node. The naive B cell becomes activated to divide and expand into a large clonal population of mature effector B lymphocytes or plasma cells that secrete immunoglobulin specific to that antigen. On binding, immunoglobulin may aid complement fixation or act to block viruses or microbial toxins from themselves binding to host cell receptors. Other antibodies may agglutinate pathogens preventing them accessing potential attachment sites and aiding clearance. Alternatively the antibody will bind the antigen and mark the pathogen for detection and ingestion by phagocytes or the generation of antibody-dependent cell mediated cytotoxicity. Key to these cell mediated processes is the Fc region of the immunoglobulin molecule that binds specifically to an Fc receptor (FcR) on the surface of macrophages, neutrophils, dendritic and NK cells, as well as B lymphocytes, leading to their activation. FcRs belong to different classes (e.g. Fc α R, Fc γ R, Fc ϵ R) according to which antibody type (IgA, IgG, IgE) they bind, with each class having its own individual members too, such as Fc γ RI, Fc γ RII(A,B,C) and Fc γ RIII(A,B). The antibody subtype and cell type will determine the effect of binding from phagocytosis, when IgG binds to Fc γ RI on the macrophage to degranulation when IgE binds to Fc ϵ RI on Eosinophils (Janeway 2001). As the response to the infection continues the B cells undergo repeated antigen stimulation and produce immunoglobulin with greater antigen binding affinity (affinity maturation). Not all naive B cells become effector cells after antigen encounter and some instead multiply and differentiate into memory B cells, which are not involved in the response but survive, ready to rapidly generate any future, antigen specific, secondary immune response (reviewed in (Alberts 2002)).

1.1.2.2 T lymphocytes

T cells derive from bone marrow precursor cells that migrate to and develop in the thymus. Here, through a process of clonal selection akin to that in B cells, they are programmed to recognise a foreign antigen by means of a specialised, T cell receptor (TCR). The TCR is made up of highly variable immunoglobulin like domains called TCR α and TCR β chains, which bind to antigen but can also recognise 'self' surface major histocompatibility complex (MHC) molecules that belong to the host. In addition to the $\alpha\beta$ TCR there are invariant accessory chains CD3 γ , CD3 δ , and CD3 ϵ which together form the CD3 complex. It is completed by a fourth accessory ζ chain which has a large cytoplasmic domain for signal transduction (Janeway 2001). T cells belong to two main classes and can be distinguished by the presence of co-receptors that enhance the signalling of the main T cell receptor; T helper cells express the CD4 co-receptor and cytotoxic T cells express CD8. A T helper cell (CD4⁺ T cell) is only functional once it has been activated by contact with an antigen presenting cell. For this contact to be successful two distinct signals must be transmitted between the cells. The first involves sharing of the foreign antigen between a class II MHC on the antigen presenting cell and the TCR on the T-cell, which triggers receptor clustering. The cytoplasmic domains of the TCR then associate with a tyrosine kinase, Fyn. CD4 amplifies the signal from the TCR when it engages MHC class II, utilising the tyrosine kinase Lck. These two receptor associated tyrosine kinases lead to the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of the TCR and initiate a signalling cascade that propagates to the nucleus (reviewed in (Isakov 1997)). The second signal provides co-stimulation and serves to amplify the first through binding of B7 proteins (CD80 and CD86) on the antigen presenting cell with CD28 on the T cell. This activates the naïve T cell and allows it to differentiate to an effector T cell and stimulate its own proliferation in an autocrine manner by generating the cytokine IL-2 (Janeway 2001). This response is kept in check by production of another protein, cytotoxic T-lymphocyte-associated protein (CTLA)-4 which binds CD28 and blocks co-stimulation, making

the T-cell anergic (reviewed in (Rudd, Taylor et al. 2009)). Prolonged T cell activation is dependent on continued antigenic stimulation, which subsides as the infection is cleared. In addition sustained activation will in time lead to a down regulation of activity as it results in susceptibility to T cell apoptosis. Repeated TCR stimulation leads to the up regulation of Fas (CD95) on the activated T cell which on binding of its cognate ligand (FasL, also expressed by T cells) triggers apoptosis, a process termed activation-induced cell death (AICD, reviewed in (Zhang, Xu et al. 2004)).

Activated T helper cells serve to help stimulate macrophages, enhancing killing of intracellular pathogens or B cells to produce antibody; which of these responses predominates depends on the type of cytokine message produced by the antigen presenting cell at the time the naive T helper cell is activated. In general intracellular bacteria stimulate antigen presenting cells to produce the cytokine IL-12 which pushes T helper maturation towards a macrophage activating or 'T_H1' phenotype involving the production of TNF- α and IFN- γ . Allied to this, T_H1 cells also promote the production of monocytes and the migration of monocytes and neutrophils into infected tissues. Alternatively, extracellular parasites such as helminths and protozoa stimulate antigen presenting cells to produce cytokines that favour a 'T_H2' response from the T cell that promotes antibody production from B cells and the activation of eosinophils through the secretion of IL-4, IL-5, IL-10 and IL-13 (reviewed in (Alberts 2002)).

CD8 expressing, cytotoxic T cells (CD8⁺ T cells) recognise and kill host cells infected by intracellular pathogens or viruses. This requires the TCR to recognise and binds antigen bound to Class I MHC on the surface of the target cell (Janeway 2001). Once activated the CD8⁺ T cell releases the protein perforin which opens channels in the cell membrane and enables the delivery of a cargo of proteases such as granzyme B which activates apoptotic death in the infected target cell (reviewed in (Berke 1994)). CD8⁺ T cells also expresses FasL, which induces an additional pro-apoptotic signal on binding to Fas on infected cells. Since activated CD8⁺ T

lymphocytes also express Fas they are also subject to control from AICD, scaling down the number of activated effector cells when the immune response is concluded (reviewed in (Janeway 2001; Alberts 2002)).

1.1.3 Apoptosis

A general property of cells is their capacity to instigate their own death. This physiological process is termed apoptosis and occurs in normal development and homeostasis. Classic examples are the removal of tissue between fingers in the formation of the foetal hand or the sloughing of the lining of the uterus in menstruation (reviewed in (Alberts 2002)). Apoptosis is also a controlled cellular response to danger when the survival of the cell would threaten the host, typically if the cell is infected with a pathogen or has damaged DNA, or as with activated T cells, is no longer needed. Thus it is also fundamental feature of the immune system, illustrated when defects in genes coding apoptosis proteins result in immunodeficiency, cancer and autoimmunity (reviewed in (Thompson 1995)).

Apoptosis differs from unplanned, catastrophic cell death which is termed necrosis. During necrotic death a cell swells and ruptures, releasing its contents inducing a potentially deleterious inflammatory response in the surrounding tissues (reviewed in (Alberts 2002)). By contrast, apoptosis is a pre-programmed and tightly regulated energy dependent death response that leads to the safe disposal of the cell (reviewed in (Alberts 2002)). Classically apoptotic cells are seen to shrink, develop blebs on the membrane and condense their chromatin (Kerr, Wyllie et al. 1972). The cellular membranes remain intact and prevent the loss of pro-inflammatory cell constituents to the tissues (reviewed in (Opferman and Korsmeyer 2003)). Phosphatidylserine, a phospholipid, translocates from the cytosol to the outer membrane of the cell to act as a signal for healthy phagocytes to engulf the dying cell and its membrane wrapped remnants and safely dispose of them (Verhoven, Schlegel et al. 1995). Cell death is the culmination of a cascade of intracellular catalytic activity mediated by

cysteine proteases known as caspases. These are grouped as initiator caspases 2, 8, 9 and 10 and effector/executioner caspases-3, -6 and -7 (Thornberry and Lazebnik 1998; Labbe and Saleh 2008). A common apoptotic pathway is activated by the triggering of either of two proximal pathways, known as the intrinsic and extrinsic pathways of apoptosis, depending on whether the original signal comes from within the cell or the extracellular environment (Opferman and Korsmeyer 2003).

1.1.3.1 The intrinsic pathway of apoptosis

The intrinsic pathway of apoptosis, also referred to as the mitochondrial pathway, is triggered by injury within the cell or cellular stress. This may follow the withdrawal of growth factors or IL-2, damage to DNA, for example by ultra violet (UV) radiation, a building up of abnormal protein, detected through the unfolded endoplasmic reticulum (ER) stress response, or oxidative stress (Green and Kroemer 2004). The survival or demise of the cell depends on the balance in expression and activity of pro and anti apoptotic members of a group of proteins known as the B cell lymphoma (Bcl)-2 family. The Bcl-2 family share up to four conserved sequences known as Bcl-2 homologous (BH) domains 1-4. The anti-apoptotic Bcl-2 proteins reside on the outer mitochondrial membrane and maintain its stability. They include Bcl-2, myeloid cell leukemia protein (Mcl)-1, Bcl-extra large (XL), and A1/Bfl-1 and share all four BH domains. Pro-apoptotic members share either Bcl-2 homology (BH) 1-3 domains (Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak)) or are BH3 domain only containing proteins (BH3 interacting-domain death agonist (Bid), Bcl-2-associated death promoter (Bad), Noxa and p53 upregulated modulator of apoptosis (PUMA)). Cellular stress and DNA damage, in particular through the activation of p53, leads to increased transcription and activation of these proapoptotic proteins while repressing antiapoptotic Bcl-2 members (reviewed in (Opferman and Korsmeyer 2003). On activation Bax migrates to the outer membrane of the mitochondrion and either directly forms pores or associates with the antiapoptotic Bcl-2 through its BH3 domain and inhibits its membrane stabilizing effect,

through association with a pore forming protein known as the adenine nucleotide translocator (ANT) (Vieira, Haouzi et al. 2000). Activated BH3 only members also migrate to the mitochondria but require the presence of Bax or Bak to disrupt the membrane (Cheng, Wei et al. 2001). As a consequence of mitochondrial membrane permeabilisation, cytochrome C (Cyt-C) leaks from the mitochondria into the cytosol where it associates with deoxyadenosine triphosphate (dATP) and apoptotic protease activating factor 1 (APAF-1) to form a complex known as the apoptosome. The apoptosome cleaves procaspase 9 to the initiator caspase 9 and so begins a cascade of caspase activity culminating in activation of the effector caspase 3. The cell is now committed to apoptosis and caspase 3 causes the digestion of cytoplasmic structural proteins, degradation of chromosomal DNA and promotes the phagocytosis of the cell by other cells such as macrophages, a process known as efferocytosis.

1.1.3.2 The extrinsic pathway of apoptosis

External signals for apoptosis are received when soluble or cell surface expressed ligands, TNF α , CD95L (FASL) and TNF-related apoptosis inducing ligand (TRAIL) bind to receptors of the TNF receptor (TNFR) family. Fas is the prototypical TNFR having a cell surface receptor and cytosolic death domain. Ligation causes receptor oligomerisation and so clustering of the death domains. These can now recruit the adaptor protein Fas-associated via death domain (FADD) and subsequently procaspase 8 to form a complex known as death inducing signalling complex (DISC). Procaspase 8 cleaves into caspase 8 which can directly activate caspase 3 and apoptosis. Alternatively, the caspase cascade can be initiated indirectly when caspase 8 cleaves Bid, enabling its migration to the mitochondrial membrane where it binds Bcl-2 and triggers the mitochondrial pathway of apoptosis. TNFR1 binding by TNF α ligation can similarly initiate caspase activity mediated by death domain clustering and the adapter molecule TNFR-associated death domain (TRADD). Unlike FAS, TNFR1 mediated apoptosis is only possible if the cell has also been sensitized by cellular factors that block protein synthesis. This is itself regulated by NF- κ B and JNK/AP-1 which are activated downstream of the TNFR1 by an

alternative signalling pathway involving the serine-threonine kinase receptor-interacting protein (RIP).

In conclusion, the innate and adaptive immune systems described work in concert to provide continual defence from pathogenic micro-organisms. The responses can be scaled up and down again as necessary to achieve a sufficient yet not excessive response to clear the pathogen while minimising tissue injury. Apoptotic cell death is an important part of this process.

1.2 The spectrum of pneumococcal disease

Streptococcus pneumoniae is an important human pathogen and the leading cause of bacterial upper respiratory tract infections (e.g. otitis media and sinusitis) and pneumonia. Current (2013) estimates from the UK are that there are 40,000 hospitalisations a year for pneumococcal pneumonia while in primary care there are 63,000 consultations for pneumococcal otitis media (Public Health England 2013). In severe cases the pneumococcus may also spread beyond these locations to normally sterile sites such as the blood, causing bacteraemia, the pleural space which can result in empyema, or the cerebrospinal fluid and meninges, leading to meningitis. For example, 10%-30% of cases of pneumococcal pneumonia are also accompanied by bacteraemia (Blasi, Mantero et al. 2012). Such conditions are collectively referred to as invasive pneumococcal disease (IPD). Overall there are between 5000 and 6000 cases of IPD in the UK each year, of which 5.5% are due to meningitis (Public Health England 2013). Worldwide the heaviest burden of pneumococcal disease is on the developing world with an estimated 1.6 million deaths per year, more than half of these in children <5 years old (WHO 2005).

Individuals at increased risk of IPD are those at extremes of age (<2 or >65 years of age) (Robinson, Baughman et al. 2001). In Europe and the United States, the overall incidence of IPD is between 11 and 23.2 per 100,000 population rising to near 60 per 100,000 in those over

65 years (Robinson, Baughman et al. 2001; Kyaw, Christie et al. 2003). Other individuals with reduced immune competence who are at risk of IPD are asplenic individuals (including those with sickle cell anaemia), diabetics, alcoholics, those with chronic pulmonary, renal, liver or cardiovascular disease, those with primary immunodeficiencies (e.g. immunoglobulin deficiency) and those with acquired immunodeficiency secondary to cancer, immunosuppressive medication or HIV-1 infection. Pneumococcal pneumonia and subsequent IPD is also more likely following influenza virus infection (reviewed in (Brundage 2006)). Despite the widespread availability of antimicrobial drugs with activity against *S. pneumoniae*, mortality from IPD has remained constant at around 20% over the last 60 years (Harboe, Thomsen et al. 2009; Rello, Lujan et al. 2010). While antibiotic resistance is important, most deaths generally occur despite appropriate antibiotic treatment (Rello, Lujan et al. 2010; Blasi, Mantero et al. 2012). Since factors independent of antimicrobial susceptibility such as host response and pneumococcal virulence factors are associated with poor outcome it is likely that the this persisting mortality is related to the consequences of sub-optimal host immune responses and the capacity of the pathogen to subvert these responses. This will be discussed further when the pathogenesis of pneumococcal diseases is considered.

1.2.1 Vaccination against *Streptococcus pneumoniae*

A significant advance in prevention of pneumococcal disease has been the introduction of anti-pneumococcal vaccination. A 23 valent pneumococcal polysaccharide vaccine (PPV23), which covers 95%-90% of IPD strains, has been available since the 1980's. It is generally used for those aged over 65 or those at increased risk of pneumococcal disease due to co-existing medical conditions. Randomised controlled trial and observational evidence report a protective efficacy of 74% (95% CI 56–85%) in preventing IPD (Moberley, Holden et al. 2008). However, efficacy against all cause pneumonia (a common surrogate for pneumococcal pneumonia given that causative pathogens are infrequently isolated) is weaker at 29%, and has only been demonstrated to make significant impact in low income countries (Moberley,

Holden et al. 2013). Importantly PPV23 is not associated with reductions in all cause mortality (Moberley, Holden et al. 2013). More recently, 7 valent (PCV7) then 13 valent pneumococcal conjugate vaccines (PCV13) have been introduced for immunisation of children less than 2 years of age. Though covering a smaller range of strains, they produce stronger antibody responses in this age group than the poorly immunogenic PPV23 since they generate T-cell dependent responses. IPD from vaccine serotypes has dramatically decreased in this age group since uptake of PCV7 and, importantly, has also decreased in older age groups, as a consequence of herd immunity (Blasi, Mantero et al. 2012; Public_Health_England 2013; Public_Health_England 2013). Nevertheless, vaccination has limitations; as stated, protection among those >65 years is limited, efficacy in immunocompromised groups is unproven and effects on mortality are inconclusive. Those adults most at risk for IPD get least benefit from vaccination (e.g. those living with HIV, discussed below). Emerging data also suggest the presence of serotype replacement, meaning that non-vaccine serotypes exploit the ecological niche left by reduction of colonizing strains and their increased contribution to IPD is likely, to some extent, to offset the herd effect (Harboe, Benfield et al. 2010; Moberley, Holden et al. 2013).

1.3 The pathogenesis of *Streptococcus pneumoniae* infection

Streptococcus pneumoniae is an encapsulated, facultatively anaerobic Gram positive coccus. It typically appears as paired diplococci on a Gram film and on blood agar colonies are α -haemolytic. Bacteria are catalase positive and usually optochin sensitive. The polysaccharide capsule of *S. pneumoniae* varies in its chemical composition and net charge and over 90 serologically distinct capsular serotypes have been recognised. The composition of the capsule endows strains with differing virulence properties. Additionally, the pneumococcus possesses protein virulence factors including pneumolysin, neuraminidase, hyaluronidase and autolysin

(Kadioglu and Andrew 2004). Together these are key determinants of pathogenicity, influencing colonisation and invasive potential (Paterson and Orihuela 2010).

1.3.1 Colonisation

The major reservoir for *S. pneumoniae* is humans, where it usually colonises the nasopharynx asymptomatically. It is transmitted by coughing and sneezing. 4-15% of adults and up to 40% of children in industrialised nations carry the pneumococcus, depending on the population studied and capsular serotype that predominates (Austrian 1986; Regev-Yochay, Raz et al. 2004). These rates increase in crowded situations, particularly hospitals and day centres (reviewed in (Mook-Kanamori, Geldhoff et al. 2011)).

Pneumococcal carriage is known to precede the development of disease in an individual, and to colonise effectively *S. pneumoniae* must evade host barriers to adherence and bacterial replication. Lysozyme, produced by the host to cleave bacterial cell wall peptidoglycan, is rendered ineffective by two pneumococcal enzymes peptidoglycan N-acetylglucosamine-deacetylase A (PdgA) and an O-acetyltransferase (Adr) which deacetylate the peptidoglycan and prevent the action of lysozyme. Pneumococcal IgA protease confers resistance to mucosal IgA1, promoting attachment (Kilian, Mestecky et al. 1980; Weiser, Bae et al. 2003). In the respiratory tract the mucocilliary escalator may be overcome by the toxin pneumolysin (Ply), which decreases epithelial cell ciliary beating, secreted exoglycosidases (e.g. neuraminidase A) or by the capsule itself which prevents entrapment in mucus (Nelson, Roche et al. 2007; Mook-Kanamori, Geldhoff et al. 2011). That some capsular serotypes are better adapted to colonise the upper airway is reflected in the varied prevalence of serotypes in colonisation studies (Tocheva, Jefferies et al. 2011). However, not all strains that colonise effectively go on to cause invasive disease while some invasive isolates are infrequently found in colonisation studies. These differences are due, in part, to characteristics of their capsule; colonising strains such as 6A and 23F are more easily coated with complement and are more readily phagocytosed by

AM while the invasive strains 4 and 7F better adhere to the epithelium and resist AM phagocytosis by virtue of their more accessible surface adhesions (Weinberger, Trzcinski et al. 2009; Hyams, Yuste et al. 2010; Sanchez, Hinojosa et al. 2011). The relative ability of a pneumococcal serotype to colonise / cause disease is expressed as the case carriage ratio. For example serotype 11C has emerged as a more frequently carried serotype since the adoption of PCV7 but it does not cause invasive disease. Conversely, serotypes 8, 12F and 22F are infrequently carried but are causing IPD (Flasche, Van Hoek et al. 2011).

1.3.2 From colonisation to disease

S. pneumoniae may be micro-aspirated from the nasopharynx into the lower airway, spread via the airway and across contiguous structures to create localised foci of infection (e.g. otitis media, sinusitis, pleural space disease) or invade into the blood and across the blood brain barrier to infect the meningeal space. In each setting the likelihood of invasive disease is determined by the outcome of the encounter between the pneumococcus, with its array of virulence factors, and immune host defence mechanisms. The pathogenesis of pneumococcal pneumonia, the commonest presentation of pneumococcal disease, has been studied in most detail and presents a paradigm for understanding the pathogenesis of pneumococcal infection in general. Distinct events during the pathogenesis of pneumococcal meningitis will also be highlighted.

Certain capsular serotypes are less easily bound by the soluble factor SP-D (Kadioglu and Andrew 2004). Capsule can also influence complement activity; by impairing C-reactive protein (CRP) activity and IgG binding to reduce classical pathway activation or by decreasing C3b degradation on the bacterial surface to limit the alternative pathway (Hyams, Yuste et al. 2010; Sanchez, Hinojosa et al. 2011). Ply also protects against complement; it activates the classical pathway in the absence of immunoglobulin leaving less complement available for bacterial surface coating (Yuste, Botto et al. 2005). Pneumococcal surface proteins (Psp) A and C are

also implicated in resistance to both the classical and alternative pathways (Dave, Carmicle et al. 2004; Yuste, Botto et al. 2005). The importance of complement for pneumococcal defence is further illustrated by the observations that C3 deficient (affecting all three complement pathways) patients suffer recurrent pneumococcal bacterial infections (Paterson and Orihuela 2010), C1q deficient mice (affecting the classical pathway) develop higher bacterial counts in the lung parenchyma, as a smaller proportion of bacteria are bound in the absence of the classical pathway, and the intensity of C3 binding is reduced in factor B deficient mice (affecting the alternative pathway) (Brown, Hussell et al. 2002). As suggested already, IgG found in the bronchoalveolar lavage (BAL) fluid of healthy individuals plays an equally important role in the opsonisation of pneumococci (Eagan, Twigg et al. 2007).

Ultimately, complement and immunoglobulin contribute to opsonic phagocytosis of pneumococci by the alveolar macrophage by means of complement (CR1 and CR3) and immunoglobulin (FcγRIIA and FcγRIII, but not FcγRIIB) receptors (Ali, Lee et al. 2003; Clatworthy and Smith 2004; Endeman, Cornips et al. 2009). The AM also phagocytose non-opsonised pneumococci by way of the scavenger receptors (SR) SR-A and MARCO (Arredouani, Yang et al. 2004; Arredouani, Yang et al. 2006). Again capsule serotype also plays a role in resisting non-opsonic phagocytosis, although this appears to be more important for facilitating colonisation than invasive disease (Weinberger, Trzcinski et al. 2009).

1.3.3 Alveolar macrophage control of *Streptococcus pneumoniae* infection

The importance of AM in defence against pneumococcal disease is illustrated by mouse models of pulmonary infection where low inocula of pneumococci are instilled into the trachea. The AM are able to clear the bacteria and infection resolves without neutrophil recruitment or bacteraemia occurring (Dockrell, Marriott et al. 2003). AM ingestion of opsonised bacteria facilitates fusion of the *S. pneumoniae* containing phagosome with a lysosome to create a phagolysosome where the ingested bacteria are killed (Jonsson, Musher

et al. 1985; Gordon, Irving et al. 2000). Impairment of this process results in invasive disease; when the mice were depleted of AM they had higher numbers of bacteria in the lung and needed to recruit neutrophils to control infection (Dockrell, Marriott et al. 2003). In keeping with this model, metal ions in welding fumes, which both inhibit AM phagocytosis and cause macrophage apoptosis thus providing a functional AM defect, result in welders having an increased rate of IPD and lobar pneumonia (Coggon, Inskip et al. 1994; Antonini, Lawryk et al. 1999; Antonini, Leonard et al. 2005). In the phagolysosome of the AM pneumococci are killed by mechanisms that include generation of NO and potentially also reactive nitrogen species (RNS) (Marriott, Ali et al. 2004; Marriott, Hellewell et al. 2007; Bewley, Pham et al. 2011). However, AM do not have as complete an arsenal as professional phagocytes like the neutrophil, lacking for instance myeloperoxidase and therefore the more potent halogenated ROS. Furthermore, macrophages need to be activated before they generate the microbicidal molecules required for bacterial killing. This is achieved both through bacterial sensing by macrophage pattern recognition receptors and stimulation from CD4⁺ and CD8⁺ T cells (Mantovani, Sica et al. 2004; Mosser and Edwards 2008). These factors, as well as the fact that many fold more neutrophils are involved when clearing bacteria, mean that the capacity of the AM to kill pneumococci is finite and can be overwhelmed.

Mouse experiments with higher inocula of pneumococci illustrate the consequences when the AM killing capacity is overwhelmed (Bergeron, Ouellet et al. 1998; Dockrell, Marriott et al. 2003). Despite partial bacterial clearance, the macrophage generates a neutrophil chemotactic and pro-inflammatory cytokine response releasing IL-8, TNF- α , IL-6 and IL-1. Higher levels of these cytokines are detectable both in lung and serum. Bacteria grow in the alveoli and neutrophils are recruited. By 24-48 hours tissue injury is visible and there follows recruitment of monocytes and lymphocytes into the lung. Three to four days post infection alveolar architecture is lost, associated with high levels of NO and malondialdehyde (MDA, generated from ROS) in the BAL fluid. As the infection progresses, higher proportions of mice become

bacteraemic as tissue damage aids translocation of bacteria from the alveolus to the bloodstream (reviewed in (Calbo and Garau 2010)). Thus the neutrophilic response now crucial to successful resolution of the infection and control of bacterial replication involves significant collateral damage as powerful antimicrobial systems such as NADPH oxidase-dependent generation of ROS also harm lung parenchyma (Marriott, Jackson et al. 2008).

In pneumococcal infection, the AM has a further killing strategy which supplements phagolysosomal killing. The AM initiates its own apoptosis which destroys persisting pneumococci by release and reaction of ROS with NO during apoptotic cell death (Dockrell, Lee et al. 2001; Dockrell, Marriott et al. 2003; Bewley, Pham et al. 2011). This is a host mediated process, triggered by pneumolysin and involving TLR4 ligation, that through a series of signalling steps leads to dynamic changes in the levels of anti-apoptotic Bcl-2 family member Mcl-1 and induction of the intrinsic, mitochondrial pathway of apoptosis (Marriott, Bingle et al. 2005; Srivastava, Henneke et al. 2005). Crucially, this apoptotic response can both control the pneumococci and limit the AM generated proinflammatory signal, averting the need for the neutrophilic inflammatory response and the consequent damage to the lung.

Macrophage pattern recognition receptors (PRR), particularly toll like receptors (TLRs) are important for the AM recognition of and response to *S. pneumoniae*. TLR 2 is ligated by cell wall lipoteichoic acid (LTA) and structural differences in LTA can underlie differences in virulence between pneumococcal strains (Yoshimura, Lien et al. 1999; Paterson and Orihuela 2010). Pneumolysin recognition by TLR4 also helps in defence against the pneumococcus and TLR4^{-/-} mice are more susceptible to pneumococcal disease (Malley, Henneke et al. 2003; Srivastava, Henneke et al. 2005). TLR9, which recognises unmethylated CpG dinucleotides, additionally plays a role in activating phagocytosis as evidence by increased disease in TLR 9^{-/-} mice (Albiger, Dahlberg et al. 2007). Ultimately, the intracellular signalling triggered by these receptors activates NF-κB mediated transcription of proinflammatory cytokines. The

importance of TLRs and their contribution to a sufficient yet not over exaggerated immune response is illustrated by the observations that single TLR knockout mice have more subtle defects in cytokine responses than combined TLR or adaptor protein knockouts (Lee, Scanga et al. 2007) and that individuals with heterozygotic variants of the TLR adaptor molecule toll-interleukin 1 receptor domain containing adaptor protein (TIRAP), which mediates TLR2 and TLR4 downstream signalling, are more susceptible to IPD (along with malaria and tuberculosis) (Khor, Chapman et al. 2007). Other PRRs are also important; nucleotide-binding oligomerisation domain (NOD) 1^{-/-} mice are more susceptible to pneumococci (Clarke, Davis et al. 2010); while some capsular strains that cause invasive disease are able to avoid NOD like receptor (NLR) dependent IL-1 β secretion (Witzenrath, Pache et al. 2011). Once again the balance of inflammatory response is critical; the strains evading NLR are more invasive because they induce a weaker inflammatory response but this also results in lower mortality (reviewed in (Dockrell, Whyte et al. 2012)). The principle that levels of inflammation must be carefully controlled is exemplified by the cytokine TNF- α where too much or too little cytokine is harmful. AM are the main source of TNF- α and high TNF- α levels result in excess neutrophil activation and lung injury, yet anti- TNF- α molecules worsen pneumonia in mouse models which have increased bacterial counts in blood, fewer neutrophils and more death (Takashima, Tateda et al. 1997; Kirby, Raynes et al. 2005). Similarly, anti-TNF- α therapies have been associated with increased risk of IPD in humans (Baghai, Osmon et al. 2001; Colombel, Loftus et al. 2004).

1.3.4 Pneumococcal bacteraemia and meningitis

Microaspiration may be sufficient to deliver pneumococci to the distal lung with invasive disease then following as a consequence of the virulence factors and host mediated inflammatory damage allowing translocation across the epithelium lining the lower respiratory tract. However, to cause invasive upper respiratory tract or meningeal infection pneumococci must cross upper airway epithelial surfaces or the endothelial blood brain barrier which have

important histological and anatomical differences compared to the lower airway. The key steps in this process have been described in a recent review (Mook-Kanamori, Geldhoff et al. 2011); the epithelial binding sites of the pneumococcal cell wall are covered by its thick capsule. Having successfully colonised the nasopharyngeal epithelium, the capsule undergoes phase transformation, decreasing its polysaccharide to become thinner, and exposes cell wall phosphorylcholine residues and other cell wall adhesion molecules (Weiser, Austrian et al. 1994; Cundell, Gerard et al. 1995). This allows the pneumococcus to bind to surface glycoconjugates and the platelet activating factor (PAF) and polymeric immunoglobulin (pIgR) receptors on the epithelial cells. Once bound, *S. pneumoniae* takes advantage of pIgR and PAF receptor recycling to become internalised to the basal membrane of the epithelial cell (Zhang, Mostov et al. 2000; Radin, Orihuela et al. 2005). Inter epithelial migration can also occur following the degradation of tight junctions by pneumococci bound to plasminogen (Attali, Durmort et al. 2008). Now the pneumococci are able to bind and degrade the extracellular matrix using pneumococcal adhesion and virulence (Pav) A and PavB and secreting hyaluronidase (Mook-Kanamori, Geldhoff et al. 2011). Pneumococci also cross the specialised endothelial cells of the blood brain barrier by using the PAF receptor recycling mechanism and binding to laminin of the basement membrane (Ring, Weiser et al. 1998). Alternatively, pneumococci may also be able to disrupt endothelial tight junctions to cross intercellularly.

Once in the cerebrospinal fluid (CSF) complement is activated and antigen presenting cells, including meningeal macrophages and microglial cells, recognise *S. pneumoniae* through TLR2, TLR4 and NLRs (Klein, Obermaier et al. 2008; Liu, Chauhan et al. 2010). There follows production of proinflammatory cytokines TNF- α , IL-6 and IL-1 β , a key consequence of which is an increase in the permeability of the vascular endothelium and influx of neutrophils, protein and fluid into the CSF and brain. While these responses are critical to the clearance of the invading bacteria, they also cause significant parenchyma damage which, along with the

release of microbial factors like pneumolysin that cause direct cytotoxicity, leaves a significant number of survivors with significant neurodisability (reviewed in (Koedel, Scheld et al. 2002)).

1.3.5 Adaptive immune responses to *Streptococcus pneumoniae* in the lung

The importance of adaptive B cell and humoral immune responses to *S. pneumoniae* are made clear from the vaccine efficacy studies discussed above and reflect the clinical evidence of increased susceptibility in agammaglobulinaemic patients (reviewed in (Austrian 1984) and (Rijkers, Sanders et al. 1993)).

A role for T cell mediated adaptive immunity in the defence against pneumococcal infection is implied by the increased risk of IPD in individuals with hyper-IgE-syndrome who have defects of cell mediated immunity and those that are HIV-1-seropositive, in whom risk is inversely correlated with CD4⁺ T cell counts (Hirschtick, Glassroth et al. 1995; Dworkin, Ward et al. 2001; Milner, Brenchley et al. 2008). It has been argued that epidemiological data from children and infants implicate a greater role for cellular than humoral responses in naturally acquired protection against colonisation with pneumococci (Malley 2010). In this population, pneumococcal disease incidence falls about 2 years before protective anticapsular immunoglobulin levels are naturally developed but when T-cell responses are known to mature (Wedderburn, Patel et al. 2001; Malley 2010). The proliferative and cytokine responses of CD4⁺ T cells from adenoidal explants (post adenoidal surgery) to pneumolysin (i.e. protein rather than capsule polysaccharide) have been found to be greater in children who are not colonised with pneumococci compared to their colonised counterparts, showing the importance of these T cell responses in protecting against colonisation (Zhang, Bagrade et al. 2007). There is also evidence for the role of T cell immunity involvement during carriage among adults; in a higher carriage prevalence population in The Gambia, adults have both effector memory and resting memory T cell responses to pneumococcal protein antigens. Although not correlating with carriage in individuals *per se*, these responses were more

pronounced in rural dwelling volunteers where carriage rates were more than two fold higher than those from urban areas (Mureithi, Finn et al. 2009). Supporting these findings are animal models, which demonstrate that protection of mice against pneumococcal carriage following mucosal exposure to killed whole cell vaccine, is dependent on CD4⁺ T cells and not humoral responses (Basset, Thompson et al. 2007). More specifically in mice this protection may be mediated by IL-17 and T helper (Th) 17 T cells, which activate macrophages to mediate clearance during initial episodes of colonisation, but prime neutrophil recruitment during secondary bacterial colonization to achieve bacterial clearance (Malley, Trzcinski et al. 2005; Lu, Gross et al. 2008; Zhang, Clarke et al. 2009; Davis, Nakamura et al. 2011). These observations have been used to support the hypotheses that T cell immunity is more important than humoral immunity in determining carriage (which is driven by responses to pneumococcal protein rather than polysaccharide), that current/recent carriage episodes result in effector T cell memory while previous or repeated colonisation episodes stimulate resting T cell memory, and that Th17 cells recognise pneumococcal antigen and protect against colonisation, possibly by recruiting and activating phagocytes with IL-17 (Mureithi, Finn et al. 2009; Malley 2010).

T cells also function in the response to pneumococcal infection in the lung; in mice there is a rapid T cell recruitment to areas of pneumococcal invasion where they become activated (Kadioglu, Gingles et al. 2000). In keeping with this, MHC Class II knockout, CD4⁺ T cell deficient mice develop a higher burden of pneumococci in their lungs following intranasal inoculation (Kadioglu, Coward et al. 2004). However, this finding was not replicated by Le Messurier et al. using the same mouse model, pneumococcal strain (D39) and infective dose, where mouse survival was unchanged or even increased and cyclosporine and antibody mediated depletion or inhibition of CD4⁺ T cell activation yielded similar results (LeMessurier, Hacker et al. 2010). A suggested explanation is that the inflammatory response, which involved greatly reduced IL-1 β , IL-6, IL-10 in these models, is better matched to the requirements of the infection so that

the bacteria can be effectively cleared (LeMessurier, Hacker et al. 2010). It has subsequently been demonstrated that the Th17 - IL17 response, so helpful in protecting against carriage, might itself be responsible for harmful effects in the more constrained environment of the distal airway where excessive inflammation could have serious consequences to the precarious balance of gas exchange in alveolus; Weber et al. found no change in survival in CD4^{-/-} mice following *S. pneumoniae* serotype 3 nasal inoculation, but observed that CD8^{-/-} mice suffered greater bacterial dissemination, lung inflammation and lethality than wild type which was attributed to increased Th17 cell numbers, IL-17 and neutrophil recruitment (Weber, Tian et al. 2011). For this serotype, at least, CD8⁺ T cells may have a role in modulating the damaging effects of the inflammatory response to *S. pneumoniae* (Weber, Tian et al. 2011). Evidence from mouse models further demonstrates that excessive T cell activation may have further adverse consequences; deficiency of FasL reduces induction of apoptosis in activated T cells and an enhanced population of activated T cells results in reduced neutrophil competence, causing decreased bacterial clearance during established pneumococcal pneumonia (Marriott, Daigneault et al. 2012).

To conclude, the pathogenic features of pneumococcal infection and disease are the net result of an interaction between pneumococcal virulence determinants and multiple facets of the innate and adaptive immune response. Experimental and clinical evidence demonstrate the consequences of deficiency in each of these responses but also that the immune reaction must be finely tuned as excessive responses are also detrimental. HIV-1 and how it contributes to the clinical epidemiology of pneumococcal disease and causes specific defects in each layer of the immune response against *S. pneumoniae* is considered next.

1.4 HIV-1 infection

1.4.1 The natural history of HIV-1 infection

HIV-1 infection is a global pandemic. Recent estimates from UNAIDS in 2011 give a prevalence of 34 million (31.4 million–35.9 million) people living with HIV-1 (known as HIV-1-seropositive), with an annual incidence of 2.5million (2.2 million–2.8 million) new cases. In 2011 1.7 million [1.5 million–1.9 million] people died from acquired immune deficiency syndrome (AIDS) - related causes (UNAIDS 2012). Worldwide, the most common route of transmission of the virus is sexual via unprotected vaginal intercourse. Other routes are sexual spread through unprotected anal intercourse, vertical transmission from mother to child, transmission through intravenous drug abuse via contaminated needles or contaminated blood transfusion products and accidental exposures in health care settings such as needle stick injuries (WHO 2013).

Following exposure 50 – 70% of people experience an acute clinical syndrome (Fauci, Pantaleo et al. 1996); primary HIV-1 infection is a short lived illness of up to four weeks involving fever, lymphadenopathy, myalgia and a maculopapular rash though other symptoms and signs may occur (Kahn and Walker 1998). The severity is often mild and the sufferer does not consult medical advice. It is also fairly non-specific in character being similar to other acute generalised viral infections such as infectious mononucleosis or influenza, and so the syndrome is frequently not recognised as due to HIV-1 infection (Rosenberg, Caliendo et al. 1999).

There then follows an asymptomatic phase that may last several years (Alcabes, Munoz et al. 1993). During this period there is ongoing viral replication and immune activation, so although apparently clinically latent, the virus is not dormant or inactive like, for instance, herpes viruses. After the asymptomatic phase symptoms or illness may develop as an increasingly dysfunctional immune system fails to prevent or control infectious or neoplastic disease or as HIV-1 leads to direct damage to the nervous system. HIV-1 infected individuals may at first suffer infections that are common in the general population such as bacterial respiratory

infections, vaginal thrush or reactivation of dormant herpes zoster infection but at increased frequency. Eventually, as immunosuppression becomes more severe, infections or malignancies are seen which are rarely seen in those with intact immune systems including Kaposi's sarcoma, CMV retinitis, oesophageal candidiasis, PCP pneumonia and intracerebral toxoplasmosis, all of which were early indicators of the HIV/AIDS pandemic (Lerner and Tapper 1984; Hanson, Chu et al. 1995). The development of any of these opportunistic infections or AIDS associated malignancies define AIDS, occurring at a median 8-10 years following seroconversion (Alcabes, Munoz et al. 1993; O'Brien, Blattner et al. 1996). Diseases resulting from the direct pathenogenic effects of the HIV-1 virus, for example HIV-1 encephalopathy, may also occur at this time, as the load of replicating virus is often very high by the later stage of disease. The progression of HIV-1 disease can be measured by the pattern of opportunistic diseases that occur, which forms the basis of the CDC and WHO classification systems of HIV-1 (WHO 2007; Schneider, Whitmore et al. 2008).

The speed of progression of HIV-1 infection towards AIDS is a function of both immune impairment and the detectable level of HIV-1 RNA in the plasma (Phillips and Pezzotti 2004). After an initially high level viraemia in the order of 10^6 copies per mL of plasma (cpm), the level of virus drops to $10^3 - 10^4$ cpm within several weeks as a result of a relatively vigorous cell mediated and humoral host response (Fauci, Pantaleo et al. 1996). This level of viral RNA, a balance between viral replication and immune control, is then maintained during the clinically latent phase and is known as the viral set point. How high or low the viral set point is determines the speed of progression towards AIDS. Thus, most individuals with HIV-1 RNA set points of <1000 cpm can continue in the asymptomatic phase for 12 years or more while more than 80% of those whose viral load remains > 100,000 cpm develop AIDS by 2 years post infection (O'Brien, Blattner et al. 1996; Phillips and Pezzotti 2004). As a marker of HIV-1 induced immune impairment the $CD4^+$ T lymphocyte cell count (CD4 count) correlates with the risk of AIDS and death, and while the plasma levels of HIV-1 RNA (the viral load) gives

information on the rate of disease progression the CD4 count provides information on the extent to which disease has progressed. The CD4 count is also used to determine the time of introduction of antiretroviral medication and along with the viral load is used to monitor responses to therapy (Mellors, Munoz et al. 1997; Williams, Churchill et al. 2012).

Without HAART 90% of individuals died within 2-4 years of the onset of AIDS (Hanson, Chu et al. 1995). With administration of HAART to reduce plasma viral load to undetectable levels the progression of HIV-1 infection can be halted and CD4 counts increase. Once restoration of immune function is established the risk of opportunistic disease risk recedes and individuals gain a vastly improved life expectancy (Sterne, Hernan et al. 2005).

1.4.2 HIV-1 structure

HIV-1 is a retrovirus and belongs to the genus lentivirus. Lentiviruses include several viruses that typically cause chronic infections involving a long period of clinical latency accompanied by ongoing viral replication. Examples are Visna-Maedi in sheep, simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV) (Dimmock, Easton et al. 2007).

Virions of HIV-1 consist of a lipoprotein membrane, enveloping a matrix protein and within that a capsid protein that houses 2 copies of HIV-1 ribonucleic acid (RNA). Incorporated in the membrane are glycoprotein complexes, composed of trimers of glycoprotein (gp) gp120, which is external, and gp41 which spans the membrane. gp120 can be detached easily as it is only weakly bound to gp41 and hence is present free in the serum of HIV-1-seropositive individuals (Oh, Cruikshank et al. 1992). Also within the virion are 3 viral enzymes required for replication; a reverse transcriptase (RT), an integrase and a protease (Dimmock, Easton et al. 2007).

The viral genome contains 3 major genes; gag (group antigen), pol (polymerase) and env (envelope) flanked by long terminal repeat (LTR) regions at the 5' and 3' ends of the RNA. HIV-1 gag codes for the capsid, env for the glycoproteins and pol for the enzymes (reverse

transcriptase, protease, integrase and RNase H). The LTR enables integration of the viral genome into host DNA. In addition 6 "accessory" genes, so called as they have been found not to be essential for replication *in vitro*, are nef (negative factor), rev (regulator of expression of virion proteins), tat (trans-activator), vif (viral infectivity factor), vpr (viral protein R), vpu (viral protein U) (Dimmock, Easton et al. 2007). Tat encodes a transcriptional regulator of the LTR, and with rev is also responsible for regulation of viral gene expression and RNA transport to the cytosol after viral gene transcription (reviewed in (Karn and Stoltzfus 2012)). Nef has functions that interfere with aspects of the antiviral immune response including T cell activation and the surface expression of CD4 and MHC class I (Kerkau, Bacik et al. 1997). These functions help HIV-1 evade immune responses such as CD8⁺ T cell killing, and deletions in nef have been associated with long term non progression (LTNP) of HIV-1 (Kirchhoff, Greenough et al. 1995; Learmont, Geczy et al. 1999). vpr allows viral replication in non dividing cells such as the macrophage (reviewed in (Kogan and Rappaport 2011)). A key role of vif is to antagonise the naturally occurring host cell restriction factor apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (APOBEC3G) that would otherwise cause hypermutation of proviral DNA through its cytidine deaminase activity (Sheehy, Gaddis et al. 2002). This results in inhibition of replicative capacity but vif induces ubiquitination and proteasomal degradation of APOBEC3G allowing the virus to escape this restriction. vpu antagonises another host viral restriction factor known as tetherin. Without vpu, new cytosolic HIV-1 virions are bound by tetherin, which prevents budding and release of the particle from the cell (Neil, Zang et al. 2008).

1.4.3 HIV-1 replication

As with other retroviruses, HIV-1 lacks the capacity to replicate its RNA without utilizing the necessary host cell machinery inside the cell. The primary receptor necessary for HIV-1 attachment and entry is the CD4 molecule, to which the virus binds using gp120. The binding of gp120 to CD4 generates a conformational change in its V3 loop to promote co-receptor

binding. As a consequence a conformational change in gp41 is induced which enables it to insert into the host cell, achieve membrane fusion and deliver the viral capsid into the cytosol (Dragic, Litwin et al. 1996; De Clercq and Schols 2001). However, CD4 alone is not sufficient for HIV-1 binding and entry and a chemokine receptor, either CCR5 or CXCR4 acting as a second viral co-receptor, must be present (Gorry and Ancuta 2011). Different viral isolates will preferentially use one or the other of these, or sometimes both, and can be differentiated according to their propagation efficiency in macrophage and lymphocyte based cell-line cultures; monocyctotropic isolates use CCR5 (M-tropic), T cell tropic isolates use CXCR4 (T-tropic). A common deletion in the CCR5 gene, $\Delta 32$, has been found to render the 1% of individuals carrying homozygous deletions resistant to infection with CCR5 utilising HIV-1 infection, while heterozygous carriers are more likely to be long term non progressors (Dean, Carrington et al. 1996; Liu, Paxton et al. 1996). M-tropic viruses are the predominant isolates until advanced stages of infection when CXCR4 using T-tropic strains become more frequent, possibly as these are better adapted to infecting naive T cells and are thus better able to replicate when memory T-cells are significantly depleted as they expand the range of T-cells within which they can replicate (reviewed in (Gorry and Ancuta 2011)).

Within the cytosol the viral enzyme reverse transcriptase converts viral RNA into proviral double stranded DNA. Using a mechanism common to most retroviruses vpr, integrase and host proteins are formed into a 'pre-integration complex' which is transported to the nucleus, allowing insertion of the proviral DNA into the host genome (Dimmock, Easton et al. 2007). Successful integration of the proviral DNA depends on the host cell being activated. Activation is also required for the subsequent transcription of integrated viral DNA, at least in part because NF- κ B activation and binding to the LTR is needed for viral transcription (reviewed in (Bieniasz 2012)). Activation occurs through stimuli such as T cell receptor engagement by antigen, activation during infection (particularly for macrophages) or by gp120 binding to CD4. If the cell remains un-activated, as can occur with macrophages or quiescent T cells,

integration may not take place and these cells with un-integrated DNA may persist for extended periods, so called pre-integration latency (Chun, Carruth et al. 1997). While pre-integration latency contributes to a reservoir of inducible productive infection when the cell becomes activated, it has been demonstrated that in T cells this form of proviral DNA is highly labile so it is probable that the major contributor to the RNA reservoir is latent integrated proviral DNA (Zhou, Zhang et al. 2005). In the activated cell viral transcription can begin, initially with the production of proteins coded by rev and tat, the HIV-1 regulatory proteins, which in turn facilitate transcription of precursors of structural and enzymatic proteins from gag, pol and env. HIV-1 protease now starts to cleave these precursor proteins both as the virus is assembled and after budding. During assembly, viral particles move to the surface membrane and new virions are formed as the cell membrane buds off, incorporating viral particles within the viral envelope that are derived from the host cell membrane (NIAID 2012). In untreated HIV-1 infection approximately 10^9 virus particles are produced a day (Perelson, Neumann et al. 1996). Retroviral transcription is inherently error prone and 5 -10 transcription errors occur per replication cycle in T cells, with an even greater rate in macrophages (Preston, Poiesz et al. 1988; Levy, Aldrovandi et al. 2004). The resulting viral progeny are thus highly varied and include many replication incompetent virions but also others with mutations that, by chance, confer resistance to the selective pressure exerted by the immune system (viral escape mutants) or antiretroviral drugs.

1.4.4 The immune response to HIV-1 infection

In the majority of cases HIV-1 is acquired via the genital or rectal mucosa as stated before. While the inoculum may contain many virus particles it is likely that, for most, infection is initiated by only a single 'founder' virus (Keele, Giorgi et al. 2008). Initial mucosal infection involves macrophages, dendritic cells (including Langerhans cells), and CD4⁺ T cells (Morrow, Vachot et al. 2007). From here antigen presenting cells carrying HIV-1 migrate to draining lymphoid tissues to present antigen to the adaptive cellular immune system (Lore, Smed-

Sorensen et al. 2005). However, HIV-1 is well adapted to the environment it encounters within the lymphoid tissue; here there is close cell to cell contact between antigen presenting cells and CD4⁺ T cells, the latter having become activated by the locally high levels of pro-inflammatory cytokines, and HIV-1 is able to transfer directly from the antigen presenting cell to infect the T cell. With the infection of CD4⁺ T cells viral production increases rapidly and this is reflected in a steep increase in the plasma viral load (Fauci, Pantaleo et al. 1996).

Meanwhile, and still early on in the course of infection, resting memory T cells and macrophages are becoming non-productively infected, creating the reservoir of latent HIV-1 as discussed previously (Chun, Engel et al. 1998; Sloan and Wainberg 2011).

In most cases, the HIV-1 viral load will begin to drop from its peak within a few weeks of infection. This is associated with the development of a CD8⁺ T cell response as virus-infected cells are recognised and this response is thought to result in elimination of these cells (reviewed in (Fauci, Pantaleo et al. 1996)). Additionally, the activated CD8⁺ T cells produce cytokines such as IFN- γ , that render nearby cells resistant to productive viral infection, and the chemokines macrophage inflammatory protein-1 alpha (MIP-1 α), MIP-1 β and regulated on activation normal T expressed and secreted (RANTES) which are natural ligands of the CCR5 receptor and block HIV-1 gp120 binding (Cocchi, DeVico et al. 1995). The strength of these responses will determine the decline in viraemia and thus the viral set point (Fauci, Pantaleo et al. 1996). The failure to achieve total control is in part due to the appearance of viral escape mutants and in part due to down regulation of MHC I by nef leading to inefficient presentation of epitopes on the infected cells in the absence of the appropriate class I stimulus (Kerkau, Bacik et al. 1997). Individuals differ in the strength of their CTL responses, by virtue of their differing HLA alleles and capacity to present viral antigen. These differences are associated with varying clinical courses of infection; thus LTNP demonstrate greater numbers of HIV-1 specific CD8⁺ T cells with broader specificity, a lower or even undetectable viral set point and

preservation of CD4⁺ T cell counts, which ensures they do not develop the clinical features associated with significant immunosuppression (Harrer, Harrer et al. 1996).

Individuals with HIV-1 also develop a vigorous antibody response to HIV-1 (Gurtler 1996).

Immunoglobulin and complement form immune complexes with circulating virus which become trapped in the reticulo-endothelial system and further contribute to the control of the initial viraemia (reviewed in (Fauci, Pantaleo et al. 1996)). However, neutralizing antibody (NAb) responses with the potential to prevent HIV-1 binding and fusion with target cells take several months to develop and are too slow to prevent infection becoming established.

Additionally, both heavy glycosylation of HIV-1 env and the trimerisation of the env gp120-gp41 complex serve to shield epitopes from antibody binding (reviewed in (Burton, Desrosiers et al. 2004)). LTNP generally have broader and more persistent HIV-1 neutralising responses but it is not clear if these are causal in slowing progression (Carotenuto, Looij et al. 1998). A more fundamental problem is that genetic changes in HIV-1 and the development of escape mutants make the antibody response ineffectual (Richman, Wrin et al. 2003). These difficulties also present major hurdles to producing an effective neutralising antibody vaccine against HIV-1 and efforts continue to find conserved, yet accessible epitopes to direct antibody against (Burton, Desrosiers et al. 2004; Johnston and Fauci 2007).

1.4.5 Consequences of HIV-1 for the immune system

The hallmark of HIV-1 infection is a progressive decline in peripheral blood CD4⁺ T cell number. There is an initial fall both in mucosal and plasma CD4⁺ T cell counts during the peak viraemia that follows initial infection with variable recovery of the circulating counts, which may only be partial in some patients. The counts then slowly decline during the clinically latent phase and with the progressive loss of control of viral replication, CD4⁺ T cell decline accelerates. At the early stages it is memory CD4⁺ T cells that are lost, particularly at mucosal sites but later naive subsets are also affected (Roederer, Dubs et al. 1995). Although HIV-1 infection is directly

cytotoxic to CD4⁺ T cells it is indirect factors that are chiefly responsible for their loss. Only 0.01–1% of circulating CD4⁺ T cells are directly infected with HIV-1 but a much larger proportion become activated (as a result of the generalised immune activation caused by HIV-1) and these undergo activation induced cell death by apoptosis (Haase, Henry et al. 1996; Patki, Zielske et al. 2000). Increased expression of adhesion molecules in the activated lymphatic system leads to sequestration of CD4⁺ T cells, which may be reflected clinically as a persistent generalised lymphadenopathy (Bucy, Hockett et al. 1999). CD4⁺ T cell production from the bone marrow is reduced at later stages of disease either because of HIV-1 infection of progenitor cells or opportunistic infections such as *Mycobacterium avium* complex (MAC). Furthermore, there is evidence that CD4⁺ T cell development in the thymus may be impaired as those who fail to restore CD4 counts on HAART have been found to have thymic failure (Teixeira, Valdez et al. 2001). Thus the decline in CD4⁺ T cell counts reflects the combinatorial effects of decreased production, sequestration and increased destruction due to direct and indirect effects of HIV-1 (reviewed in (Douek 2003)).

HIV-1 infection is also associated with a generalized activation of the immune system which has been demonstrated to correlate with clinical progression; there are increased levels of activation markers on CD8⁺ and CD4⁺ T cells and B cells and increased levels of pro-inflammatory cytokines TNF- α , IL-1 β and IL-6 and chemokines MIP-1 α , MIP-1 β and RANTES (reviewed in (Appay and Sauce 2008)). In fact, the clinical usefulness of CD4 count in predicting prognosis notwithstanding, immune activation is likely to be the key driver of pathogenesis in HIV-1. For instance, CD38 expression on CD8⁺ T cells predicts the CD4⁺ T cell decline (Giorgi, Liu et al. 1993). In primate SIV infections, rhesus macaques have high levels of immune activation and progress to AIDS whereas sooty mangabes do not demonstrate comparable immune activation and show no CD4⁺ T cell decline or disease progression (Silvestri, Sodora et al. 2003). The drivers of immune activation in HIV-1 are not fully understood but are likely to include; translocation of microbial products such as LPS from a CD4⁺ T cell depleted intestinal mucosa;

loss of regulatory responses following Treg (regulatory T cell) depletion; innate immune stimulation by viral gene products such as gp120, nef and tat; and autoactivation through molecular mimicry between HIV-1 and self proteins such as HLA, as exemplified by epitopes from gp120 C2 and C5 domain peptide binding regions which can stimulate autoreactive T lymphocyte activation (reviewed in (Cadogan and Dalglish 2008)). As stated above one of the consequences is activation induced CD4⁺ T cell loss. The influx of pathogen associated microbial proteins like LPS from the gut and elevated levels of pro-inflammatory cytokines also induce up-regulation of programmed death (PD)-1 on monocytes which, following ligation by PD-L1 expressed on other cell types, produce IL-10 and reduced CD4⁺ T cell expansion (Said, Dupuy et al. 2010). Activation of CD8⁺ T cells leads to their differentiation into memory cells, but the repeated antigenic stimulation of HIV-1 specific CD8⁺ cells, which otherwise control the virus, results in exhaustion of their replicative capacity again through up-regulation of PD-1 expression. This also correlates positively with higher viral load and inversely with CD4⁺ T cell counts (Day, Kaufmann et al. 2006). Meanwhile, inflammation induced fibrosis of lymphatic tissue, involution of the thymus, and loss of bone marrow progenitor pool capacity means there is also a reduction in naïve T cell regeneration to replace these depleted and exhausted cells (reviewed in (Appay and Sauce 2008)).

HIV-1 infection is also associated with chronic humoral immune system activation. It is characterised by hyperactivation and polyclonal expansion of B cells, that react to both viral and non viral antigens, which results in generation of high levels of non specific antibodies and produces hypergammaglobulinaemia (Shirai, Cosentino et al. 1992; Shen and Tomaras 2011). The hypergammaglobulinaemia persists even in LTNP (Titanji, De Milito et al. 2006). Despite the enhanced activation, there is a paradoxical reduction in antigen specific memory B cell numbers, which correlates with the decline in CD4⁺ T cell numbers and the B cells are hyporesponsive, as illustrated by the failure to develop protective responses to immunization (Steinhoff, Auerbach et al. 1991; Moir, Malaspina et al. 2001).

Thus HIV-1 infection elicits an immune response that is able to partially control the early viraemia. However, a lack of broad NAb responses, the high mutation rate of the virus favouring escape from the selective pressure of the immune system, down regulation of HLA molecules and viral latency within quiescent T cell and macrophage reservoirs ultimately contribute to a failure to eliminate the virus. Persistent viral replication and a vigorous but incompletely effective immune responses is associated with pathological chronic activation of the immune system which through CD4⁺ T cell loss and CD8⁺ T cell exhaustion, as well as a hyporesponsive humoral response, underlies the immunopathogenesis of HIV-1 infection, the clinical progression to AIDS and ultimately death.

1.5 Macrophages in HIV-1

Tissue macrophages can be infected by HIV-1. Macrophage tropism is a common feature of many-lentiviruses, and contributes significantly to the pathogenesis of this virus family. In sheep, Visna-Maedi predominantly infects macrophages, rather than T lymphocytes, yet causes an AIDS like wasting syndrome (Gendelman, Narayan et al. 1986). Similarly, SIV infected macrophages sustain high virus loads and pathogenic infection whereas SIV strains that are unable to productively infect macrophages fail to cause high level viraemia or pathogenesis in rhesus macaques (Whetter, Ojukwu et al. 1999; Igarashi, Brown et al. 2001). In humans, macrophages in the genital and rectal mucosa are a key cell where initial HIV-1 infection becomes established (reviewed in (Meltzer and Gendelman 1992; Morrow, Vachot et al. 2007)). The virus binds and fuses with the cell by means of the same gp120/gp41 interaction with CD4 and CCR5 as described previously for CD4⁺ T cells. These HIV-1 infected macrophages are an important source of virus replication and contribute to CD4⁺ T cell infection with HIV-1 (Eckstein, Sherman et al. 2001; Swingler, Brichacek et al. 2003; Groot, Welsch et al. 2008). They also contribute to CD4⁺ T cell depletion (Badley, Dockrell et al. 1997; Orlikowsky, Wang et al. 1997). Similarly HIV-1 infected microglia, the tissue macrophages of the central

nervous system, are strongly implicated in the death of neuronal cells in the pathogenesis of HIV encephalopathy (HIVE) (Aquaro, Calio et al. 2002).

Given their prominent role in HIV-1 pathogenesis, a surprisingly small proportion of macrophages are actually infected with HIV-1 *in vivo*. Highest infection rates have been reported in the brain at 1-10 per 100 cells (Koenig, Gendelman et al. 1986; Stoler, Eskin et al. 1986), with lower estimates of 60 per 100,000 cells in intestinal lamina propria in AIDS (Smith, Fox et al. 1994). In the lung as few as 3 per 100,000 AM have been observed to contain integrated HIV-1 DNA (Lewin, Kirihara et al. 1998). However, the number infected and the overall level of HIV-1 RNA or DNA detected from purified *ex vivo* AM may be greater with advanced HIV-1 disease, particularly with lymphocytic interstitial pneumonitis (Chayt, Harper et al. 1986; Jeffrey, Israel-Biet et al. 1991; Clarke, Gates et al. 1994; Sierra-Madero, Toossi et al. 1994; Lewin, Kirihara et al. 1998). *In vitro* studies find that monocytes are less permissive to HIV-1 infection possibly as a consequence of type 1 IFN responses to HIV-1 (which are not seen in macrophages), that upregulate host restriction factors such as TRIM5 α or APOBEC3G (Stremlau, Owens et al. 2004; Williams and Burdo 2009). Nevertheless, HIV-1 DNA has been detected in up to 125 per 100,000 peripheral blood monocytes, verifying that some monocytes are also infected (McElrath, Pruett et al. 1989; Spear, Ou et al. 1990; McElrath, Steinman et al. 1991; Lewin, Kirihara et al. 1998).

In the modern era, with widespread availability of HAART, many research groups have focused on an additional pathenogenic role of the macrophage as a reservoir of HIV-1; as with resting T lymphocytes, non replicating HIV-1 can persists within macrophages even with antiretroviral therapy (Aquaro, Calio et al. 2002; Crowe, Zhu et al. 2003; Contreras, Lenasi et al. 2006).

Although there is some evidence that antiretroviral compounds may not penetrate macrophages as effectively as T cells, the integrated HIV-1 DNA is untouched by HAART and contributes to the reservoir of HIV-1 (Crowe and Sonza 2000). Furthermore, unlike activated

CD4⁺ T cells, even productively infected macrophages remain relatively resistant to the cytopathic effects of HIV-1 and can harbour infective virions for prolonged periods in their cytoplasm (Meltzer and Gendelman 1992; Sharova, Swingler et al. 2005; Cassol, Alfano et al. 2006). Crucially in HIV-1 infection, macrophages not only fail to die but actually exhibit a prolonged lifespan as a result of specific interactions between the virus, its proteins and the pathways that regulate host cell apoptosis (Lum and Badley 2003). These longer lived HIV-1 infected macrophages are believed to contribute to the slower, second phase of viral decay with HAART following the initial rapid elimination of virus (Perelson, Essunger et al. 1997) and to be one source of re-emergent virus following discontinuation of therapy (Chun, Davey et al. 2000).

1.5.1 Macrophage apoptosis and its circumvention by HIV-1

One option for the macrophage on becoming infected with a pathogen is to trigger its own demise through the induction of apoptosis, and so prevent productive infection (reviewed in (Behar, Divangahi et al. 2010)). There are many examples of pathogens that have evolved counter regulatory measures to undermine this defence strategy. These include intracellular bacteria such as *Mycobacterium tuberculosis* (Behar, Martin et al. 2011) and *Legionella pneumophila* (Banga, Gao et al. 2007) and the protozoal *Leishmania* spp. (Akarid, Arnoult et al. 2004) among others (reviewed in (Ashida, Mimuro et al. 2011)). Viruses, which absolutely rely on the host to support their replication, are particularly adept at preventing apoptosis in a variety of host cells, for instance EBV carries a gene that resembles human Bcl-2 (Vaux, Haecker et al. 1994) while in monocytes/macrophages cytomegalovirus (CMV) upregulates Mcl-1 (Chan, Nogalski et al. 2010). There have been several lines of enquiry into the mechanisms by which HIV-1 perturbs the apoptotic process in macrophages. Unbiased gene expression analyses show that in monocytes and macrophages, in contrast to CD4⁺ T cells, there is upregulation of anti-apoptotic gene expression resulting from differential modulation of p53, TNF- α and extracellular-signal-regulated kinase mitogen-activated protein kinase (ERK

MAPKinase) signalling pathways in response to HIV-1 (Giri, Nebozyhn et al. 2009). This anti-apoptotic gene expression profile is seen both in monocytes/macrophages isolated from HIV-1-seropositive individuals and when healthy MDM are exposed to HIV-1 *in vitro* (Coberley, Kohler et al. 2004; Vazquez, Greenwell-Wild et al. 2005; Giri, Nebozyhn et al. 2009; Van den Bergh, Florence et al. 2010). For example, immediate-early response gene (IEX-1L), Bcl-xL and p21 were upregulated in 7 day MDM by HIV-1_{BAL} (Vazquez, Greenwell-Wild et al. 2005).

A number of mechanistic studies have shown that HIV-1 changes the activity of the Bcl-2 family members with consequences for the regulation of the intrinsic pathway of apoptosis, reflecting the strategies of the herpes viruses above; higher levels of the anti-apoptotic proteins Bcl-2 and Bcl-xL and decreased expression, with or without inactivation, of the pro-apoptotic proteins Bax and Bad have been observed following HIV-1 infection of MDMs (Zhang, Li et al. 2002; Guillemard, Jacquemot et al. 2004). More specifically, *nef* plays a key role inducing Bcl-xL (Choi and Smithgall 2004) and inactivating Bad through hyper-phosphorylation (Wolf, Witte et al. 2001; Olivetta and Federico 2006). Additionally, exogenous *tat* leads to Bcl-2 upregulation (Zhang, Li et al. 2002) and HIV-1 *env*/gp120, through engagement with CCR5 on MDM, upregulates Mcl-1 and Bcl-2-related protein A1 (A1, also known as Bfl-1) transcription (Swingler, Mann et al. 2007). HIV-1 has been demonstrated to activate the phosphoinositide 3-kinase (PI3K)/Akt pathway in primary human macrophages and monocytic cell lines (Chugh, Bradel-Tretheway et al. 2008; Patel, Swan et al. 2009). This pathway regulates survival of differentiated macrophages through Mcl-1 (Liu, Perlman et al. 2001). The advantage for HIV-1 is that by activating PI3K/Akt in an infected macrophage, the cell is rendered resistant to stress induced death (for example from NO) and ongoing HIV-1 replication is enhanced (Chugh, Bradel-Tretheway et al. 2008). Compared with U937 cells U1 cells, a subclone of the promonocytic U937 cell line with inducible integrated proviral HIV-1 DNA, exhibit decreased apoptosis in response to hydrogen peroxide (H₂O₂) or staurosporine (Fernandez Larrosa, Croci et al. 2008) and preservation of the mitochondrial inner transmembrane potential when

treated with TNF- α and cyclohexamide (Pinti, Biswas et al. 2003). HIV-1 also blocks proteasomal activity in MDM (Haorah, Heilman et al. 2004) and while the functional consequences of this have not been explored in depth, impaired proteasomal activity would be predicted to further contribute to the maintenance of rapidly turned over proteins such as Mcl-1 (Nijhawan, Fang et al. 2003).

Extrinsic pathways of apoptosis may also be perturbed by HIV-1. The M-CSF induced signalling pathway can be activated by gp120 and downregulates the TNF-related apoptosis-inducing ligand (TRAIL) receptor TRAIL-R1 (DR4) blocking an extrinsic, death receptor pathway (Swingler, Mann et al. 2007). HIV-1 also interferes with death receptor signalling. Using HIV-1 expressing specific nef mutations it was identified that regions of nef, distinct from those which inactivate Bad as described above, also inhibit apoptosis signal-regulating kinase (ASK)-1 in lymphocytes, a kinase that plays a role in Fas signalling (Geleziunas, Xu et al. 2001; Olivetta and Federico 2006). Monocytes from HIV-1-seropositive individuals show enhanced resistance to the pro-apoptotic stimuli cadmium chloride (which activates p38MAPK, downstream of Fas) and FasL (Giri, Nebozyhn et al. 2009), with the latter effect also demonstrated in U1 cells (Pinti, Biswas et al. 2003). Another mechanism by which HIV-1 may protect against apoptosis is through production of an HIV-1 derived microRNA which downregulates pro-apoptotic host factors (Klase, Winograd et al. 2009).

Despite this evidence of macrophage resistance to apoptosis, in specific circumstances HIV-1 may in fact be responsible for increasing macrophage apoptosis. HIV-1 infected MDM are susceptible to apoptosis when exposed to leucine zipper TRAIL, a potent trimeric form of TRAIL, while mock-infected MDM remain resistant. This is in contrast to the lack of activity observed with soluble TRAIL described by others in response to HIV-1 env / gp120 (Lum, Pilon et al. 2001; Swingler, Mann et al. 2007). Recombinant human (rh) TRAIL was identified by other groups as able to induce apoptosis in MDM infected with env pseudotyped virus or HIV-1

in vitro (Huang, Erdmann et al. 2006; Zhu, Shi et al. 2011). Zhu et al. attributed this susceptibility to apoptosis to down regulation of TRAIL decoy receptors DcR1 and DcR2 and intracellular FADD-like IL-1 β -converting enzyme (FLICE)- inhibitory protein (c-FLIP) a negative regulator of TRAIL-induced signalling (Zhu, Shi et al. 2011). Huang and colleagues reported that HIV-1 infection *decreased* Akt phosphorylation, in contrast to many other reports, thus enhancing rather than reducing apoptosis. Moreover they established that adding constitutively active Akt-1 blocked rhTRAIL-induced apoptosis in HIV-1 infected MDM (Huang, Erdmann et al. 2006). They went further to suggest that HIV-1 mediated Akt inhibition impairs inactivation of the transcription factor FOXO3A leading to upregulation of the pro-apoptotic Bcl-2 family protein PUMA (Cui, Huang et al. 2008). Importantly though, other than the leucine zipper TRAIL study, these studies have shown relatively low levels of apoptosis by widely recognised apoptosis assays and only low levels of fold induction of apoptosis when compared to control macrophages (Lum, Pilon et al. 2001; Huang, Erdmann et al. 2006; Cui, Huang et al. 2008).

Supporting the notion that specific circumstances dictate whether HIV-1 leads to a pro-apoptotic or anti-apoptotic outcome are data from studies using HIV-1 vpr. vpr induces apoptosis in monocytes and promonocytic cell lines (Busca, Saxena et al. 2012). It achieves this when its C terminal region binds to the adenine nucleotide translocator of the inner mitochondrial membrane causing its permeabilisation (Jacotot, Ravagnan et al. 2000). However, once differentiated into macrophages, vpr no longer induces apoptosis and this is attributed to greater levels of anti-apoptotic Bcl-2 family members; Bcl-xL and Mcl-1 (Busca, Saxena et al. 2012). In contrast, resistance to apoptosis in monocytes from asymptomatic HIV-1-seropositive individuals (Elbim, Pillet et al. 1999) and promonocytic U937 cells is relatively transient; have been shown to upregulate Bcl-2 transcription after HIV-1 infection, a response that follows an initial downregulation at the protein level secondary to oxidative stress of initial infection (Aillet, Masutani et al. 1998). More recent work supports the view that

observed differences in apoptotic response may be related to the phase of HIV-1 infection being studied; monocytes from healthy human volunteers were infected with R5 tropic HIV-1 24 hours following isolation and allowed to differentiate for 5 days into MDM in the presence of HIV-1 (to model acute HIV-1 infection) or were isolated from rhesus macaques during the acute phase SIV infection (Laforge, Campillo-Gimenez et al. 2011). The MDM were observed to be sensitised to TRAIL or FasL mediated apoptosis and showed reduced anti-apoptotic Mcl-1 but increased pro-apoptotic Bax and Bak. Significantly though, after the acute phase of infection (14 days) monocytes from the macaques no longer exhibited the pro-apoptotic phenotype (Laforge, Campillo-Gimenez et al. 2011). This dynamic balance of pro and antiapoptotic factors has also been demonstrated in a genomic and proteomic analysis (Brown, Kohler et al. 2008). Two days after MDM infection with R5 tropic virus there was upregulation of pro-apoptotic Bad, Bid, ASK-1 and caspase-7 with downregulation of anti-apoptotic Bcl-2. However, this balance of expression was reversed by seven days post infection (Brown, Kohler et al. 2008).

In summary, HIV-1 infection of the macrophage is central to its pathogenesis and is a mechanism through which HIV-1 can persist during HAART and contribute to the viral reservoir. Furthermore, many lines of enquiry show that perturbation of macrophage apoptosis is a recurring theme in HIV-1 infection that promotes HIV-1 replication and persistence but may have additional consequences for the regular immune function of this cell.

1.6 Invasive pneumococcal disease and pneumococcal pneumonia in HIV-1

1.6.1 The epidemiology of *Streptococcus pneumoniae* infection in HIV-1

There is a clear epidemiological association between pneumococcal disease and HIV. The first signals of an increased incidence of both IPD and pneumococcal pneumonia were reported

within only a few years of the emergence of the HIV pandemic (Polsky, Gold et al. 1986; Witt, Craven et al. 1987). Subsequent studies of individuals living with untreated HIV-1 infection found IPD to be as much as 100 times more common with incidence rates from 1 to 24 per 1000 patient years (py) compared to 0.1 per 1000py in HIV-seronegative individuals in the comparable settings (Janoff, Breiman et al. 1992; Hirschtick, Glassroth et al. 1995; Jordano, Falco et al. 2004; Heffernan, Barrett et al. 2005; Grau, Ardanuy et al. 2009). This relationship is also seen with bacterial meningitis during HIV-1 infection, where there is similarly a greater than 100 fold greater incidence in pneumococcal meningitis in HIV-1-seropositive individuals (Almirante, Saballs et al. 1998). For bacterial pneumonia more broadly, which is most commonly due to *S. pneumoniae* infection, rates are higher in HIV-1-seropositive individuals, for example one study observed 5.5 cases per 100py in HIV-1-seropositive individuals compared with 0.9 per 100py in seronegative controls (Dworkin, Ward et al. 2001). *S. pneumoniae* has consistently been found to be the most common cause of pneumonia not due to *Pneumocystis jirovecii* (PCP); in HIV-1-seropositive individuals the pneumococcus accounts for between 32% and 82% of infections where organisms are isolated, both in the pre- and the post-HAART eras (Polsky, Gold et al. 1986; Witt, Craven et al. 1987; Le Moing, Rabaud et al. 2006; Curran, Falco et al. 2008; Madeddu, Porqueddu et al. 2008). Rates of HIV-1 associated IPD are substantially greater in resource poor settings at 4250 per 100,000py and proportionally more non-pneumonic pneumococcal disease is seen (meningitis, sinusitis or occult bacteraemia)(Gilks, Ojoo et al. 1996; Gordon 2004). These numbers are at least in part due to the higher prevalence of pneumococcal disease, increased rates of HIV-1 in young children and the less developed health infrastructure in these settings.

As with opportunistic infections like PCP, the importance of an intact adaptive immune response to pneumococcal infection is demonstrated by an inverse correlation between the incidence of pneumococcal disease and the absolute CD4 count (Hirschtick, Glassroth et al. 1995). However, whereas the incidence of PCP rapidly declines as CD4 counts improve,

individuals with a normal CD4 count early in the course of HIV-1 infection or following HAART driven recovery still have a significantly elevated risk of IPD compared with the background population (Dworkin, Ward et al. 2001; Barry, Zetola et al. 2006). Again, the risk of bacterial pneumonia in HIV-1 infection mirrors this and is as high as 2.3 episodes per 100py for those with normal range CD4 counts of $>500 \text{ cells}\mu\text{L}^{-1}$ compared with 0.9 per 100py (Hirschtick, Glassroth et al. 1995). Likewise, hospitalisation for non-PCP pneumonia in the latter HAART era (2005-207) was 5.8 fold higher in those with CD4 counts of $>500 \text{ cells}\mu\text{L}^{-1}$ compared with HIV-seronegative controls (Sogaard, Lohse et al. 2008). HAART reduces the incidence of IPD significantly (odds ratios of 0.37 – 0.5) (Dworkin, Ward et al. 2001; Grau, Pallares et al. 2005; Heffernan, Barrett et al. 2005). While it is likely that much of this reduction is secondary to CD4 count recovery, the SMART study, which compared intermittent with continuous HAART, found a 55% increased incidence of IPD and pneumonia in the intermittent therapy arm and this correlated with HIV-1 viral load independently of the CD4 count (Gordin, Roediger et al. 2008).

Thus, both replicating virus and suppressed CD4 counts independently contribute to the risk of pneumococcal disease in HIV-1 but do not explain the entire excess risk. Despite the widespread availability and use of HAART with consequent improvements in average CD4 counts, pneumococcal disease continues to be more common in the current late HAART era; a 35 fold or greater incidence of IPD in HIV-1-seropositive populations (423 v 9 per 100,000py) (Grau, Pallares et al. 2005; Heffernan, Barrett et al. 2005) (Kyaw, Rose et al. 2005), 20 fold as many hospitalisations for bacterial pneumonia (Sogaard, Lohse et al. 2008) and 20 times as many cases of bacterial meningitis (Domingo, Suarez-Lozano et al. 2009) than in those who are seronegative.

A potential explanation for the higher incidence of IPD and pneumococcal pneumonia in HIV-1-seropositive individuals despite HAART would be that other risk factors, not directly related to HIV-1 infection, are over-represented among this population; and that while these additional

factors were always present, it is only since the HAART era that their contribution to IPD risk has become apparent. Intravenous drug use (IVDU) and cigarette smoking, risk factors for IPD, are more common in some HIV-1 cohorts (Mathers, Degenhardt et al. 2008; Tesoriero, Gieryc et al. 2010; Lifson and Lando 2012). IVDU, smoking and alcohol abuse have been identified as additional risk factors for IPD in HAART era HIV-1 cohorts (Grau, Pallares et al. 2005; Heffernan, Barrett et al. 2005; Kyaw, Rose et al. 2005; Gordin, Roediger et al. 2008; Rodriguez-Barradas, Goulet et al. 2008; Sogaard, Lohse et al. 2008). Additionally medical comorbidities (liver cirrhosis, COPD, haematological malignancy, splenectomy) also bring independent risk and appear to be more frequently seen in IPD cases now than the pre HAART era (Grau, Pallares et al. 2005). However, among intravenous drug users HIV-1 infection increases the risk of pneumonia 4 fold even in the absence of AIDS (Selwyn, Feingold et al. 1988; Mientjes, Spijkerman et al. 1996) and in one of the largest comparisons of IPD incidence among HIV-1-seropositive and seronegative individuals in the HAART era fewer than 11% were ever IVDU yet IPD was still substantially more common in the HIV group (Sogaard, Lohse et al. 2008). Intriguingly, mortality rates with IPD or pneumococcal pneumonia in the HAART era have risen, in one report from 9% to 25% (Grau, Ardanuy et al. 2009), and it has been speculated that could be the result of the restoration of a more aggressive immune response (Jordano, Falco et al. 2004).

Taken together, these clinical observations indicate that HIV-1 infection adversely effects the immune response to the pneumococcus, increasing both the risk of infection and potentially also the severity of disease. Furthermore, given that increased susceptibility persists even when CD4 counts are restored with HAART, it is likely that this immune impairment to *S. pneumoniae* is more complex than can be explained by the effects of reduced CD4⁺ T cell function alone or the over representation of other risk factors for *S. pneumoniae* such as smoking and IVDU. This strongly suggests the presence of additional, and as yet unrecognised, factors underlying the pathogenesis of IPD in HIV-1-seropositive individuals receiving HAART.

1.6.2 HIV-1 and *Streptococcus pneumoniae* pathogenesis

1.6.2.1 Pneumococcal carriage in HIV-1

Pneumococcal pneumonia and IPD is preceded by a period of bacterial colonisation of the nasopharynx, although for some strains causing IPD the upper airway interaction is transient and the strains rarely cause asymptomatic colonisation. It has been suggested that the greater risk of IPD might be a consequence of increased carriage rates in HIV-1-seropositive individuals, whereby HIV-1 associated immune defects impair *S. pneumoniae* clearance from the nasopharynx. An alternative possibility is that different health seeking behaviour among those with HIV-1, such as more frequent hospital visits, might involve greater exposure to *S. pneumoniae* and consequently an increased likelihood of carriage.

The strength of the evidence for increased colonization rates of *S. pneumoniae* among HIV-1-seropositive individuals varies. Higher colonisation rates have been reported for HIV-1-seropositive children up to 5 years old at 76% versus 66% for controls in Kenya (Abdullahi, Karani et al. 2012), while lower rates have been observed in younger (< 6 months old) HIV-1-seropositive infants in Tanzania (Kinabo, van der Ven et al. 2013). However, the lack of well controlled epidemiological carriage surveys makes it impossible to draw firm conclusions about the effect of HIV-1 on *S. pneumoniae* colonisation in the African paediatric population, where carriage of *S. pneumoniae* is particularly high anyway in children under 5 years (Hill, Akisanya et al. 2006). There are a greater number of studies in adults with HIV-1. Two studies from the pre-HAART reported no significant difference in pneumococcal carriage rates between HIV-1-seropositive and adults in the USA. However, the numbers were small and both showed trends towards higher carriage rates in HIV-1 than control individuals with 14% versus 9% in one and 20% versus 10% in the other, respectively (Janoff, O'Brien et al. 1993; Rodriguez-Barradas, Tharapel et al. 1997). A study from Nairobi, Kenya observed 28% carriage in HIV-1-seropositive adults compared to 16% in controls (Paul 1997). These are similar to carriage rates detected in adults with both HIV-1 and a previous history of IPD in Uganda

(French, Nakiyingi et al. 2000). Reports of two Malawian adult cohorts with no previous IPD history and asymptomatic, WHO Stage I HIV-1 observed lower carriage rates at 14% and 19%, which were not significantly different from the carriage rates in the respective seronegative control groups (16% and 13%) (Glennie, Sepako et al. 2011; Glennie, Banda et al. 2013). By contrast, symptomatic HIV-1-seropositive individuals with advanced, WHO stage IV HIV-1 infection had considerably higher carriage rates at 38%, implying the difference between the Kenyan and Malawian studies may be due to the stage of HIV-1 clinical disease progression (Glennie, Banda et al. 2013). In keeping with this, a study among HIV-1-seropositive South African mineworkers, where the median CD4 was $290 \text{ cells}\mu\text{L}^{-1}$, found that lower CD4 counts were also associated with increased carriage (Pemba, Charalambous et al. 2008). Overall *S. pneumoniae* carriage rates, at 8.8%, were lower for this cohort but no HIV-seronegative control group was reported (Pemba, Charalambous et al. 2008). Notably, the carriage rates in the mineworkers roughly doubled for those living with a child or reporting recent hospitalisation (Pemba, Charalambous et al. 2008), in keeping with similar evidence from non HIV-1 studies (Hendley, Sande et al. 1975). Importantly colonisation in the Glennie study from Malawi was not found to decrease in those established on ART for 6-12 months (33%) or even 18 months (52%), despite a rise in median CD4 counts from $218 \text{ cells}\mu\text{L}^{-1}$ to $328 \text{ cells}\mu\text{L}^{-1}$. Furthermore, HIV-1-seropositive individuals carried a broader range of both invasive and non-invasive serotypes compared to controls (Glennie, Banda et al. 2013). In contrast, carriage rates among a Brazilian cohort of HIV-1-seropositive adults did not correlate directly with CD4 count or viral load, but were lower in those individuals that had been stable on ART for a year or longer (Nicoletti, Brandileone et al. 2007). These apparently different findings with may reflect that this Sao Paulo based cohort had earlier access to ART than the Malawi cohort so were less likely to suffer advanced HIV-1 before ART; more than two thirds had CD4 counts greater than $200 \text{ cells}\mu\text{L}^{-1}$ and for 30% counts were greater than $500 \text{ cells}\mu\text{L}^{-1}$ (Nicoletti, Brandileone et al. 2007).

Possible immune mechanisms that could underlie differential carriage rates with HIV-1 have also been investigated. T cell mediated immunological memory has been seen to be relevant to protection against colonisation in HIV-seronegative populations (Mureithi, Finn et al. 2009), as discussed already. The findings that T cells from the peripheral blood of colonised HIV-1-seropositive individuals demonstrate impaired pneumococcal antigen-specific proliferation, and a defect in pneumococcal-specific T-cell IFN- γ production, compared to HIV-seronegative controls suggest that impaired T cell immunity may in part be responsible for loss of control of colonisation (Glennie, Banda et al. 2013). Glennie and colleagues have shown reduced pneumococcal specific memory T cell responses in the HIV-1-seropositive cohort, with senescence of the functional effector memory subset and decreased proliferation of the central memory population from which these cells would ordinarily be replenished (Glennie, Sepako et al. 2011).

Taken together, these data show that carriage is increased in some HIV-1-seropositive populations and that there is a relationship with immune impairment. Additionally increased exposure to *S. pneumoniae*, be it in hospital, through contact with young children or simply from living in a region with high prevalence of *S. pneumoniae* disease like sub-Saharan Africa, also contributes to *S. pneumoniae* carriage in those with HIV-1. However, though it remains unclear whether the immune restoration with ART is or is not sufficient to allow clearance of colonising *S. pneumoniae*, it seems unlikely that levels of colonisation are sufficiently greater than those of seronegative individuals to adequately explain the excess risk of IPD described in HIV-1-seropositive individuals with good CD4 recovery on ART, particularly in regions where pneumococcal carriage prevalence in the general population is not as high as in Africa.

1.6.2.2 Immunoglobulin, HIV-1 and *Streptococcus pneumoniae*

As already outlined, humoral immunity plays an important role in the defence against *S. pneumoniae* and in HIV-1 infection there is B cell hyperactivation, a non-specific

overproduction of antibody and reduced antigen specific memory B cell numbers. Reduced titres of anti-pneumococcal capsular polysaccharide IgG have been found both in the serum (Janoff, O'Brien et al. 1993; Carson, Schut et al. 1995; Janoff, Fasching et al. 1997; Titanji, De Milito et al. 2006) and in response to *ex vivo* B cell stimulation with *S. pneumoniae* polysaccharide antigen in primary and chronic HIV-1 infection (Titanji, De Milito et al. 2006). Of interest, some type-specific IgG have been found to be elevated in the serum of HIV-1-seropositive Ugandans (type 3 and type 9) (Takahashi, Oishi et al. 2003) and both the serum and BAL fluid of Malawians with HIV-1 compared with seronegative controls (*S. pneumoniae* type 1) (Eagan, Twigg et al. 2007). However, irrespective of the absolute levels of IgG, impaired functional opsonic activity and opsonophagocytic activity of IgG from both lung and blood have been described (Eagan, Twigg et al. 2007).

HIV-1-seropositive individuals do mount significant antibody responses to PPV23, but these are neither as broad nor as high as those in seronegative controls (Carson, Schut et al. 1995; Janoff, Fasching et al. 1997; French, Gilks et al. 1998). The antibody responses are greater in those with higher CD4 at time of vaccination but decline over time and remain abnormal, even in individuals receiving ART, especially in those with lower CD4 counts (Hung, Chang et al.). A meta-analysis of prospective, randomised controlled PPV23 trials in developed settings during the pre-HAART era did not show any protective benefit against pneumococcal pneumonia in HIV-1-seropositive subgroups in contrast with HIV-seronegative groups (Fine, Smith et al. 1994). However, retrospective case-control studies did suggest some benefit of PPV23 in HIV-1 in preventing IPD (including pneumococcal pneumonia) (Dworkin, Ward et al. 2001) (Breiman, Keller et al. 2000). Some HAART era, observational PPV23 studies have also found reduced incidence of IPD (including pneumococcal pneumonia) (Penaranda, Falco et al. 2007) and also, surprisingly, reduced incidence of pneumonia in general even though the vaccine is not generally thought to reduce pneumonia but rather to mainly effect IPD in at risk groups (Rodriguez-Barradas, Goulet et al. 2008; Teshale, Hanson et al. 2008). Reductions in IPD

severity and mortality in HIV-1-seropositive adults who received PPV23 are also reported (Imaz, Falco et al. 2009). Currently PPV23 is used in HIV-1 if the CD4 count is $> 200 \text{ cells}\mu\text{L}^{-1}$ (Geretti, Brook et al. 2008). Of particular importance is the discovery that despite developing raised anti-pneumococcal titres post PPV23, Ugandan adults with HIV-1 were not protected from IPD, but more susceptible to all cause pneumonia (French, Nakiyingi et al. 2000). The reason for the excess disease with vaccination, which was still apparent in extended follow up of the cohort, is not clear. One postulated mechanism is that there was vaccine induction of polysaccharide responsive B-cell clones leading to exhaustion and a subsequent selective defect in pneumococcal immunity following re-challenge, reflecting the hyperactivity of B lymphocytes and loss of antigen specificity in HIV-1 in general described above (Brichacek, Swindells et al. 1996; French, Nakiyingi et al. 2000; Watera, Nakiyingi et al. 2004).

In adults with HIV-1, PCV7 has been shown to produce similar levels of anti- *S. pneumoniae* antibody to PPV23 (Feikin, Elie et al. 2001) (Miuro, Kayhty et al. 2005). The increase in IgG is not as large as in HIV-seronegative individuals, particularly when CD4 cell counts are low but, unlike PPV23, vaccination also results in good mucosal responses in the lung as demonstrated by increasing capsule specific IgG levels in BAL fluid at 1 and 6 months (Gordon, Kayhty et al. 2007). Prime-boost strategies with PCV7 then PPV23 further strengthen antibody responses in adults (Kroon, van Dissel et al. 2000; Lesprit, Pedrono et al. 2007). Significantly, a 2 dose strategy of PCV7 has been shown to significantly reduce recurrent pneumococcal disease caused by vaccine serotypes in HIV-1-seropositive adults in Malawi by 75% (French, Gordon et al. 2010). In this study half of IPD events began as pneumonia (as in the PPV23 trial) which may imply that PCV7 protection came through greater induction of mucosal immunity than was the case with the PPV23 trial (French, Nakiyingi et al. 2000). Impaired IgG response to pneumococcal conjugate vaccination is also seen in HIV-1-seropositive children (Madhi, Adrian et al. 2007). Nevertheless they too can still acquire protection; a PCV9 vaccine reduced IPD incidence by 53% in HIV-1-seropositive children in South Africa (Klugman, Madhi et al. 2003),

albeit that efficacy against vaccine serotypes was lower than in HIV-seronegative children and there was no evidence of any reduction against radiologically confirmed pneumonia. This was disappointing since one of the benefits of PCV in comparison to PPV, at least in children, has been a reduction in pneumonia, presumably because of the induction of mucosal immunity (Madhi, Levine et al. 2008).

The levels and protective efficacy of antibody to protein rather than capsular targets on *S. pneumoniae* such as pneumococcal surface protein A (PspA) or pneumolysin (Ply) have also been investigated in HIV-1; serum titres of anti-Ply IgG and BAL titres of both anti-Ply IgG and anti-PspA IgG are higher in HIV-1-seropositive patients compared to HIV-seronegative controls (Etuwewe, Swann et al. 2009; Collins, Batrawy et al. 2013). The higher levels may represent repeated antigenic stimulation due to greater carriage of *S. pneumoniae* and, for the BAL compartment at least, were not a consequence of the non-specific systemic polyclonal hypergammaglobulinaemia seen in HIV-1 as ratios of *S. pneumoniae* specific IgG were still high (Collins, Batrawy et al. 2013). Thus, as seen with IgG directed against capsular antigen, apparently appropriate immunoglobulin levels do not correlate with protection from clinical disease.

Overall, these studies in HIV-1-seropositive individuals imply that while the humoral immune system retains capacity to produce anamnestic responses to *S. pneumoniae*, either a functional or quantitative (at least for polysaccharide responses) problem persists with the immunoglobulin. It is also likely that the humoral responses to pneumococcal protein and capsular antigens are not the only determinant of IPD risk in HIV-1.

1.6.2.3 *Streptococcus pneumoniae* phagocytosis and killing in HIV-1

Successful clearance of *S. pneumoniae* from the lung involves phagocytosis by AM and, as discussed when they are overwhelmed, by neutrophils. HIV-1 can affect phagocytosis both with and without the presence of opsonins, including immunoglobulin; reduced non-opsonic

ingestion has been observed for *Pneumocystis jirovecii* by AM from HIV-1-seropositive individuals in association with down-regulation of the mannose receptor (Koziel, Eichbaum et al. 1998). *In vitro*, reduced expression of the macrophage mannose receptor on macrophage cells line is mediated by HIV-1 nef and tat (Caldwell, Egan et al. 2000; Vigerust, Egan et al. 2005). Opsonic, complement-mediated phagocytosis is has also been seen to be less efficient, as demonstrated in directly HIV-1 infected MDM (Azzam, Kedzierska et al. 2006). In addition, impairment of immunoglobulin mediated, opsonic phagocytosis in HIV-1 has also been observed (Crowe, Vardaxis et al. 1994; Leeansyah, Wines et al. 2007) and appears to be related to dysfunction of FcγR signalling or Fcγ subunit expression rather than macrophage surface expression of Fcγ receptors *per se* (Capsoni, Minonzio et al. 1994; Kedzierska, Vardaxis et al. 2001; Kedzierska, Ellery et al. 2002). However, none of these studies of phagocytosis in HIV-1 have specifically looked at *S. pneumoniae* phagocytosis. It appears that for macrophages at least, HIV-1 does not result in impaired uptake of *S. pneumoniae*; *ex vivo*, AM from HIV-1-seropositive subjects were as efficient at phagocytosis of *S. pneumoniae* opsonised with IgG from healthy, PPV23 vaccinated individuals as AM from HIV-seronegative controls (Gordon, Molyneux et al. 2001). This may be explained by the fact that although the mannose receptor and FcγR1 and FcγRIII receptors are impaired in HIV-1 infection, these are not critical to the phagocytosis of *S. pneumoniae*, which can be internalised by other routes, principally FcγRIIa (Endeman, Cornips et al. 2009), the function of which appears preserved during HIV-1 infection (Kedzierska, Ellery et al. 2002).

Following internalization by the macrophage, the phagosome and lysosome fuse to form a phagolysosome, where *S. pneumoniae* are killed, as previously described. Defects in microbial killing by macrophages have been demonstrated in HIV-1 infection but not consistently, which may be due to variation in the type of pathogen, macrophage model and strain of virus studied (Nottet, de Graaf et al. 1993; Cameron, Granger et al. 1994; Gordon, Gordon et al. 2007) (Eales, Moshtael et al. 1987; Baldwin, Fleischmann et al. 1990; Biggs, Hewish et al. 1995;

Chaturvedi, Frame et al. 1995; Reardon, Kim et al. 1996; leong, Reardon et al. 2000). *In vitro*, gp120 can inhibit phagolysosomal fusion (Moorjani, Craddock et al. 1996) and nef similarly inhibits autophagosome-lysosome fusion (Kyei, Dinkins et al. 2009). In circumstances where IL-10 is increased, driven by soluble HIV-1 proteins such as gp120, tat or vpr, macrophage ROS production has been shown to be reduced and result in impaired killing of *P. jirovecii* (Muller, Rollag et al. 1990; Koziel, Li et al. 2000). Importantly however, no impairment of *S. pneumoniae* killing has been reported in macrophages.

As discussed already, the capacity of the macrophage to control pneumococcal infection can be overwhelmed and necessitate a neutrophil response to clear infection. Neutrophil counts in the peripheral blood are often reduced in HIV-1 infection (Brettle 1997; Kuritzkes 2000). In AIDS opportunistic disease and suppression of bone marrow function both contribute to reduced production. More generally, there is accelerated loss due to either antibody mediated destruction or increased apoptosis, in part mediated by nef and associated with enhanced expression of Fas (Pugliese, Cantamessa et al. 1999; Salmen, Teran et al. 2004). Of note is the finding that neutrophil apoptosis is accelerated in pathenogenic (Rhesus macaque) but not non-pathenogenic (African green monkey) SIV infection (Elbim, Monceaux et al. 2008). Chemotaxis and activation of neutrophils may also be impaired as in HIV-1 neutrophils show reduced expression of the complement 5a receptor, reduced IL-8 and altered responsiveness to IL-8, potentially affecting degranulation and chemotaxis (Meddows-Taylor, Martin et al. 1999; Meddows-Taylor, Pendle et al. 2001). However, the literature is inconsistent with respect to alterations in neutrophil phagocytic function and respiratory burst following HIV-1 infection, which have been described as reduced, normal or increased, and as with macrophages, observations may depend on the stage of HIV, neutrophil model employed and the pathogen in question (Lazzarin, Uberti Foppa et al. 1986; Baldwin, Gasson et al. 1988; Bandres, Trial et al. 1993; Dobmeyer, Raffel et al. 1995; Munoz, Salmen et al. 1999). Neutrophil microbicidal killing has been observed to be reduced in one model that could be reversed with

G-CSF therapy for HIV-1-seropositive individuals (reviewed in (Pitrak 1999)). None of these *in vitro* studies have specifically looked at neutrophil responses to *S. pneumoniae*. A single clinical study of IPD in HIV-1-seropositive Kenyan females did find a significant positive correlation between peripheral blood neutrophil and CD4 counts at presentation, documenting that neutrophil responses were blunted in those with HIV-1 infection (Gilks, Ojoo et al. 1996). However, a causal relationship between quantitative or qualitative neutrophil dysfunction and increased incidence or severity of IPD or outcomes in HIV-1 has, to date, not been demonstrated.

In conclusion, despite evidence that HIV-1 infection is associated with impairments of macrophage and neutrophil function in certain circumstances, these have not been shown to extend to any deficit in the phagocytic killing of *S. pneumoniae* by macrophages or neutrophils.

1.7 Aims

It is clear that invasive pneumococcal disease continues to be a significant predicament for HIV-1 seropositive individuals. The incidence is increased and mortality is significant.

Antiretroviral treatment, even where universal coverage is achievable, has not resolved these problems and in addition pneumococcal vaccine efficacy is limited in HIV-1. Research focused on understanding the immune deficit in HIV-1 that underlies the increased risk of IPD has demonstrated impairments in adaptive immunity to the pneumococcus but there is no clear evidence for a defect in innate immune responses. Moreover, these studies do not fully explain the reasons for the increased rates of IPD in HIV-1 seropositive individuals on antiretroviral therapy.

Macrophage apoptosis-associated killing of *S. pneumoniae* has emerged as an important determinant of successful, innate defence against the pneumococcus. Meanwhile, there exists a body of evidence showing that macrophage apoptosis is impeded by HIV-1. I was interested to see if HIV-1 causes any impairment of macrophage apoptosis in the context of pneumococcal infection and whether this might reduce apoptosis-associated bacterial killing.

I hypothesised that:

HIV-1 modulates the macrophage apoptotic response to *S. pneumoniae* resulting in enhanced susceptibility to IPD in HIV-1-seropositive individuals

On this basis the aims of my doctoral work were:

1. To examine the effect of HIV-1 infection on macrophage apoptosis and bacterial killing following pneumococcal challenge
2. To observe whether any defects extend to *ex vivo* alveolar macrophages from HIV-1-seropositive individuals on fully suppressive antiretroviral therapy
3. To elucidate the possible mechanisms by which this happens

Chapter 2. Materials and Methods

2.1 Cell culture and differentiation

2.1.1 Cell lines

2.1.1.1 U937 and U1 cell lines

The U937 and U1 cell lines were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA. U937 is a promonocytic cell line from a histiocytic lymphoma and the U1 cell line is a U937 subclone latently infected with HIV-1 (Folks, Justement et al. 1987). Cells were maintained in T25 tissue culture flasks (Nunc) at 37°C in 5% CO₂ in the containment level 3 laboratory (CL3), University of Sheffield Medical School. Cells were passaged every 3-4 days by splitting 1 in 10 in growth medium: Roswell Park Memorial Institute (RPMI) 1640 medium (Lonza) supplemented with 10% heat inactivated, ultra-low endotoxin, fetal calf serum (HIFCS; Bioclear) and 2 mmolL⁻¹ L-glutamine (Gibco BRL). Healthy cells were counted in a disposable haemocytometer (KOVA® GLASSTIC® Slide, Hycor Biomedical Ltd.) using trypan blue exclusion to exclude non viable cells (see section 2.1.4). U937 or U1 cells from synchronised passages were seeded at a density of 1 x10⁶ mL⁻¹ in 24 (1 mL) or 12 well (2 mL) tissue culture plates (Costar). To differentiate the cell lines toward a macrophage phenotype, and in the case of U1 to also activate HIV-1 transcription, cells were incubated with 50-100 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 24 or 72 hours. PMA is a phorbol ester which activates protein kinase C (PKC). PMA containing medium was then removed and replaced with fresh growth medium for a further 2 or 5 days. A range of differentiation protocols were tested, derived from published work; 50 nM PMA for 24 hours then rested in growth medium alone for 3 days (50 nM PMA1r3), 100 nM PMA for 2 days then 3 days rested (100 nM PMA2r3) and 100 nM PMA for 3 days then 5 days rested (100 nM PMA3r5) (Tachado, Zhang et al. 2005; Nicol, Mathys et al. 2008; Daigneault, Preston et al. 2010).

2.1.1.2 NP2 cell line

NP2 cells are an astrocytoma cell line that has been stably transfected with CD4 and CCR5 and express CD4 and CCR5 (kindly donated by Dr. M Noursadeghi, University College London). Cells were maintained in NP2 medium: Dulbecco's Modified Eagle Medium (DMEM) (Lonza) with 10% HIFCS and 2 mmol⁻¹ L-glutamine, 1 µgml⁻¹ puromycin (Sigma-Aldrich) and 100 µgml⁻¹ geneticin (G418 Sigma-Aldrich) at 37°C, 5% CO₂ in the tissue culture laboratories, University of Sheffield Medical School (TCL). NP2 cells were passaged weekly: medium was removed from flasks and the adherent cells washed twice with PBS. 1 mL of trypsin-versene (Lonza) was added and the flask returned to the incubator for 2-3 minutes. The flask was tapped and checked to ensure cells were detaching then 9 mL of working medium (DMEM + 10% HIFCS) was added and the mixture pipetted vigorously to break up any clumps. Cell suspensions were then diluted 1 in 10 in NP2 medium and placed in a new flask.

2.1.1.3 Ghost cell line

Ghost cells are a human osteosarcoma cell line that act as an indicator of HIV-1 infection (Programme EVA Centre for AIDS Reagents, NIBSC, UK). They express CD4 and CCR5 and contain a green fluorescent protein (GFP) gene which is controlled by an HIV-2 LTR promoter, expressed during HIV-1 infection via Tat transactivation (Janas and Wu 2009). Ghost cells were maintained in the TCL in T25 flasks at 37 °C, 5% CO₂ in parent medium: DMEM + 10% HIFCS, 100 unitsml⁻¹ penicillin (Lonza), 100 µgml⁻¹ streptomycin (Lonza) and 500 µgml⁻¹ geneticin (G418, Sigma-Aldrich). 100 µgml⁻¹ hygromycin (Calbiochem) and 1 µgml⁻¹ puromycin (Sigma-Aldrich) were added to the parent medium for GFP and chemokine receptor selection in subsequent passages (selection medium). Cells were passaged 1 in 10 every 2-3 days until passage 14-16 using trypsin-versene to detach the cells as described in section 2.1.1.2.

2.1.2 Human peripheral blood cells

2.1.2.1 Peripheral blood mononuclear cell isolation

Blood was donated by healthy volunteers recruited from staff and students of STH or The University of Sheffield and HIV-1-seropositive patients recruited from the HIV treatment clinics of STH. STH research directorate sponsored the study (STH14625) and ethical approval was granted by The South Sheffield Research Ethics Committee (07/Q2305/7). Volunteers gave written, informed consent. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by Ficoll-Paque™ (GE Healthcare Life Sciences) density centrifugation in the TCL. Up to 25 mL of blood was carefully layered onto 12.5 mL of Ficoll-Paque™ in a 50 mL centrifuge tube (Starstedt), then centrifuged at 500g (1500 rpm) for 23 minutes with no brake (FALCON 6/300). The flocculent layer containing mononuclear cells was transferred to a new 50mL centrifuge tube, made up to 50mL with phosphate buffered saline (PBS, Oxoid) and centrifuged at 300g (1000 rpm) for 10minutes with the brake on. The pellet was washed in PBS and centrifuged again in the same manner.

2.1.2.2 Human monocyte-derived macrophages

The PBMC pellet was resuspended in 10 mL of AB medium: RPMI 1640 medium and 2 mmolL⁻¹ L-glutamine containing 10% human AB serum (First Link (UK) Ltd). Cells were counted using a haemocytometer (see section 2.1.4), resuspended at 2 x 10⁶ mL⁻¹ then incubated in 24-well tissue culture plates, with or without glass coverslips, 6 wells plates or T25 flasks. After 24 hours, medium containing non adherent cells was removed, and the remaining adherent cells were cultured in growth medium, changed every 3-4 days, at 37 °C, 5% CO₂ and allowed to differentiate into macrophages for fourteen days. The final concentration of the monocyte-derived macrophages (MDM) was measured using a haemocytometer after removing the medium, washing twice with PBS, gently scraping adherent cells from the base of the well with a rubber cell scraper (Sarstedt), and resuspending the cells in 1mL of PBS. Typically this yielded approximately 2 x 10⁵ ml⁻¹ MDM.

2.1.2.3 Human lymphoblasts

Peripheral blood lymphocytes (PBL) were purified from PBMC by performing 2 plastic adherence steps to remove monocytes; the previously isolated PBMC were resuspended at $2 \times 10^6 \text{ mL}^{-1}$ in RPMI containing 10% AB serum in a T75 tissue culture flask (Nunc) and incubated for 2 hours at 37°C , 5% CO_2 in the TCL. Flasks were gently agitated and medium containing non adherent wells was transferred to a second T75 flask and incubated overnight. The non adherent PBL were then centrifuged at $500g \times 10$ minutes in a 50 mL centrifuge tube and then resuspended in a T75 flask at $1 \times 10^6 \text{ cells mL}^{-1}$ in growth media with $20 \mu\text{mL}^{-1}$ interleukin (IL)-2 (PeproTech) and $0.5 \mu\text{g mL}^{-1}$ phytohemagglutinin (PHA, Sigma-Aldrich) for 3 days to activate them into lymphoblasts. Activation was confirmed visually by the presence of cell aggregation.

2.1.3 Human alveolar cells

2.1.3.1 Recruitment of volunteers

HIV-1-seropositive and seronegative volunteers were recruited for bronchoscopy and bronchoalveolar lavage (BAL) in order to obtain alveolar macrophages and lymphocytes. HIV-1-seropositive volunteers were recruited by advertisement and direct invitation from the HIV treatment clinics of the STH directorate of communicable diseases and the wider South Yorkshire HIV Clinical Network clinics. Control volunteers were recruited from the staff and students of STH and The University of Sheffield. All volunteers were given at least 24 hours to read written information sheets and then had a face to face interview with a study investigator. To be enrolled volunteers had to be non smoking adults with no active or chronic lung disease. Selection involved clinical history and examination, review of clinic notes for HIV-1-seropositive volunteers and spirometry and peripheral blood analyses. Full inclusion and exclusion criteria for enrolment are set out in Table 2-1. Three groups of HIV-1-seropositive patients were enrolled: those on non nucleotide reverse transcriptase inhibitor (NNRTI) based antiretroviral therapy (ART), those on protease inhibitor (PI) based ART and those who were antiretroviral therapy naive. A sample size was calculated from preliminary *in vitro* work which

had demonstrated a 17% difference in the rates of apoptosis of differentiated U937 and U1 cells following *S.pneumoniae* challenge. It was calculated that a 17% difference in macrophage apoptosis following challenge with *S. pneumoniae* could be detected with a power of 80% and $p < 0.05$ if there were at least seven in each group. The study protocol was approved by the scientific advisory board of the STH Clinical Research Facility (CRF), the STH research directorate and through the University of Sheffield internal scientific review system. The study sponsor was STH (STH15411) and the study was granted portfolio status by the South Yorkshire Comprehensive Local Research Network (UKCRN11811). Ethical approval was granted by the NRES Committee Yorkshire & The Humber - South Yorkshire (11/YH/0217).

Table 2-1 Inclusion and Exclusion criteria for study enrolment

Inclusion criteria
HIV seropositive adults aged 18-69 years who are either
a) Antiretroviral naive
b) Already receiving a first line antiretroviral regime using a backbone of 2 nucleoside/nucleotide analogues and either a protease inhibitor (lopinavir, atazanavir or darunavir boosted with ritonavir) or efavirenz, with no prior alterations
HIV seronegative controls aged 18-69 years
Exclusion criteria
Febrile illness or other symptoms of acute infectious illness within two weeks
Receipt of any vaccine within two weeks
Malignancy
Active Hepatitis B or C infection (defined as detectable HB surface antigen/DNA or HCV RNA)
Ongoing anaemia or any symptoms (shortness of breath, chronic fatigue, chest pain or pallor) suggestive of possible anaemia
Donated >200 ml of blood for any reason within the last 6 months
Abnormal clotting screen
Pregnancy or breast feeding
Current participation in any clinical trial
Inability to communicate in English or convey willingness to participate
Active lung condition including; infection, malignancy, asthma, Chronic Obstructive Pulmonary Disease, Interstitial lung disease, FEV1 <70% predicted

2.1.3.2 Bronchoscopy and bronchoalveolar lavage

Bronchoscopy and lavage was carried out in the Sheffield Teaching Hospitals endoscopy suite by a consultant respiratory physician. Volunteers were taken through consent for the procedure and an IV cannula was secured. Lidocaine local anaesthetic jelly and spray were

used to anaesthetise the nasopharynx. Midazolam sedation was offered to all volunteers. A fibre optic endoscope (Olympus) was passed into the trachea by intubating through the nostril then further anaesthetic was applied to the vocal chords, carina and bronchial branches. The medial and lateral segments of the right middle lobe were each intubated in turn and 2 x 50 mL sterile saline was instilled and then aspirated under 23 kPa suction pressure in each segment up to a total of 200ml as tolerated. Lavage fluid was collected in a trap (Argyle™, Coviden) that had been pre cooled in ice and then kept on ice for transfer to the CL3. Following extubation, volunteers were monitored for two hours in the endoscopy recovery suite before discharge.

2.1.3.3 Human alveolar macrophage and lymphocyte isolation from bronchoalveolar lavage fluid

The volume of BAL fluid was documented and then sieved through sterile gauze into a 50mL falcon centrifuge tube. Sealed tubes were inverted twice to mix the contents and then 5 x 1 mL aliquots were removed and stored at -80 °C. The remaining BAL fluid was centrifuged at 400g x 10 minutes. 30 mL of supernatant was reserved and frozen at -80 °C and the rest discarded. The pellets were immediately disrupted by vortex to avoid cell clumping and then resuspended in 10 mL of antibiotic medium; RPMI + 10% AB serum + 40 μmL^{-1} penicillin (Lonza) + 40 $\mu\text{g mL}^{-1}$ streptomycin (Lonza) + 0.5 $\mu\text{g mL}^{-1}$ amphotericin (Fungizone™, GIBCO). 100 μL was aliquoted into a microcentrifuge tube (Eppendorf) and diluted 1:1 with HIFCS with low LPS. 50 μL of 4% paraformaldehyde (PFA, Fisher Scientific) was then added and the tube reserved for making a cytospin slide. A 50 μL aliquot of this suspension was then diluted 1:10 in PBS then 10 μL diluted 1:1 with 0.4% Trypan Blue (Sigma)(see 2.1.4). Alveolar macrophages were counted with a KOVA™ slide using an inverted bright field microscope (Olympus CK2) with a 25 x lens, final magnification 250 x, to accurately distinguish epithelial cells and lymphocytes from live and dead (taking up trypan blue) AM. BAL fluid samples that contained visible red blood cells were subjected to Ficoll-Paque density centrifugation to remove red cells (as described in section

2.1.2.1) after the cytospin aliquot had been removed. Antibiotic medium was added to resuspended BAL cells to achieve a final suspension of cells with AM at a density of 2×10^5 cells mL^{-1} . Cells were incubated overnight at 37°C , 5% CO_2 in 6 well, 24 well and 96 well cell culture plates (Costar). The following day medium with non adherent cells was removed and reserved and fresh RPMI + 10% HIFCS without antibiotics was added. The non adherent cells from each donor were pooled then prepared for flow analyses of the lymphocytes as described in section 2.6.5. The adherent AM were allowed to incubate for a further 24 hours before being used for *S. pneumoniae* challenge experiments. For each donor, one well was incubated with anti-p24 / β galactosidase secondary antibody (see section 2.2.3.1) to detect intracellular HIV-1 p24 antigen as a measure of the rate of HIV-1 infection of AM.

2.1.4 Counting cell numbers

As glass is not permitted in the CL3 a disposable plastic counting chamber was used to count cells (KOVA® GLASSTIC® Slide, Hycor Biomedical Ltd., UK). 50 μL of cell suspension was added to 450 μL of PBS and 10 μL allowed to enter the 6.6 μL chamber by capillary action. The average number of cells per small square of an 81 square grid were counted using an Olympus CK2 inverted bright-field microscope (Olympus) with a 10 x lens and 10 x eye piece to give 100x magnification. This number was then multiplied by 90 (as per manufacturer's instructions) and then by 10 (to account for dilution) to give the number of cells μL^{-1} then by 1000 to obtain cells mL^{-1} . In all other TCL cells were counted in a haemocytometer (Weber Scientific International) using the same type of microscope and magnification. The number of cells in four 4 x 4 grids was calculated, divided by 4 and then multiplied by the dilution factor of 10 to give cells μL^{-1} and then by 10,000 to give cells mL^{-1} . To determine Trypan Blue exclusion 10 μL of diluted cell suspension was mixed with 10 μL of 0.4% Trypan blue vital dye (Sigma) in a microcentrifuge tube before transfer to the haemocytometer / KOVA™ slide. Dead cells were identified as those that took up the Trypan blue dye and stained blue (Strober 2001).

2.2 HIV-1 propagation

2.2.1 HIV-1 propagation in lymphoblasts

Lymphoblasts were centrifuged and re-suspended in 1 mL HIV-1_{BAL} (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA) $\pm 10 \mu\text{g mL}^{-1}$ polybrene (Millipore), incubated in the CL3 facility for 2 hours at 37°C, 5% CO₂, then diluted to 1×10^6 cells mL⁻¹ in growth media + 20 $\mu\text{g mL}^{-1}$ IL-2 (PeproTech). After 3 days incubation, suspensions of HIV-1 infected lymphoblasts were centrifuged at 400g x 5 minutes and all but 1 mL of the supernatant was filtered through a 0.45 μm syringe filter (MF-Millipore™, Millipore) into an ultracentrifuge (UC) tube (Beckman Coulter). The virus containing medium was carefully underlayered with 5 mL of the 25% weight for weight sucrose (Fisher Scientific) and PBS solution, then topped up with PBS within the UC bucket. Buckets were carefully balanced to within 100 mg and loaded into an ultracentrifuge (Optima K-100 XP, Beckman Coulter) in the CL3. These were then centrifuged for 2 hours at 23,000 g. Supernatant was removed and purified virus was re-suspended in 10 mL AB media and stored in 1 mL aliquots in liquid nitrogen (LN). Lymphoblasts from a second donor were re-suspended at $1 \times 10^6 \text{ mL}^{-1}$ and added to the remaining 1 mL of HIV-1 containing supernatant and the propagation / purification cycle repeated a total of 4 times.

2.2.2 HIV-1 propagation in MDM

MDM, isolated from healthy volunteers as above, were re-suspended in T25 flasks (Nunc) in RPMI + 10% heat inactivated autologous human serum supplemented with 20 ng mL^{-1} macrophage colony-stimulating factor (M-CSF, R&D Systems). After 3 days non adherent cells were removed and the cultures were refreshed with growth medium (without M-CSF). After a total incubation time of 7 days flasks were transferred to the CL3. Here the medium was removed and 1 mL of lymphoblast propagated HIV-1_{Bal}, at a concentration of $1 \times 10^{5-6}$ infectious units (IU) mL⁻¹, or medium (for sham control) was added and flasks were incubated

at 37°C, 5% CO₂. After 16 hours the inoculum was removed and 10 mL of growth medium added. At 7, 14, 21 and 28 days following virus inoculation, 9 mL of medium was removed and replaced with fresh growth medium. Virus containing medium was filtered and ultracentrifuged through 25% sucrose as before (see 2.2.1) to yield purified, macrophage-passaged HIV-1 or sham which was stored in 1 mL aliquots in LN.

2.2.3 HIV-1 titration

2.2.3.1 NP2 cell line titration method

HIV was titrated using the NP2 cell line titration method previously developed by Noursadeghi et al. (Noursadeghi, Tsang et al. 2009). NP2 cells were washed x 2 with PBS then detached from flasks with trypsin-versene as described (see 2.1.1.2). Cells were resuspended in fresh NP2 medium at a concentration of $1.5 \times 10^5 \text{ mL}^{-1}$ and cultured in volumes of 200 μL per well in a 96 plate (Costar) for 24 hours at 37 °C, 5% CO₂. The plates were transferred to the CL3 where medium was removed and the wells then inoculated in duplicate with 8 serial log₁₀ dilutions of purified HIV-1_{BAL} from lymphoblast or MDM propagations and incubated for 3 days at 37 °C, 5% CO₂. Medium was then removed and the wells washed with PBS before being fixed and permeabilised in an ice cold mixture 1:1 of pure acetone and methanol for 5 minutes and then washed x 3 in PBS. Each well was incubated with PBS containing 2% HIFCS and 1:25 p24 antibody (IgG1 κ monoclonal antibody to HIV-1 gag p24, Programme EVA Centre for AIDS Reagents, NIBSC, UK) for 2 hours at room temperature. Cells were then washed in PBS x 3 and then incubated with PBS containing 2% HIFCS + 5 $\mu\text{g mL}^{-1}$ goat anti-mouse antibody conjugated to β galactosidase (IgM+IgG+IgA (H+L) pooled antisera, Southern Biotechnology Associates) for 1 hour at room temperature. The cells were then incubated overnight at 37 °C in a galactosidase substrate solution of 0.5 mg mL^{-1} 5-bromo-4-chloro-3-indolyl—galactopyranoside (X-gal) (Melford) in PBS containing 3 mmol L^{-1} potassium ferricyanide (FLUKA), 3 mmol L^{-1} potassium ferrocyanide (FLUKA), and 1 mmol L^{-1} magnesium chloride (Sigma). Blue stained cells

positive for p24 were counted by microscope (Figure 2-1) and a virus titre of focus forming / infectious units per mL was derived using the following formula; IU mL⁻¹ = dilution factor of well with the lowest countable number of blue cells / number of blue cells counted (averaged for both duplicates).

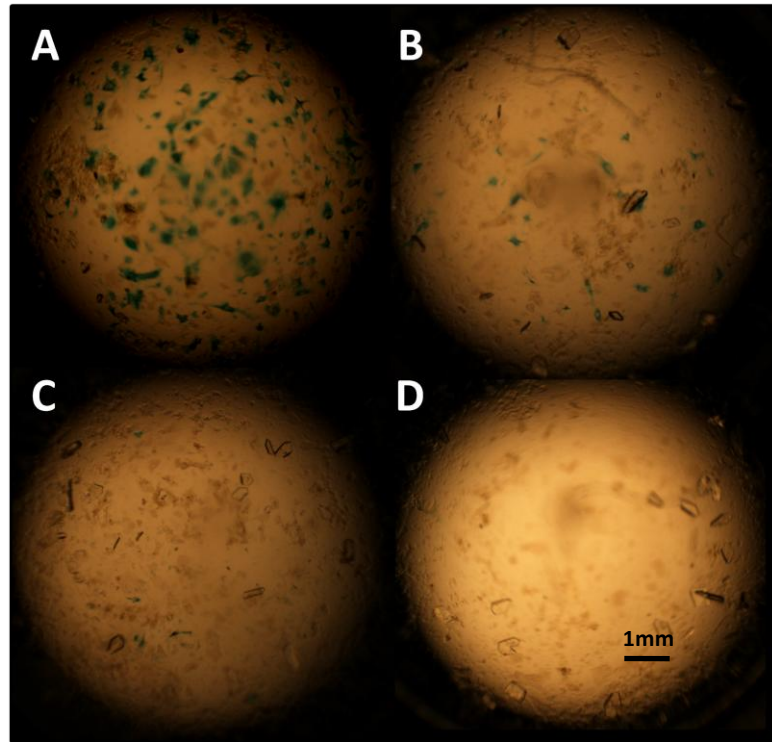


Figure 2-1 Photomicrographs of four wells of NP2 cells stained for HIV-1 p24

Wells of a 96 well plate were seeded with 3×10^4 NP2 cells then inoculated with serial \log_{10} dilutions of purified HIV-1_{BAL} for 3 days. Cells were permeabilised, fixed and then stained with anti-HIV-1 p24 and a β galactosidase conjugated secondary antibody. Images show wells at 40 x magnification with dilutions of HIV-1_{BAL} at (A) 10^{-3} , (B) 10^{-4} , (C) 10^{-5} and (D) medium alone. p24 positive cells stain blue.

2.2.3.2 Ghost cell titration method

Ghost cells were resuspended at 1×10^4 in ghost cell parent medium (as above 2.1.1.3) and incubated for 24 hours in volumes of 2mL per well of a 12-well tissue culture plate (Costar). Cultures were removed to the CL3 where they were washed in PBS x 2 and inocula of 25 μ L, 50 μ L and 100 μ L of each viral stock to be measured were added to individual wells, which were then brought up to a final volume of 300 μ L per well with parent medium. Typically three separate virus stocks were tested per plate along with three negative control (medium only) well totalling 12 wells. Cells were incubated overnight for 16 hours at 37°C, 5% CO₂ and then medium was replaced. After a further 48 hours cells were gently washed with PBS x 2, detached with 250 μ L of trypsin-versene, incubated for 2-3 minutes in the incubator and then resuspended in 1mL of DMEM +10% FCS. Cell counts were performed on one of the negative control wells using the KOVA™ cell counting chamber. Cells from individual wells were transferred to microcentrifuge tubes (Eppendorf) and centrifuged for 10 mins at 500g at 4°C. Each pellet was resuspended in 600 μ L of PBS with 2% PFA, thoroughly pipetted and left on ice for 1 hour in the dark. Next cells were washed in PBS, centrifuged as before then resuspended in 200 μ L FACS buffer: PBS with 0.01% bovine serum albumin (BSA, First Link). Flow cytometry was performed on the FACSCalibur™(BD) in the University of Sheffield Medical School core facility, capturing 10,000 events. Forward scatter (FSC) and side scatter (SSC) plots were used to exclude debris and gate the intact cells as described (see 2.6.1). GFP expression was measured with the 488 nm blue laser and the 515/45 nm filter (FL1-H channel). GFP positive cells were identified from this population as those events with a greater than 1 Log₁₀ increase in fluorescence intensity compared with negative control cells (Figure 2-2). Virus titre was then calculated based on the following formula: Infectious Units (IU)/mL= (cell number) x (% of GFP-positive cells) x (dilution factor) (Janas and Wu 2009).

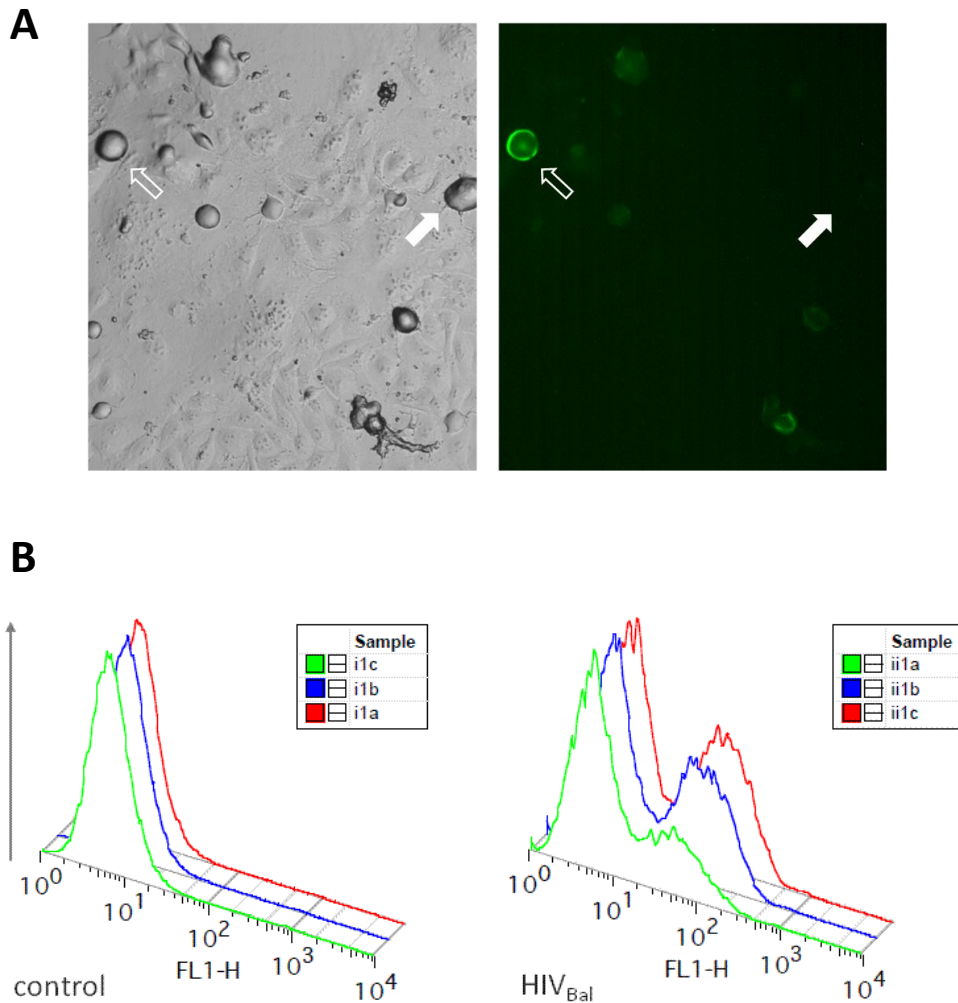


Figure 2-2 Photomicrographs and histograms of GFP expression in ghost cells indicating HIV-1 infection

Ghost cells were incubated with 25 μ L, 50 μ L and 100 μ L of HIV_{BAL} or medium alone for 3 days then cells were analysed for GFP expression by fluorescent microscopy and flow cytometry. Events with $\geq 1 \text{ Log}_{10}$ increase in FL1-H intensity than control were considered GFP positive. (A) Brightfield (left panel) and FITC filter images (right panel) of ghost cells showing the presence (empty arrow) or absence (white arrow) of green fluorescent protein expression (GFP). (B) Histograms of control (left) and HIV inoculated cells (right) with GFP expressing proportions; 25 μ L (23.5% positive, green line), 50 μ L (40.6% positive, blue line) and 100 μ L (45.4% positive, red line).

2.3 *Streptococcus pneumoniae* culture and cell challenge

2.3.1 *Streptococcus pneumoniae* culture

Streptococcus pneumoniae serotype 2 strain D39 (NCTC7466) were cultured in 20 mL brain-heart infusion broth (BHI, Oxoid Unipath) with 20% HIFCS in the University of Sheffield Medical School containment level 2 laboratory (CL2). Growth curves were constructed from hourly measurement of the optical density (OD) by spectrophotometer (Jenway) and the number of colony forming units (cfu) per mL of broth using the Miles and Misra viable count technique (see 2.3.2) every hour in parallel. Subsequently OD readings were used to determine the point of mid log growth at which time broth was aliquoted and immediately frozen in 1 mL aliquots at -80 °C, typically at a concentration of 5×10^8 cfumL⁻¹. *S. pneumoniae* colonies were confirmed by their typical α -haemolytic appearance on Columbia blood agar (CBA) plates (Oxoid) and optochin sensitivity as demonstrated by growth inhibition with an optochin disk (Oxoid).

2.3.2 Miles and Misra viable bacterial count

To accurately count the number of viable bacteria in a suspension the Miles and Misra technique was used (Miles, Misra et al. 1938). Eight serial log₁₀ dilutions of the suspension were made by adding 100 μ L of the suspension to 900 μ L of PBS in a microcentrifuge tube and then mixing for 10 seconds with a bench top vortex. Three 10 μ L drops of each dilution were then dropped onto each of four quadrants of two CBA plates, allowed to dry and then incubated at 37°C, 5% CO₂ overnight for 18-20 hours. The number of colonies of *S. pneumoniae* per quadrant was counted and the viable count calculated using the formula: cfumL⁻¹ = (number of colonies in quadrant / 30) x 1000 x dilution factor.

2.3.3 *Streptococcus pneumoniae* opsonisation with human serum

S. pneumoniae aliquots were rapidly thawed, centrifuged at 7600g and washed x 2 in PBS. Pellets were resuspended in RPMI plus 10% human serum pooled from three volunteers

previously vaccinated with the 23 valent polysaccharide pneumococcal vaccine, and in whom a documented vaccine response has been demonstrated. The bacteria were incubated for 30 minutes at 37°C, 5% CO₂ in the CL2, then centrifuged at 3000g x 3 minutes, washed x 2 in PBS and resuspended in RPMI + 10% HIFCS and kept on ice. Miles Misra viable counts were performed on aliquots of opsonised bacteria for every infection to confirm the final multiplicity of infection (MOI). To confirm binding of antibody after opsonisation treatments, both serum treated and untreated *S. pneumoniae* were washed in PBS and 10⁶ bacteria resuspended in 100 mL of 0.1% PBS-azide (Lonza). Bacteria were incubated with 1 mg of fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG Fc (Dako) for 15min at 20 °C. Samples were then centrifuged at 2500g for 2 min, then the pellet was washed and resuspended in 400 mL of 0.5% PFA. Bacteria were analyzed using a LSRII™ (BD Biosciences) flowcytometer. FSC/SSC plots were used to identify the bacterial population (see 2.6.1). 10,000 events from this population were analyzed for FITC expression with the 488 nm blue laser and the 530/30 filter (Figure 2-3).

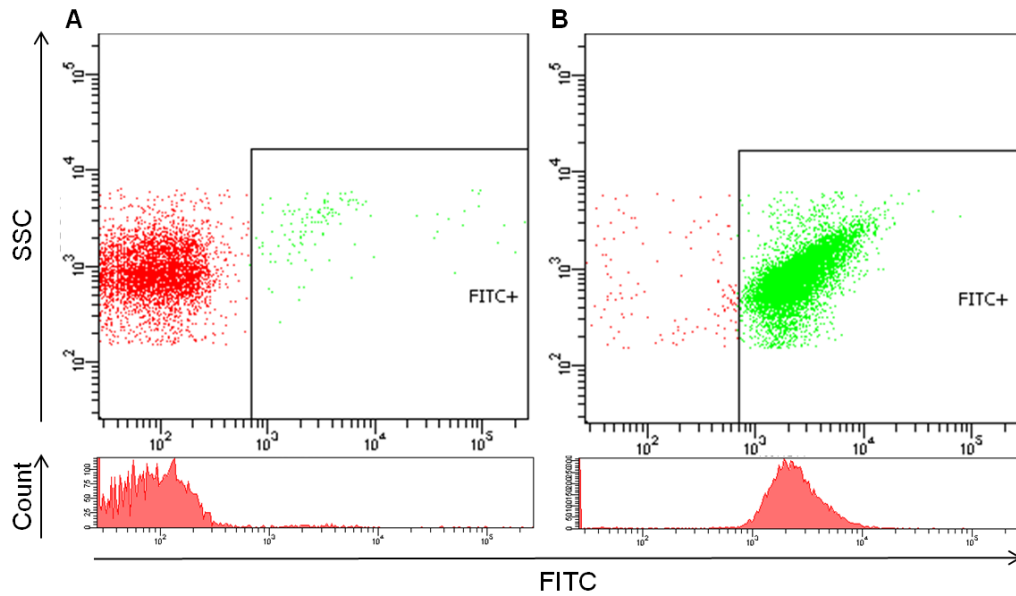


Figure 2-3 Human serum from PCV-23 vaccinated subjects opsonises D39 *Streptococcus pneumoniae*

Dot plots and histograms showing FITC expression by D39 that had been incubated for 30 minutes with PBS (A) or with anti-Spn human immune serum (B) washed and then incubated with FITC-conjugated goat anti-human IgG Fc. SSC side scatter

2.3.4 Cell challenge with *Streptococcus pneumoniae*

All pneumococcal challenge experiments were performed with opsonised *S. pneumoniae* serotype 2 (D39). Wells containing cells were washed x 1 in PBS. Cells were then inoculated with medium for mock infection or *S. pneumoniae* at MOI of 10, topped up to a final volume of 1 mL with growth medium and immediately placed on ice to facilitate bacterial adherence. After 1 hour, tissue culture plates were transferred to an incubator at 37 °C, 5% CO₂. After a further 3 hours adherent cells were washed x 2 with PBS and then incubated with fresh, bacteria free, growth medium for up to a total of 20 hours. Cells from a representative well were counted prior to infection to confirm cell numbers, typically 2 x 10⁵ mL⁻¹ and this informed the volume of the bacterial inoculum to achieve an MOI of 10. As a positive control for

apoptosis, two uninfected wells were treated with 1 μM staurosporine (STS) (Calbiochem) at the 4 hour time point and incubated for a further 16 hours.

2.3.5 Gentamicin protection assays

To determine the number of viable, internalised bacteria in monocytes/macrophages at 4 hours post infection wells were washed x 2 in PBS then returned to the incubator for 30 minutes in fresh medium containing 40 units mL^{-1} benzyl penicillin (Crystapen™, Genus Pharmaceuticals) and 20 $\mu\text{g}\text{mL}^{-1}$ gentamicin (Cidomycin™, Sanofi) or 20 $\mu\text{g}\text{mL}^{-1}$ gentamicin alone. Wells were again washed x2 in PBS and incubated for 12 minutes with 200 μL sterile filtered 2% saponin (Sigma) before being made up to 1mL with PBS and scraped and pipetted vigorously to break up cell membranes and release bacteria into the medium. Miles and Misra viable counts were performed. To determine the number of viable intracellular bacteria at later time points whilst preventing ongoing bacterial replication in the medium, other wells were returned to the incubator following penicillin/gentamicin treatment in medium containing 0.7 $\mu\text{g}\text{mL}^{-1}$ vancomycin (Sigma) until the desired time point. Wells were again washed x2 with PBS and incubated for 12 minutes with 2% saponin to lyse cells and then viable counts were performed as before.

2.4 MDM incubation with HIV-1 gp120

MDM from human donors were treated with recombinant HIV-1_{LAI/IIIIB} envelope glycoprotein gp120 (Programme EVA Centre for AIDS Reagents, NIBSC, HPA, UK) at concentrations of 10 ngmL^{-1} and 100 ngmL^{-1} from 1 hour prior to and then throughout the challenge with opsonised *S. pneumoniae* / mock infection.

2.5 MDM infection with HIV-1

For MDM infections, macrophage passaged HIV-1_{BAL} was removed from the liquid nitrogen store and rapidly thawed in a water bath. Virus was resuspended in growth media at 10⁵ IU mL^{-1} . 7-14 day old MDM from human donors were washed x2 with PBS and then inoculated with

200 μ L per well (24 well plates) or 80 μ L per well (96 well plate) of this viral suspension and incubated at 5% CO₂ 37°C for 16 hours in the CL3. Wells were then topped up with 1 mL / 200 μ L growth medium respectively and incubated for a further 7 days. Rates of macrophage cell infection were measured by detecting the presence of intracellular HIV p24 antigen using the anti-p24 / β -galactosidase secondary antibody as described for NP2 HIV-1 titration (see section 2.2.3.1). Alternatively, HIV-1 infected MDM were identified using confocal fluorescence microscopy. MDM were seeded onto coverslips then infected with HIV-1_{BAL} and incubated with anti-p24 as above. Coverslips were then incubated with an Alexa 488 conjugated goat anti-mouse IgG (H+L) secondary antibody (Molecular Probes™, Life Technologies), inverted and sealed with nail polish and image capture was performed with a Zeiss LSM 510 confocal microscope with a Zeiss 63 \times /1.4 oil objective and processed with AxioVision 4.7.2 software.

2.6 Flow cytometry

2.6.1 Flow Cytometry

Measurements were made in the University of Sheffield Medical School Flow Cytometry core facility using a two laser, 4 colour FACSCalibur™ (BD Biosciences) and CellQuest™ PRO version 4.0.2 software (BD Biosciences) or a four laser, 13 colour LSRII™ (BD Biosciences) and BD FACSDiva version 6.1.1 software (BD Biosciences). Forward scatter (FSC) and side scatter (SSC) were used to exclude debris and to identify cell populations by measuring the size and granularity or internal complexity of events respectively. 10,000 gated events were recorded unless otherwise stated. Analyses of data were performed using FlowJo™ software version 9.3.2 (Tree Star, Inc.).

2.6.2 Antibodies

To identify cell types and cell surface molecules fluorophore conjugated antibodies (Table 2-2) were used at 0.1-0.25 μ g per 10⁵-10⁶ cells in 100 μ L of 0.1% BSA in PBS (FACS buffer) and incubated for at least 30 minutes at 4 °C as per the manufacturers' instructions. Washes were

with FACS buffer and centrifugation at 400g x 10 minutes unless otherwise stated and performed in 1.5 mL flow cytometer tubes. To avoid non specific antibody binding, cells were initially resuspended in 100 μ L FACS buffer and incubated with recombinant human IgG (sigma-Aldrich) for 10-20 minutes at 4°C to block Fc receptors. To control for any residual non specific binding of antibodies, a second aliquot of the same cells were incubated with isotype control antibodies with the same fluorophores and the difference in fluorescence intensity between the specific antibody and isotype control was measured. Following incubation with antibodies, cell suspensions were centrifuged at 500g (Heraeus), washed, and pellets resuspended and fixed in 200 μ L FACS buffer with 1% PFA prior to removal from the CL3. Cells were stored at 4 °C until run on the flowcytometer.

Table 2-2 Fluorophore labelled antibodies

Antibody	Fluorophore	Species	Isotype	Clone	Manufacturer
anti-Human CD3	PE	Mouse	Mouse IgG ₁ kappa	SK7	eBiosciences
anti-Human CD38	FITC	Mouse	Mouse IgG ₁ kappa	HB7	eBiosciences
anti-human CD4	APC	Mouse	Mouse IgG _{2a}	S3.5	Invitrogen
anti-human CD8a	Brilliant Violet 421	Mouse	Mouse IgG1 kappa	RPA-T8	Biolegend
anti-human CD206	APC	Mouse	Mouse IgG ₁ kappa	19.2	eBiosciences
anti-human CD163	PE	Mouse	Mouse IgG ₁ kappa	GHI/61	eBiosciences
anti-human CD80	PE	Mouse	Mouse IgG ₁ kappa	2D10.4	eBiosciences
anti-human CD200r	PE	Mouse	Mouse IgG ₁ kappa	OX108	eBiosciences
LIVE/DEAD® Fixable Dead Cell Stain	blue fluorescent reactive dye	-	-		Invitrogen

2.6.3 Cell viability staining

Cell viability was confirmed during flow analyses with a LIVE/DEAD® Blue Fixable Dead Cell Stain Kit (L23105, Molecular Probes, Invitrogen). This contains an amine reactive viability dye (ViD) which reacts with free amines both in the cell interior and on the surface. As the dye is non cell permeable and only dead cells have permeable membranes, dead cells take up more ViD resulting in increases of 1 Log₁₀ or greater in fluorescence intensity compared with live

cells (Perfetto, Chattopadhyay et al. 2006). Cells were prepared and incubated with 1 μ L LIVE/DEAD[®] Blue per 10⁵⁻⁶ cells in the same manner described in section 2.6.2

2.6.4 Measurement of alveolar macrophage polarisation

On the day following BAL, adherent AM were washed twice in ice cold FACS buffer then gently removed with a cell scraper and resuspended in FACS buffer. Cells suspensions were divided into five equal volumes and labelled with (1) unstained, (2) anti-CD206-allophycocyanin (APC), (3) anti-CD206-APC and anti-CD80- phycoerythrin (PE), (4) anti-CD206-APC and anti-CD163-PE and (5) anti-CD206-APC and anti-CD200r-PE and with corresponding APC and PE isotype controls (as described in section 2.6.2). Cell surface expression of these markers was then measured using the FACSCalibur[™]. FSC and SSC detectors were set while running unstained samples and then FL2-H (excited by 488 nm blue laser, detected by 575/26 BP filter) and FL4-H (excited by 635 nm red laser, detected by 650 LP filter) detectors were set using isotype control and antibody labelled samples. The settings were saved and used for subsequent sample analyses. Data were analyzed with FlowJo[™] software version 9.3.2 (Tree Star, Inc.). Gates were set on FSC/SSC to exclude debris and identify intact cells (see section 2.6.1). APC (CD206⁺) geometric mean fluorescence intensity (MFI) expression was measured on this subpopulation using the FL4-H channel. PE (CD80 / CD163 / CD200r) MFI for each conjugated antibody was measured on the CD206⁺ gated subpopulation using the FL2-H channel (Figure 2-4). Values were expressed as the ratio of the MFI of the marker (APC or PE) to the MFI of the isotype control.

2.6.5 Measurement of lymphocyte subsets and activation

24 hours after BAL fluid collection non adherent cells from all tissue culture plates were pooled in a 50mL centrifuge tube and centrifuged at 500g for 10 minutes at 4°C. The pellet was resuspended and washed x 2 in FACS buffer and then resuspended in three aliquots of 80 μ L with FACS buffer in FACS tubes. The cells were incubated (1) unstained, (2) with fluorophore

conjugated antibodies to CD3, CD4, CD8 and CD38 and LIVE/DEAD® Blue stain or (3) isotype control and LIVE/DEAD® Blue stain, and then washed and fixed (as described in section 2.6.1). In parallel anti-mouse Ig kappa and negative control compensation beads (BD™ Compbeads, BD Biosciences) were incubated with each antibody conjugate separately. Labelled cells were then analysed on the LSRII. The beads were used to set a compensation matrix, unstained cells were used to set FSC and SSC, and isotype control labelled cells used to set the red 633nm (660/20 filter, APC), blue 488 nm (575/26 filter, PE and 530/30 filter FITC), violet 405nm (450/40 filter, brilliant violet), and UV 355nm (450/40 filter UV) laser voltages and filters. These were then kept the same for each subsequent donor sample. Lymphocytes were identified on FSC/SSC plots as before. Doublet cells were excluded using a FSC-A versus FSC-H event plot. Cells with high UV 450/40 (LIVE/DEAD® Blue) intensity on the singlet cell gate were considered to be dead lymphocytes and excluded. T lymphocytes were identified as CD3⁺ cells, defined as blue 575/26⁺ events in the live cell population. Back gating was performed to confirm that the CD3⁺ cells were within the original lymphocyte gate on FSC/SSC. CD4⁻/CD8⁺ (CD8⁺ T lymphocyte) cells were defined as violet 450/40⁺ red 660/20⁻ events and CD4⁺/CD8⁻ (CD4⁺ T lymphocyte) cells were defined as violet 450/40⁻ red 660/20⁺ in CD3⁺ population (Figure 2-5). The expression of CD38 on CD8⁺ T cells was defined as the ratio of the MFI on the blue 530/30 channel for CD3⁺/CD4⁻/CD8⁺ gated events to that of isotype control.

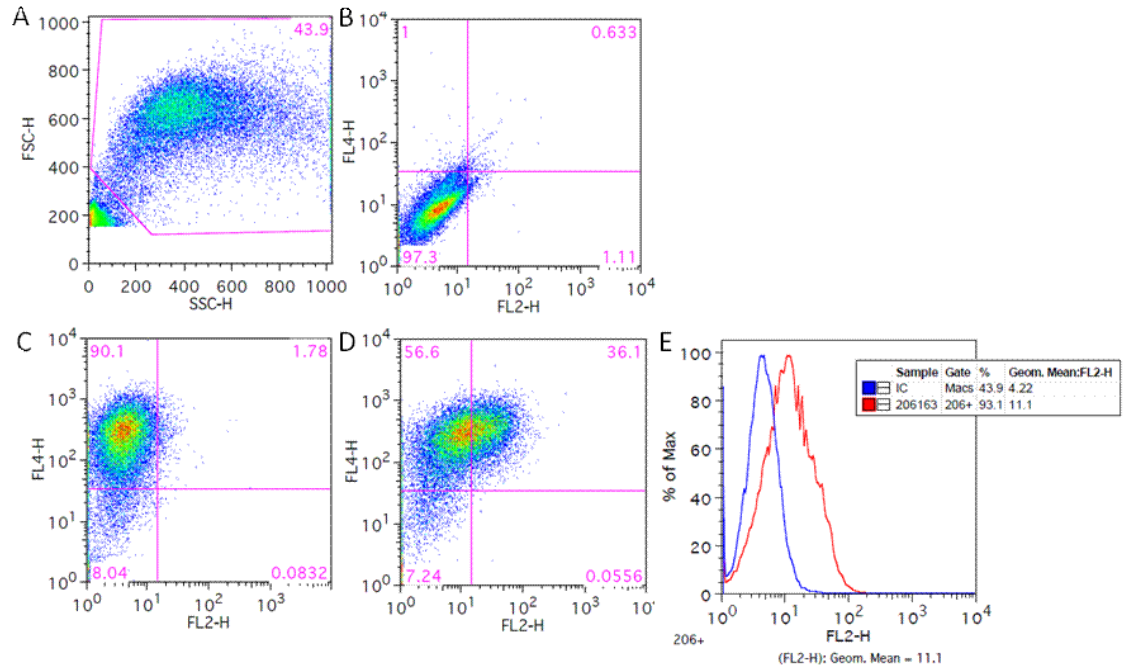


Figure 2-4 Gating strategy for measuring macrophage surface markers

Alveolar macrophages were isolated from donor BAL fluid and then labelled with anti-CD206-APC and anti-CD80-PE/ anti-CD163-PE/anti-CD200r-PE or isotype controls. Macrophages were identified on FSC/SSC (A) and red and blue laser voltages were set on this population with APC/PE isotype controls (B). CD206-APC expression was measured on the FL4 channel with PE isotype control (C) then CD163-PE expression was measured on the CD206⁺ subpopulation on the FL2 channel (D). The MFI of CD163 expression and isotype control were compared (E) to derive the GMR. CD80 and CD200r expression was measured as for CD163. FSC forward scatter, SSC side scatter, MFI geometric mean fluorescence intensity, GMR geometric mean ratio, APC allophycocyanin, PE phycoerythrin, IC isotype control

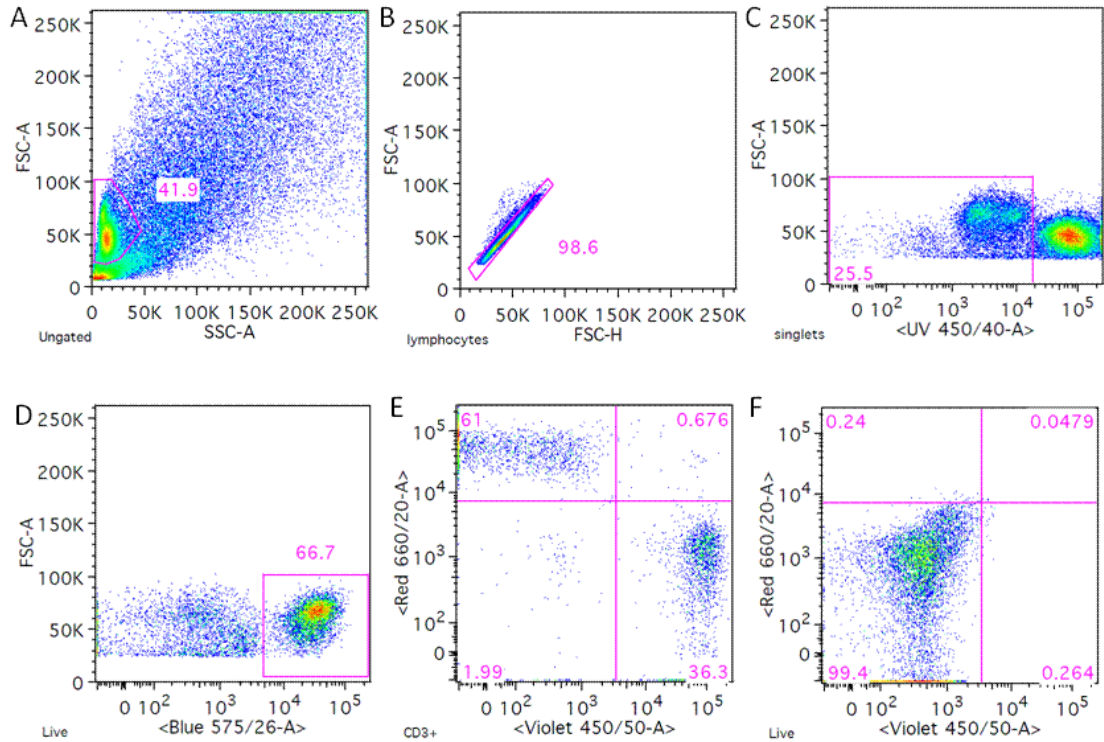


Figure 2-5 Gating strategy for measurement of BAL fluid lymphocyte subsets

Cells were isolated from BAL fluid and after 24 hours non adherent cells were washed and labelled with anti-CD3-PE, anti -CD4-APC, anti -CD8-brilliant violet, and a viability dye.

Lymphocytes were identified on FSC/SSC (A) and doublets excluded using FSC-A/FSC-H (B).

Cells expressing $>1 \log_{10}$ viability die on the UV 450/40 channel were considered dead and

excluded (C). CD3 expressing cells were identified on the blue 575/26 filter (D) and CD4⁺ T cells (APC⁺ brilliant violet⁻) and CD8 T cells (APC⁻, brilliant violet⁺) identified using the red 660/20 and violet 450/50 channels (E).

Cells incubated with PE, APC and brilliant violet isotype controls are shown for comparison (F). FSC forward scatter, SSC side scatter, APC allophycocyanin, PE phycoerythrin, IC isotype control

2.7 Measuring cell death

2.7.1 Nuclear Morphology

After 16-20 hours of infection, for wells with coverslips, media was removed and wells were gently washed with 1mL PBS which was then aspirated and replaced with 2% PFA for 1 hour. Subsequently the PFA was removed and the wells left in PBS at 4 °C overnight. Wells were aspirated to dryness and the glass coverslips were removed, inverted onto glass slides (Thermo Scientific) in 4',6-diamidino-2-phenylindole (DAPI) containing mounting medium (Vectorshield™, Vector Laboratories) and fixed in place with nail polish. When non adherent cells were to be analysed slides were made by centrifuging (Cytospin3, Standon) 150 µL of cell suspension onto glass slides at 10g (300 rpm) for 3 minutes, allowed to air dry and were fixed with methanol. Vectorshield™ medium was then dropped directly onto the slide and mounted with a glass coverslip and nail polish. Slides were read in blinded fashion to measure the rate of apoptosis by nuclear morphology. The DAPI filter of a fluorescence microscope (Leica DMRB 1000) was used with a 100 x oil emersion objective and 10 x eyepiece (1000 x magnification) to study the morphology of the nuclei of macrophages. Cells with condensed or fragmented nuclei were counted as apoptotic (Figure 2-6). At least 10 high power fields (HPF) or 300 cells (whichever the greater), starting at a set distance of 5 fields from the edge of the coverslip, were systematically counted in the same manner for each slide.

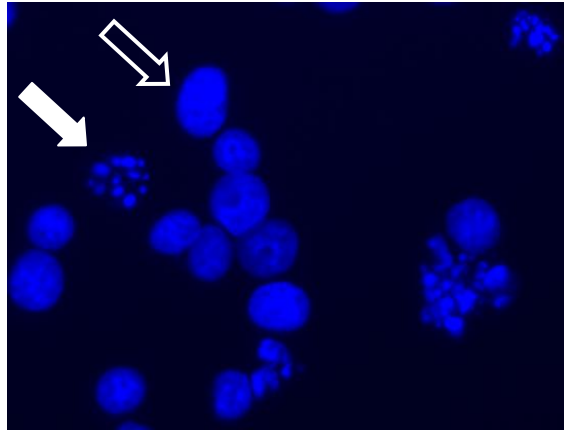


Figure 2-6 Chromatin fragmentation and nuclear condensation in DAPI stained U937 nuclei following *Streptococcus pneumoniae* challenge

U937 cells were seeded in 24 well plates at 2×10^6 and differentiated with 100nM PMA for 3 days then rested for 5 days before being challenged with *S. pneumoniae* at MOI = 10 for 20 hours. Cells were fixed and stained with 4',6-diamidino-2-phenylindole (DAPI) then examined with the DAPI filter of a fluorescence microscope at 1000 x magnification. Full arrow indicates condensed, apoptotic nucleus, empty arrow indicates non apoptotic nucleus.

2.7.2 TUNEL assay

In certain experiments, terminal deoxynucleotidyl transferase 2' –deoxyuridine, 5' – triphosphate (dUTP) nick end labelling (TUNEL, Millipore) was used to label apoptotic nuclei. In this method the 3'-OH ends of 180 bp DNA fragments, cleaved specifically during apoptosis, are labelled with digoxigenin-nucleotides by the catalytic action of terminal deoxynucleotidyl transferase (TdT) and a fluorescein isothiocyanate (FITC) conjugated anti-digoxigenin antibody is then used to identify these DNA fragments. Cells on coverslips were fixed with 2 % PFA and were permeabilised with ice cold 2:1 (100%) ethanol:acetic acid and then washed x2 with PBS. Each coverslip was then incubated at room temperature for 10 minutes with 13 µL of equilibration buffer then 11 µL of TdT enzyme - reaction buffer mix (containing nucleotides) for 1 hour at 37°C in a humidified chamber in the dark. Stop wash buffer was then applied for 10 minutes and then washed x 2 with PBS. Finally, the coverslips were incubated for 30 minutes in a humidified chamber in the dark at 37°C with 13 µL of anti-digoxigenin fluorescein conjugate all as per the manufacturer's instructions. Coverslips were then mounted with Vectorshield™ DAPI and inspected by fluorescence microscopy as described above (see 2.7.1). Cells demonstrating nuclear fragmentation and condensation were considered to be apoptotic if they also fluoresced green on the FITC filter (Figure 2-7).

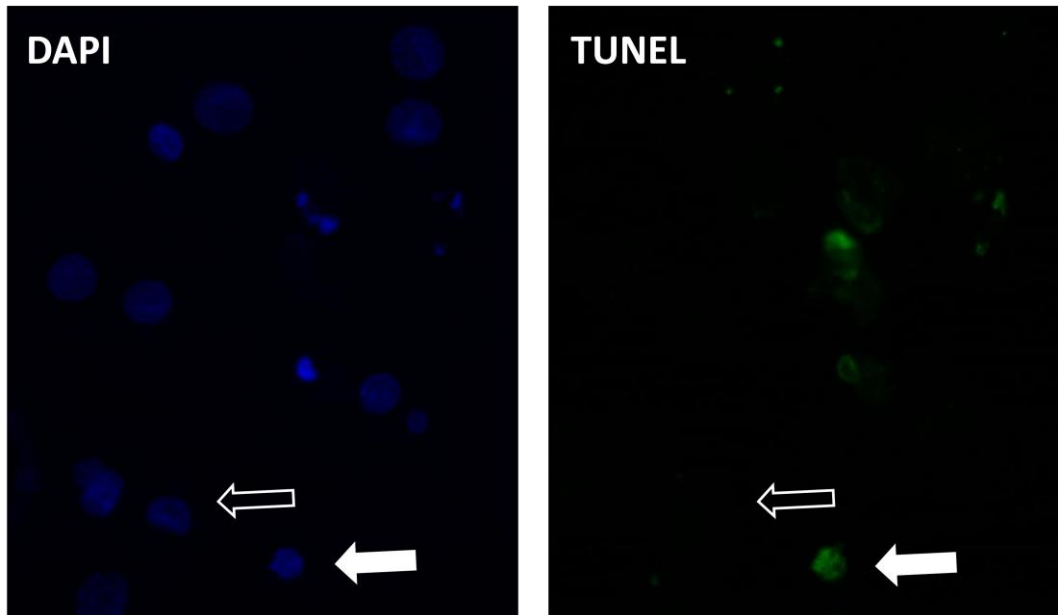


Figure 2-7 Photomicrograph of DAPI and TUNEL staining nuclei

U937 cells were seeded in 24 well plates at 2×10^6 and differentiated with 100nM PMA for 3 days then rested for 5 days before being challenged with *S. pneumoniae* at MOI = 10 for 20 hours. Cells were fixed and stained with terminal deoxynucleotidyl transferase 2' – deoxyuridine, 5' –triphosphate (dUTP) nick end labelling (TUNEL) and then 4',6-diamidino-2-phenylindole (DAPI) then examined with the DAPI and fluorescein isothiocyanate(FITC) filters of a fluorescence microscope at 1000 x magnification. Examples of TUNEL + condensed nuclei (full arrow) and TUNEL – non apoptotic nuclei (empty arrow) are shown.

2.7.3 Measurement of Caspase 3/7 activity

2.7.3.1 Luminescent Caspase 3/7 assay

Sixteen hours following challenge with *S. pneumoniae* or mock infection, a luminescent Caspase-Glo™ 3/7 (Promega) assay was used to measure caspase 3 and caspase 7 activity. In accordance with the manufacturer's instructions, the reagent was made by combining Caspase-Glo™ 3/7 substrate and buffer and allowing them to equilibrate to room temperature. An equal volume of reagent (100µL) was added to each well of the 96 well plate containing cells and their medium, agitated to mix and then incubated at room temperature for 90 minutes. Luminescence was then measured for each well using a plate reader (Varioskan™ Flash Multimode Reader with SkanIt™ Software). The assay has a luminogenic substrate with an Asp-Glu-Val-Asp (DEVD) caspase 3/7 specific cleavage site. Active caspase 3/7 cleaves and releases an amino-luciferin substrate which interacts with luciferase and results in luminescence.

2.7.3.2 Fluorimetric Caspase 3/7 assay

Caspase 3 and 7 activity was also measured with a combined caspase 3 and caspase 7 fluorimetric assay kit (Sensolyte® Homogeneous AMC Caspase - 3/7 Assay Kit, Anaspec). As caspase 3 and caspase 7 cleave the same amino acid sequence Asp-Glu-Val-Asp (DEVD), this kit uses acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin (Ac-DEVD-AMC) as a substrate which on proteolytic cleavage by these caspases generates the fluorogenic indicator 7-amino-4-methylcoumarin (AMC). This fluoresces bright blue and is detected at excitation/emission spectra 354 nm/ 442nm. The kit contains a buffer which also lyses the cells allowing fluorescence to be measured in the tissue culture plate by a plate reader. Sixteen hours following challenge with *S. pneumoniae* or mock infection adherent cells from 2-4 wells were washed twice with PBS, the wells were aspirated to dryness and the cells lysed with 300µL of kit lysis buffer. The lysate was transferred to a microcentrifuge tube and kept at on a rotor at 4

°C for 30 minutes. Samples were then centrifuged for 10 minutes at 1000g and supernatants were analysed for caspase 3/7 activity using the Sensolyte® fluorimetric assay as per the manufacturer's instructions. 100 µL of each supernatant was mixed with the Ac-DEVD-AMC substrate in an opaque 96 well plate (Costar) and incubated on a plate shaker for 1 hour before fluorescence was measured on a plate reader (excitation 380 nm, emission filter 460 nm, Varioskan™ plate reader). A standard curve was generated by making 2-fold serial dilutions of 60 µM AMC from the kit to obtain 30, 15, 7.5, 3.75, 1.88, 0.94, 0.47 µM concentrations along with a water blank which were included in the 96 well plate with the supernatants. Fluorescence intensity readings were converted to relative caspase activity by interpolation from the AMC standard curve using GraphPad prism v5.04 software (GraphPad Inc.)(Figure 2-8). Supernatants were also analysed for protein content using a Pierce BCA Protein Assay (see section 2.8.2). Relative caspase 3/7 activity results were then normalised for each sample according to the protein content.

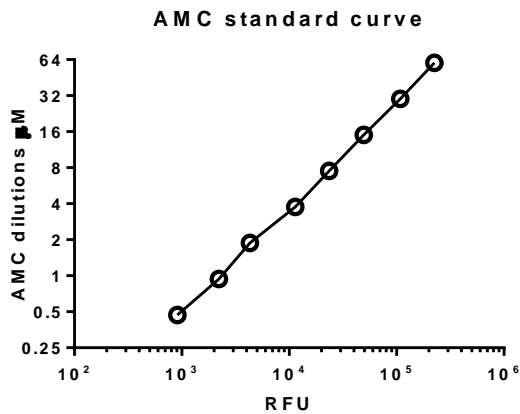


Figure 2-8 Representative AMC standard curve as used to derive relative caspase activity from RFU measurements with fluorimetric caspase 3/7 assay

Serial 2-fold AMC dilutions of known concentration and cell lysates from *S. pneumoniae* challenge experiments were treated with fluorogenic caspase 3/7 kit reagents and the RFU measured at excitation/emission spectra 380/460 nm. A standard curve was created and the AMC concentration extrapolated for samples to give a measure of caspase 3/7 activity. AMC 7-amino-4-methylcoumarin, RFU relative fluorescence units

2.7.4 Measuring hypodiploid DNA

After 16-20 hours of infection, adherent cells were removed with a cell scraper and, together with the media and any non adherent cells in the well, centrifuged at 400g for 5 minutes and then washed x 2 in PBS. They were then re-suspended in 300 μ L of ice cold PBS, then made up to a 1 mL suspension with 100% ice cold ethanol (final concentration 70% ethanol) and kept at 4 °C overnight. Cell suspensions were washed x 2 in PBS and re-suspended in 200 μ L of PBS with 0.2 mgml⁻¹ of RNase A (DNase free) (Sigma) with 20 μ gml⁻¹ propidium iodide (PI, Sigma) for 30 minutes. Samples were run on the FACSCalibur with cells identified and gated using FSC versus SSC event plots. Doublets were excluded from this population using FL2-W versus FL2-A event plots. PI intensity was measured on this population in the FL-2 channel (562-588nm) on a linear scale to enable cell cycle analysis and after gating for debris, the sub G0/G1 population of cells with accumulation of hypodiploid DNA were recorded as a proportion of the total cells, as a measure of apoptosis (Nicoletti, Migliorati et al. 1991).

2.8 Measurement of protein expression by Western Blot

2.8.1 Protein extraction

At the desired time point, typically 20 hours post infection, protein was extracted from the cells using the tri-chloroacetic acid (TCA) precipitation method (Wang, Posner et al. 1996). Cells were washed in PBS x1 and then 600 μ L of sodium dodecyl sulphate lysis buffer was added (20mM TRIS-HCl pH7.4, 5mM Ethylenediaminetetraacetic acid (EDTA), mM ethylene glycol tetraacetic acid (EGTA), 150mM NaCl and 1% SDS) along with a commercial protease inhibitor cocktail containing inhibitors of pancreas extract, metalloprotease, chymotrypsin, trypsin and papain (Complete™, Roche). After a few seconds cells were scraped vigorously with a cell scraper (Costar). 100 μ L of 100% TCA (Sigma-Aldrich) was then added to the lysate to precipitate out the DNA, which was carefully removed. The remaining lysate was centrifuged in a bench top centrifuge (Eppendorf) at 12,000g for 5 minutes, the supernatant discarded and

pellet washed in 1 ml 2.5% TCA. After a second centrifugation at 12,000g x 5 minutes the supernatant was removed and the pellet dissolved in 40 μ L 3M Tris base at 4 °C overnight. An equal volume of water was then added to dilute to 1.5M Tris and the samples then stored at -20°C.

2.8.2 Protein quantification assays

Protein concentrations were determined using commercial colorimetric kits: DC Protein Assay (Biorad) and Pierce BCA Protein Assay (Thermo Scientific). A standard curve of protein concentration was generated using duplicate serial dilutions of bovine serum albumin (BSA) in the same plate as test samples. Reagents were added as per the manufacturer's instruction and then, for the DC assay, colour formation estimated in a plate reader at a wavelength of 630 nm. For the Pierce assay, absorbance was measured at 562 nm. Protein concentrations were determined by interpolating from the standard curves using GraphPad Prism™ v6.01 (GraphPad Software Inc.).

2.8.3 SDS PAGE

Protein separation was performed using sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS PAGE). Equal quantities of protein from each sample were denatured by diluting 1:1 in reducing Laemmli buffer (Laemmli 1970) and heating to 90°C for 5 minutes. These were each then loaded into the lanes (either 10 or 15) of a 1.5 mm thick, 12% poly acrylamide gel in running buffer (0.25M Tris-Base, 1.9M glycine and 1% SDS). The electric current was applied at 80 V until the protein had passed the stacking gel and then at 180-200 V until the dye in the buffer had passed to the bottom of the resolving gel. 5 μ L of a protein ladder (Colour plus protein ladder™ S7711, New England Biolabs) was added to the first lane.

2.8.4 Western blot semi dry transfer

Protein from the SDS PAGE gel was transferred to a nitrocellulose membrane (BioRAD) by semi-dry blotting. The membrane was cut to the size of the gel and activated by soaking in

distilled H₂O. Six similarly sized pieces of filter paper were soaked in Towbin transfer buffer (25mM Tris-Base, 192mM glycine, 20% methanol). The gel was placed on top of the membrane and sandwiched between filter paper and then placed in a semi-dry transfer blotter (Trans-Blot SD transfer cell, BioRad) and the current applied at 15 V for 30 minutes (Towbin, Staehelin et al. 1979).

2.8.5 Protein detection by chemiluminescence

After transfer the presence of protein bands on the nitrocellulose membrane were confirmed using Ponceau S stain. The membranes were then washed in Tris buffered saline (TBS)-Tween then blocked with 5% milk buffer (5% w/v fat free milk in TBS containing 0.05% Tween) for 60 minutes at room temperature on a plate shaker. Membranes were then incubated overnight with primary antibodies at 1:5000 – 1:500 dilutions in 5% milk buffer. The following antibodies were used: anti-Mcl-1 (rabbit polyclonal, 1:500 S-19: sc-819, Santa Cruz Biotechnology, INC.) and anti-actin (rabbit polyclonal, 1:5000 Sigma-Aldrich), was used as a loading control for total protein. After incubation with the primary antibody, membranes were washed three times in TBS-Tween for a total of 15 minutes and then incubated with 1:5000 dilution horseradish peroxidase (HRP)-conjugated goat anti rabbit antibody (Dako) for one hour at room temperature on a shaker. The membranes were washed in TBS-Tween as before and then protein labelled with antibody was detected by enhanced chemiluminescence (ECL) and light sensitive film (Amersham).

2.8.6 Densitometry

The optical density of bands on the light sensitive film detected by western blot was measured by densitometry to enable a semi quantitative analysis of protein expression. Transparencies were scanned using a document scanner (Epson perfection V330 photo) to create a JPEG image. The density of the bands was measured using ImageJ™ software v1.440. To correct for differential loading of the gel lanes the ratio of the density of each band to the actin loading

control was measured using spreadsheet software (Excel™ Microsoft). This ratio was then compared with the control condition for each MDM *S. pneumoniae* challenge experiment, namely sham MDM, mock-infected, to derive a measure of change from control.

2.8.7 Measurement of mitochondrial superoxide

To measure the generation of mitochondrial superoxide (O_2^-) a fluorogenic dye, MitoSOX™ Red (Invitrogen) was used. This dye contains dihydroethidine which is targeted to the mitochondria and undergoes O_2^- dependent hydroxylation to 2-hydroxyethidium which fluoresces at excitation/emission spectra of 400/590nm (Robinson, Janes et al. 2008). The MitoSox dye was dissolved in dimethyl sulfoxide (DMSO) and then diluted in Hank's Balanced Salt Solution (HBSS, Gibco) without phenol red to make a 2.5 μ M staining solution as per the manufacturer's instructions. MDM were cultured in 96 well plates at a final density of 4×10^4 per well for subsequent HIV_{BAL} or sham infection followed by pneumococcal challenge experiments. At the end of infection 0.5 mL of staining solution was added to each well and incubated for 15 minutes at 37 °C in the dark. Wells were then washed three times with HBSS warmed to 37°C. 200 μ L of PBS was added to each well and the plate kept on ice for no more than 30 minutes while it was sealed, cleaned and removed from the CL3 facility. Fluorescence was then measured on a Varioskan™ Flash Multimode Reader plate reader using SkanIt™ Software (Thermo Fisher Scientific Inc.) at excitation/emission spectra of 410/590nm. As the dye is not retained after fixation, plates were analysed with unfixed cells but then immediately resealed and returned to the CL3 facility for disposal.

2.8.8 Measurement of mitochondrial density

To measure any change in the number of mitochondria a green-fluorescent mitochondrial stain MitoTracker™ Green FM (Invitrogen) was used. This localizes to the mitochondria and the density of mitochondria can be inferred from the intensity of the signal at excitation/emission spectra of 490/516 nm. MDM were cultured in 96 well plates and infected with HIV-1 or sham

then subsequently challenged with pneumococci or mock-infected as above (see 2.8.7). A 1 mM working solution was made by diluting the supplied dye in 74.4 μ L of DMSO and a staining solution made by adding 1 μ L of working solution to 2 mL of serum free RPMI 1640 medium. At the end of pneumococcal challenge, medium was removed and wells washed in PBS prior to 15 minutes incubation at 37 °C with 200 μ L of staining solution per well. The solution was then replaced with pre-warmed PBS and taken to be read on the Viroskan plate reader at the 490/516nm settings and then disposed of as above (see 2.8.7).

2.9 Statistical Methods

All data are shown as mean plus or minus the standard error of the mean unless otherwise indicated. Statistical analyses were performed using GraphPad Prism (GraphPad Inc.) version 6. Matched data from within experiments were compared with paired Student's t-test (parametric data) or Wilcoxon matched pairs test (non parametric). Alternatively, for unmatched groups where data were derived from different experiments, unpaired Students t-test or Mann Whitney test were used. One way ANOVA (parametric) and Kruskal Wallace (unmatched, non parametric) or Friedman's (matched, non parametric) tests were used where 3 or more groups were compared and Two-way ANOVA was used where two factors were being compared simultaneously. Categorical data were compared using Chi square and Fischer's exact tests. The strength of correlation was measured with Pearson's test. For flow cytometry data geometric means were calculated using FlowJo™ software version 9.3.2 (Tree Star, Inc.). All tests were 2 way and statistical significance was defined as $p < 0.05$.

Chapter 3. HIV-1 infection is associated with attenuation of macrophage apoptosis in response to *Streptococcus pneumoniae*

3.1 Introduction

Macrophages play a crucial role in the defence against potentially invasive *S. pneumoniae*, particularly in the lung; alveolar macrophages recognise, phagocytose and kill *S. pneumoniae* (Jonsson, Musher et al. 1985). An additional capability of the macrophage is the ability to engage apoptosis-associated killing that occurs after the initial phase of phagolysosomal killing and may be sufficient to contain infection without the need for neutrophil recruitment to the lung (Dockrell, Marriott et al. 2003; Marriott, Bingle et al. 2005; Bewley, Marriott et al. 2011). HIV-1 infection is associated with attenuation of apoptosis in macrophages, contributing to their persistence as a latent reservoir of HIV-1 (Crowe, Zhu et al. 2003; Giri, Nebozyhn et al. 2009). It is not known whether the perturbation of apoptosis by HIV-1 has any impact on *S. pneumoniae* associated macrophage apoptosis.

Both *in vitro* cell culture of macrophage cell lines and primary macrophages as well as *in vivo* mouse models have been used to describe the contribution of apoptosis-associated killing by macrophages to pneumococcal clearance (Dockrell, Marriott et al. 2003; Marriott, Hellewell et al. 2006; Bewley, Marriott et al. 2011). Cell lines can be expanded in culture to enable experimental protocols requiring high numbers of cells and both monocyte-derived macrophages (MDM) and cell lines are more accessible than whole animal models. The promonocytic cell line, THP-1 can be induced to differentiate towards a macrophage-like phenotype by treatment with phorbol 12-myristate 13-acetate (PMA), a phorbol ester which activates protein kinase C (PKC) (Daigneault, Preston et al. 2010) and can be used to model *S. pneumoniae* associated macrophage apoptosis (Bewley, Marriott et al. 2011). U937 is a promonocytic cell line similar to THP-1, that originates from a histiocytic lymphoma

(Sundstrom and Nilsson 1976), and can also be differentiated into a macrophage-like phenotype (reviewed in (Harris and Ralph 1985)). The U1 line is a clonal derivative of U937 cells that have been infected with the X4 HIV-1_{LAI/III B} strain. Integrated HIV-1 pro-viral DNA is latent in U1 but its expression can be induced with PMA (Folks, Justement et al. 1988). Together, U937 and U1 have been used as *in vitro* models to compare the effect of HIV-1 infection on monocyte/macrophage behaviour (Cassol, Alfano et al. 2006; Patel, Zhu et al. 2007). Published studies comparing U937 and U1 have used short periods of exposure to PMA (typically 24 to 48 hours) to activate transcription of HIV-1 in the U1 (Tachado, Zhang et al. 2005; Nicol, Mathys et al. 2008). In addition, PMA exposure for this duration starts to differentiate cells towards a macrophage like phenotype but the extent of differentiation is less well characterised (Tachado, Zhang et al. 2005; Nicol, Mathys et al. 2008). Thus the U937 and U1 cell lines present a potential macrophage model with which to study the effect of HIV-1 on *S. pneumoniae* associated apoptosis.

MDM from healthy human volunteers are a source of primary macrophages recognized as an *in vitro* model of differentiated tissue macrophages (Gantner, Kupferschmidt et al. 1997) and have also been employed as models of the interaction between macrophages and *S. pneumoniae* infection (Ali, Lee et al. 2003). MDM express CD4 and CCR5 on their surface and can be productively infected *in vitro* with CCR5 tropic HIV-1 (HIV-1_{BAL}) (Tsang, Chain et al. 2009) making it possible to interrogate of the effects of HIV-1 infection on macrophage responses to pathogens (Crowe, Vardaxis et al. 1994).

I have therefore set out to differentiate U937 and U1 cells and compare how they develop towards a macrophage-like phenotype. Next I have compared the effect of HIV-1 infection on the cell lines' apoptotic responses to *S. pneumoniae* at different stages of differentiation. Subsequently, after propagating the CCR5 tropic laboratory strain HIV-1_{BAL} *in vitro*, I have set

up cultures of productively HIV-1-infected MDM and examined the consequences of HIV-1 on the apoptotic responses of MDM to *S. pneumoniae* infection.

3.2 Results

3.2.1 U937 and U1 promonocytic cell lines can be differentiated similarly towards a macrophage-like phenotype

Both the U937 and U1 are promonocytic cell lines and need to be induced to differentiate further before they can be used as models of monocytes or macrophages. PMA induces the differentiation of promonocytic cell lines like THP-1 towards a macrophage-like phenotype that resembles MDM (Daigneault, Preston et al. 2010). As the effect of PMA on U937 and U1 differentiation has been less extensively evaluated, a range of PMA concentrations and incubation times were used to characterize and compare the differentiation of these two cell lines towards a macrophage phenotype. 1 mL of 2×10^6 cells/mL of each cell type were seeded in 24 well culture plates, treated with 0, 10, 50, 100 or 200 nM PMA for 24 hours and allowed to adhere to the plastic before the medium was then refreshed. Granularity, autofluorescence and cell survival were measured using flow cytometry and trypan blue exclusion after a further 1 day (PMA1r1) or 3 days (PMA1r3) of resting in growth medium (

Figure 3-1). Both protocols resulted in significant and similar increases in granularity of U937 and U1 cells; after a single day of resting granularity (geometric mean side scatter) increased a similar amount with all concentrations of PMA ($p < 0.0001$, 2 way ANOVA); after three days resting maximal changes in granularity were achieved with concentrations of 100 nM PMA or greater ($p < 0.0001$, 2 way ANOVA). Autofluorescence increased with PMA concentrations of 50 - 200nM in the PMA1r3 protocol, but with no difference between U1 and U937 ($p < 0.05$, 2 way ANOVA). Autofluorescence did not increase when only one day resting (PMA1r1) was used regardless of the concentration of PMA. All strategies resulted in loss of viable cells which were greater with longer resting, although the differences were not statistically significant (2 way ANOVA). The 50nM PMA1r3 protocol was judged to result in the optimal balance of

differentiation and cell loss and so was chosen as an initial differentiation protocol to prepare cells for exposure to *S. pneumoniae*.

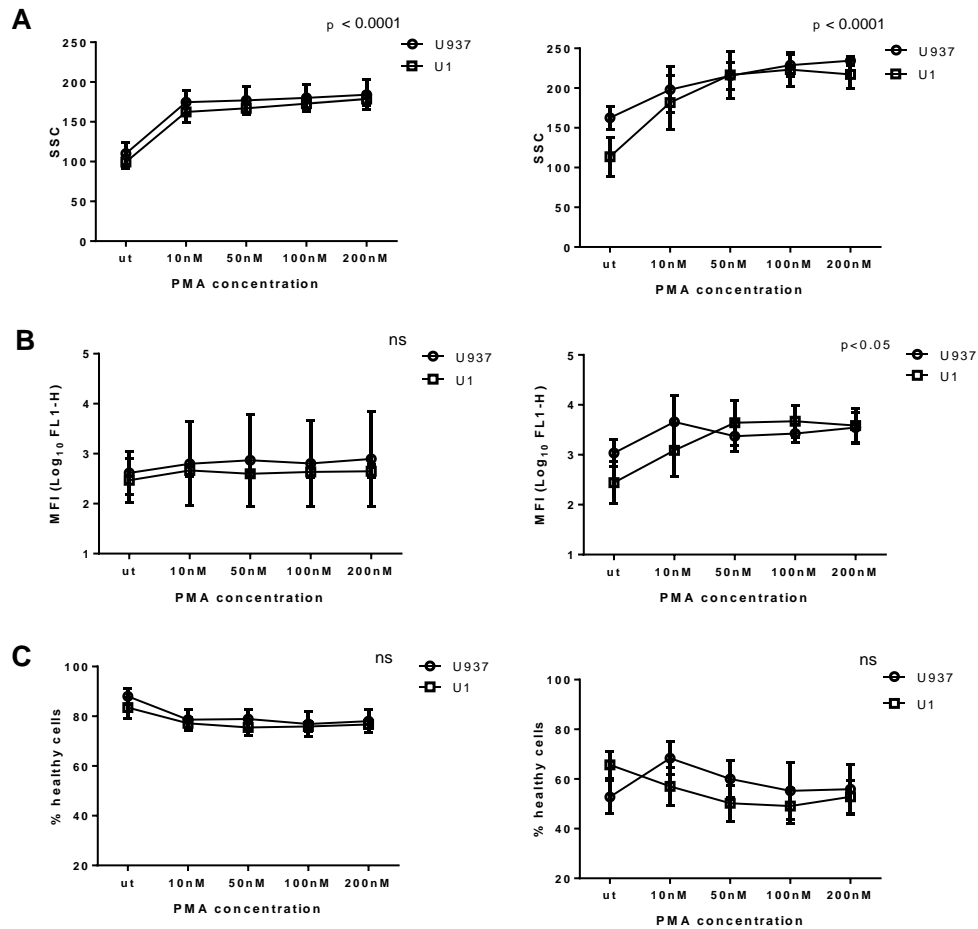


Figure 3-1 Characterisation of morphological changes in U937 and U1 following treatments with PMA.

U937 and U1 cells were treated with 10, 50, 100 or 200nM PMA or medium alone for 24 hours. The medium was then refreshed and morphological changes were measured after a further 24 hours (left hand column) or 3 days of resting (right hand column). Granularity (A) and auto-fluorescence (B) were measured using flow cytometry and cell survival (C) using trypan blue exclusion. ut= untreated, n=3-4, ns= non significant (for PMA dose) 2 way ANOVA.

3.2.2 The induction of apoptosis by *Streptococcus pneumoniae* is reduced in U1 compared with U937 cells after a brief differentiation period

U937 and U1 cells were differentiated with 50nM PMA for one day then rested for 3 days (50nM PMA 1r3). The differentiated U937 and U1 cell lines were challenged with opsonised *S. pneumoniae* serotype 2 (D39) at a multiplicity of infection (MOI) of 10 or mock-infected for up to 20 hours. After infection the number of cells remaining adherent to coverslips was significantly lower ($p=0.002$) and there was a trend for this to be more pronounced in U937 (11.5±4.1% remaining) than the U1 cells (33.3±16.6%) by 20 hours following *S. pneumoniae* challenge vs. 20 hours mock infection ($n=5$, $p=0.065$, paired t test) (Figure 3-2). After infection cells were also gently scraped from the plastic, stained with propidium iodide (PI) and subjected to cell cycle analysis by flow cytometry. Cells containing less DNA than those in growth phase (sub G0/1) were considered to be apoptotic (hypodiploid DNA). Apoptosis was compared between U937 and HIV-1-infected U1. There were significant increases in the number of cells with hypodiploid DNA for both U1 ($p<0.01$) and U937 ($p<0.0001$) after 20 hours exposure to *S. pneumoniae* compared with 20 hours mock infection and for U937 the increase in apoptosis from 16 hours to 20 hours post *S. pneumoniae* was also significant (2 way ANOVA with Bonferroni's post test) (Figure 3-3A). Comparison of the 20 hour mock-infected cells showed a trend towards a higher level of apoptosis in U1 than U937 cells at $9.3 \pm 0.7\%$ vs. $6.0 \pm 0.7\%$, although the difference was not significant ($n=3$) (Figure 3-3A). However, with exposure to *S. pneumoniae* the increase in apoptosis at each time point was significantly greater in U937 than U1 ($p<0.05$, 2 way ANOVA), with an absolute difference in the level for U937 vs. U1 cells at each time point; 12 hours (1.4%), 16 hours (4.3%) and 20 hours (8.5%) (Figure 3-3B).

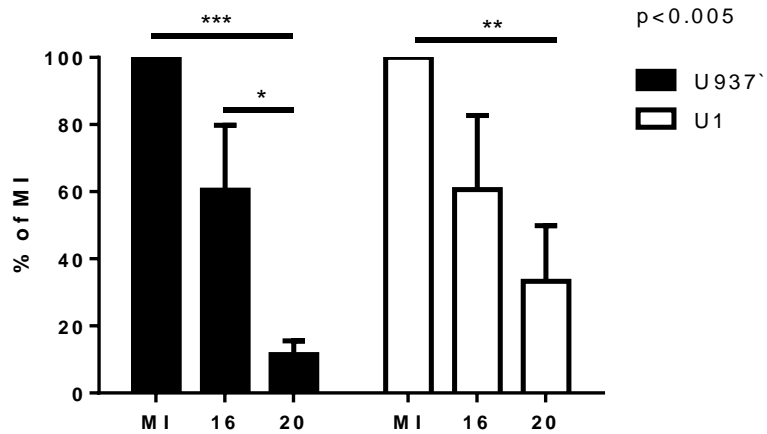


Figure 3-2 After a brief differentiation protocol there is loss of adherent cells following challenge with *Streptococcus pneumoniae*, which is less marked for U1 than U937

U937 and U1 cells were treated with 50nM PMA for 24 hours then rested for 3 days (PMA1r3) before being challenged *S. pneumoniae* or mock-infected (MI). At the indicated time points (hours), wells were washed and fixed and the numbers of adherent cells per high power field were counted by microscopy. Values represent % of adherent cells compared with paired 20 hour mock-infected replicates at each time point, n=5, ***p<0.001, **p<0.01, *p<0.05 2 way ANOVA with Bonferroni's post test.

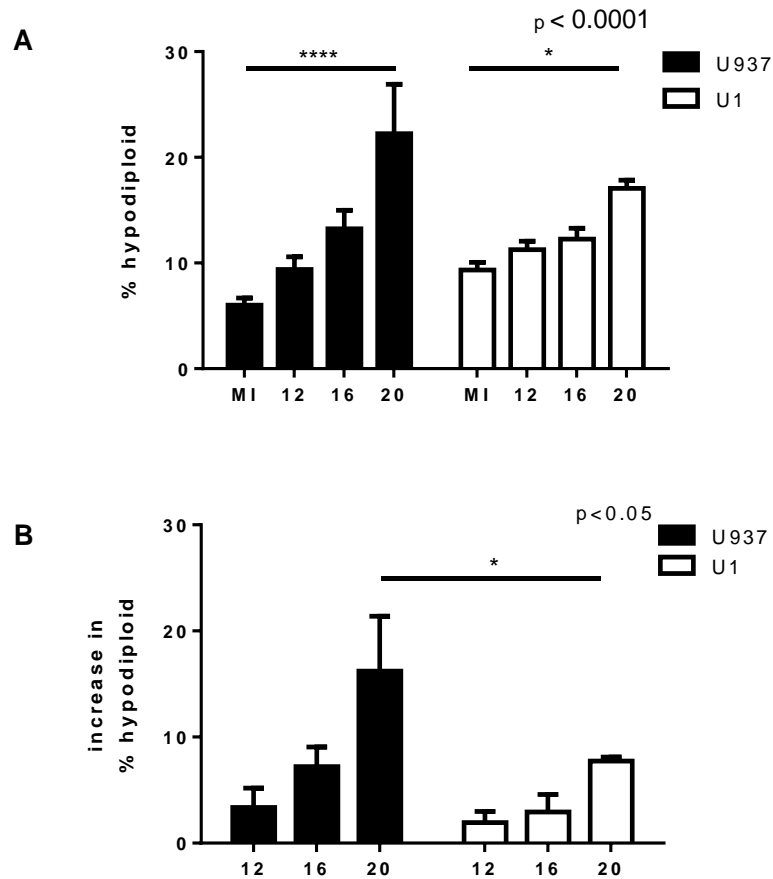


Figure 3-3 After a brief differentiation protocol U1 demonstrate less induction of apoptosis than U937 during infection with *Streptococcus pneumoniae*.

U937 and U1 cells were treated with 50nM PMA for one day then rested for 3 days (PMA1r3) before being challenged with *S. pneumoniae* or mock-infected. At the indicated time points (hours), hypodiploid DNA content of cells was measured using flow cytometry. Cells with less DNA content than cells in growth phase (sub G0/1) were considered to have hypodiploid DNA and be apoptotic. **(A)** The proportion of cells with hypodiploid DNA and **(B)** the difference between *S.pneumoniae* challenged and mock-infection. MI = 20 hours mock-infection n=3, *p<0.05, ****p<0.0001, 2 way ANOVA with Bonferroni's post test.

3.2.3 Characterisation of U937 and U1 cell lines following a more extended PMA differentiation period

To further differentiate the U937 and U1 cells further toward a macrophage-like phenotype, the differentiation protocol was extended. U937 and U1 cells were incubated with 100nM PMA for 3 days and then rested for a further 2 to 5 days in fresh growth medium, a variation on the strategy of prolonged resting to allow greater differentiation that has been shown to be effective in the THP-1 cell line (Daigneault, Preston et al. 2010) . As they differentiated, both U937 and U1 cells became significantly more granular (Figure 3-4 A, $p < 0.0001$), larger (Figure 3-4 B, 2 way ANOVA, $p < 0.01$) and developed increasing autofluorescence (Figure 3-4 C, $p < 0.0001$). Increases in size and granularity were less pronounced in U1 compared with U937 but while these morphological differences were also visible microscopically (Figure 3-4 E, F), the differences were not statistically significant. There was a decline in the number of cells during the differentiation process (Figure 3-4 D). At the end of the extended differentiation protocols, surviving cells were generally more adherent than those differentiated with the briefer protocol (data not shown). Cells differentiated for 5 days (PMA3r2) demonstrated marked increases in granularity, size and autofluorescence which were similar to the longer protocol 8 day protocol (PMA3r5) but with less cell loss. The PMA3r2 protocol was therefore used to differentiate cells further toward a macrophage phenotype for repeat *S. pneumoniae* challenge experiments.

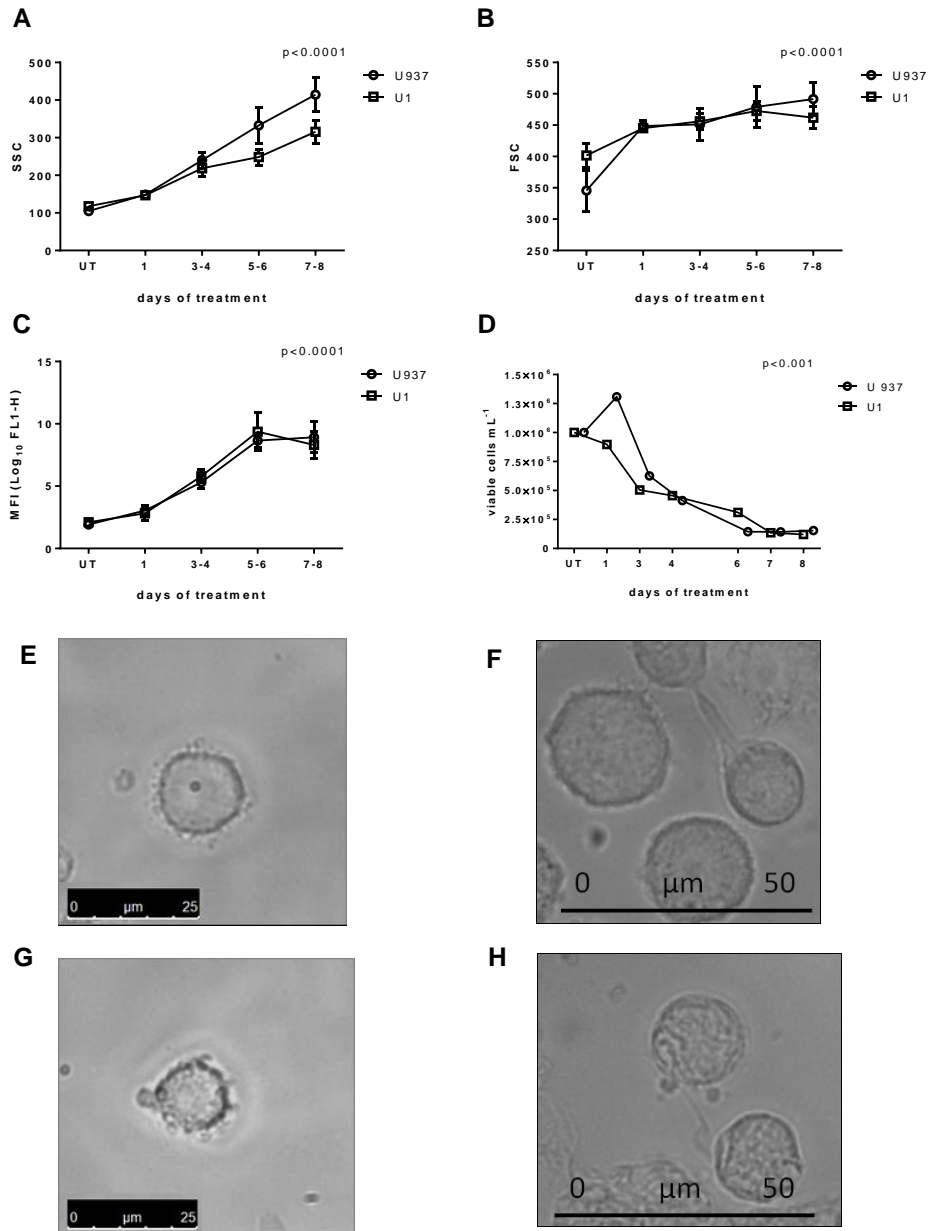


Figure 3-4 Characterisation of morphological changes in U937 and U1 following extended PMA differentiation protocols.

U937 and U1 cells were treated with 100nM PMA for 3 days and then allowed to rest in fresh medium for up to 5 days. Cells were fixed then analysed by flow cytometry for (A) cell granularity (SSC), (B) cell size (FSC) and (C) auto-fluorescence (FL1-H). (D) Cell viability rates were measured by trypan blue exclusion. Photomicrographs of U937 (E,F) and U1 (G,H) cells at day 0 and day 8. UT = untreated, n≥3, 2 way ANOVA.

3.2.4 The induction of apoptosis by *Streptococcus pneumoniae* is reduced in U1 compared with U937 cells after an extended differentiation period.

U937 and U1 cells treated with 100nM PMA for 3 days and rested in fresh media for a further 2 days were challenged with *S. pneumoniae* or mock-infected as before. At various time points following infection cells were gently scraped from the plastic, stained with propidium iodide (PI) and subjected to cell cycle analysis by flow cytometry. Cells with hypodiploid DNA were considered to be apoptotic. The gating strategy to exclude debris and perform cell cycle analysis is shown in Figure 3-5. With mock-infection only, U1 showed a trend for greater apoptosis compared with U937 with 26.1±4.7% vs. 16.9±2.5% hypodiploid DNA (n=3, ns) (Figure 3-6 A). A population of cells with decreased forward scatter was seen 20 hours following challenge with *S. pneumoniae* for both cell types. There were significant increases in the number of cells with hypodiploid DNA by 16 hours post *S. pneumoniae* challenge for U937 and by 20 hours for both cell types (Figure 3-6 A + B). The induction of apoptosis 20 hours following D39 challenge was significantly greater for U937 than U1 ($p < 0.01$, 2 way ANOVA), with a mean difference of 12.9±4.7% at 20 hours ($p < 0.05$, Bonferroni's post test)(Figure 3-6 C).

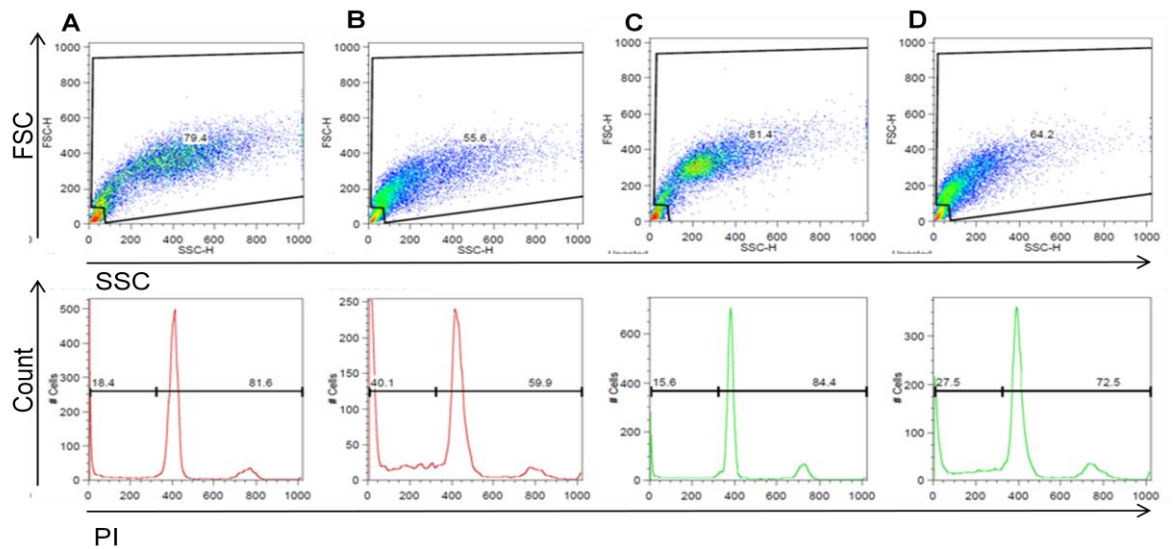


Figure 3-5 Representative flow cytometry dot plots and histograms for U937 and U1 hypodiploid DNA measurement following *Streptococcus pneumoniae* challenge or mock-infection.

A gate was set on FSC/SSC event plot to exclude debris and PI intensity was measured in 100nM PMA 3r2 differentiated cells. (A) U937 and (C) U120 hours mock-infected, (B) U937 and (D) U1 20 hours after *S. pneumoniae* challenge. Hypodiploid DNA containing cells are those to the left of the G0/1 peak on a linear scale. SSC side scatter, FSC forward scatter, PI propidium iodide

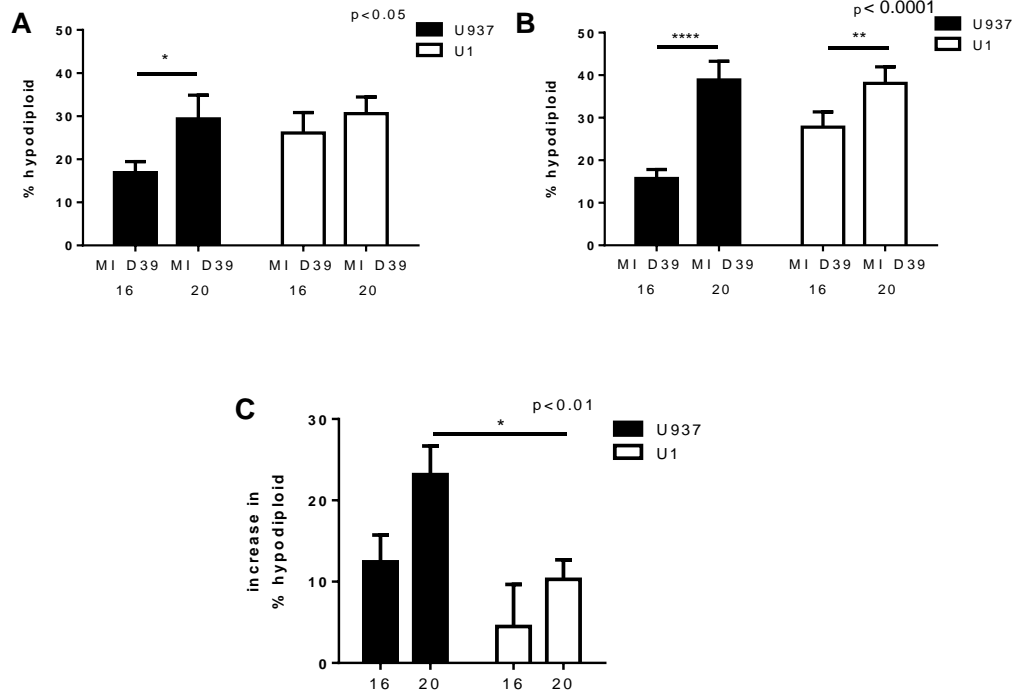


Figure 3-6 After an extended differentiation period U1 demonstrate less induction of apoptosis than U937 following challenge with *Streptococcus pneumoniae*.

U937 and U1 cells were treated with 100nM PMA for 3 days then rested for 2 days before being challenged with *S. pneumoniae* or mock-infected (MI). At the indicated time points (hours), hypodiploid DNA content of cells was measured using flow cytometry. Cells with less DNA content than cells in growth phase (sub G0/1) were considered apoptotic. **(A)** Proportion of cells with hypodiploid DNA at 16 hours and **(B)** at 20 hours. **(C)** Difference between *S.pneumoniae* challenged and 20 hours mock-infection. n= 6-8 *p< 0.05, **p<0.01, ***p<0.0001,, 2 way ANOVA, with Bonferroni's post test).

3.2.5 There is reduced induction of caspase 3/7 following challenge with *Streptococcus pneumoniae* in U1 compared with U937 cells after an extended differentiation period

S. pneumoniae associated macrophage apoptosis involves caspase 3/7 activation (Bewley, Marriott et al. 2011). Increased caspase 3/7 activity can be used to measure apoptosis (Akhter, Gavrilin et al. 2009; Daigneault, De Silva et al. 2012). U937 and U1 cells were differentiated with 100nM PMA for three days and rested for 2 days as above (PMA3r2). Lysis buffer from the kit was then added to whole wells 16 or 20 hours following mock-infection or D39 challenge and caspase 3/7 activity was measured in the lysates. Fold change in caspase 3/7 activity compared to mock-infection was calculated for each time point and cell type. The fold change in caspase 3/7 activity was significantly greater for U937 at 16 hours (3.1 fold) and 20 hours (3.5 fold) vs. 20 hours mock infection than U1 cells (n=4, two-way ANOVA with Bonferroni's post test p=0.0002)(Figure 3-7).

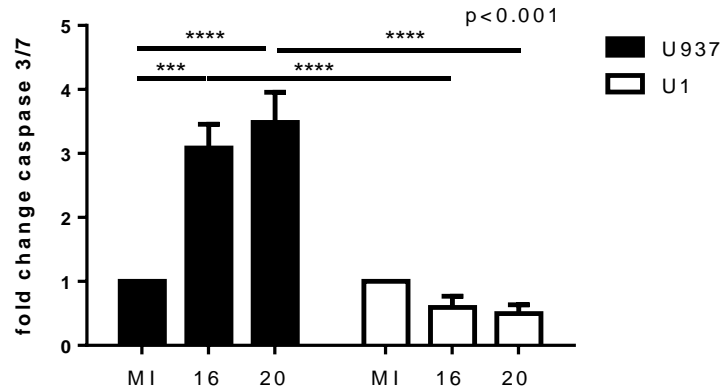


Figure 3-7 Following an extended differentiation period U1 demonstrate less induction of caspase 3/7 activity than U937 cells following *Streptococcus pneumoniae* challenge.

At the indicated number of hours following *S. pneumoniae* challenge or 20 hours mock-infection (MI) cells were lysed and caspase 3/7 activity was measured using fluorometric caspase 3/7 assay. n=4, **** p<0.0001, *** p<0.0005, 2 way ANOVA with Bonferroni's post test.

3.2.6 The induction of apoptosis by *Streptococcus pneumoniae* is reduced in U1 compared with U937 cells after a prolonged, eight day differentiation period.

THP-1 cells differentiated for as long as eight days and have been shown to have many of the characteristics of MDM and work well as a macrophage model for *S. pneumoniae* challenge experiments (Daigneault, Preston et al. 2010; Bewley, Pham et al. 2011). To reflect this prolonged protocol, U937 and U1 cells were treated with 100nM PMA for 3 days and rested in fresh media for a further 5 days (PMA 3r5), and then challenged with *S. pneumoniae* or mock-infected. Wells were washed and fixed at 16 and 20 hours after bacterial challenge. Apoptosis was measured by observation for typical changes in nuclear morphology. Hypodiploid peak analysis was not used as these more differentiated macrophages are not so easily detached by scraping without significant damage to the cells (data not shown). By 20 hours post *S. pneumoniae* challenge a similar proportion of U937 and U1 (31.6% vs. 28.8%, ns, n=4) remained adherent to the glass cover slips. However, there was a significant increase in the proportion of apoptotic nuclei for U937 at 53.7%±14.5 (p=0.0134), but not U1 at 24.2%±11.4 when compared to 20 hours mock-infection (ns, n=4). Overall, U1 showed a reduced induction of apoptosis when compared to U937 at 20 hours post challenge with *S. pneumoniae* (difference in induction = 29.5±13.2%, p=0.0442, 2 way ANOVA with Fisher's post test)(Figure 3-8).

3.2.7 Infection of monocyte-derived macrophages with HIV-1

To investigate the effect of HIV-1 on the behaviour of primary macrophages, monocyte-derived macrophages (MDM) were infected with HIV-1_{BAL}, a CCR5 / macrophage tropic strain of HIV-1 (HIV-1 MDM). HIV-1 infection of the MDM was confirmed by detection of intracellular p24 using mouse anti-HIV-1 gag (p24) monoclonal antibody and a goat anti-mouse Ig Ab conjugated to β-galactosidase. Positively staining cells were counted microscopically to calculate the proportion of infected cells. MDM were also inoculated with sham virus (supernatant from macrophage cultures that had been identically treated to those used for

HIV-1 propagation except for the exclusion of an HIV-1_{BAL} infection step), to serve as controls for both the *S. pneumoniae* challenge experiments and the HIV-1 p24 assays (control MDM). Successful infection was defined as the presence of at least 5 blue, p24 positive cells from the HIV-1_{BAL} inoculated well, when the paired negative control well had zero blue cells (Figure 3-9, upper panel). For certain HIV-1 infections of MDM, a goat anti-mouse - Alexa488 conjugated secondary antibody was used instead of the β -galactosidase conjugated antibody (Figure 3-9, lower panel). As an additional indicator of successful HIV-1 infection the cells were inspected for the presence of multinucleated giant cells (the combination of 2 or more macrophages). A giant cell was only counted as a single p24 positive cell when calculating the proportion of infected cells. In total MDM from 69 different donors were used, of which 7 (10.1%) failed to show any evidence of HIV-1 infection. The proportion of cells infected with HIV-1 varied considerably across donors and followed a bimodal distribution (see Figure 3-10). 16 (23.2%) of the donors had 100% infection rates with this protocol.

3.2.8 HIV-1 infection of MDM is not associated with altered phagocytosis of *Streptococcus pneumoniae*.

To compare early bacterial internalization and killing between HIV-1 MDM and control MDM, at 4 hours post infection viable internalized bacteria were extracted and counted using a gentamicin protection assay. There was no difference in the recovered viable count between HIV-1 and control MDM (17168 ± 2658 vs. 17789 ± 4572 CFU/mL, n=27), even though there was marked inter-donor / inter experimental variability in the number of bacteria recovered with yields ranging from 167 to 140000 cfu/mL (Figure 3-11). When a subset of donor MDM with at least 80% HIV-1 positive cells were compared with their matched controls, there was still no difference in viable counts (2028 ± 715.5 vs. 2194 ± 711.6 CFU/mL, n=6).

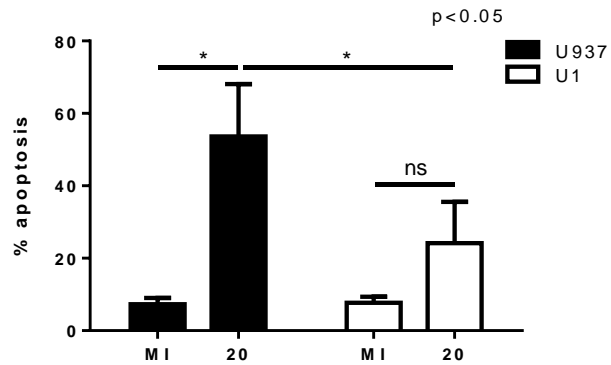


Figure 3-8 After a prolonged, 8 day differentiation protocol U1 demonstrate less induction of apoptosis than U937 following exposure to *Streptococcus pneumoniae*.

U937 and U1 cells were treated with 100nM PMA for 3 days then rested for 5 days before being challenged with *S. pneumoniae* or mock-infected. Apoptosis was measured by nuclear morphology at 20 hours. n=4, *p<0.05, 2 way ANOVA with Fisher's post test.

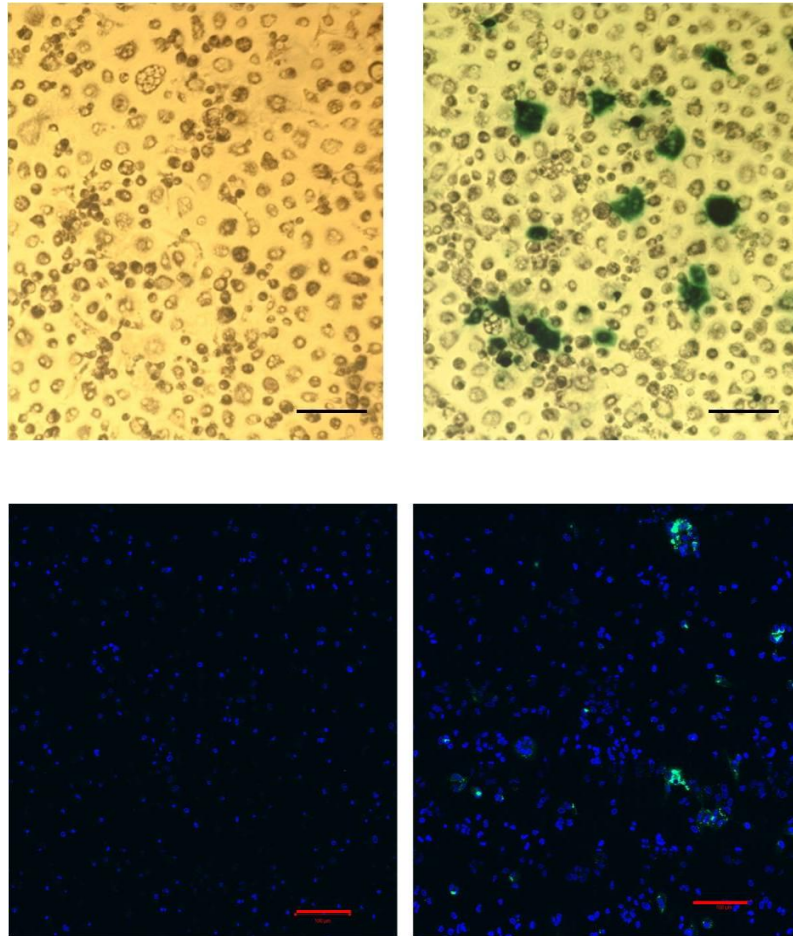


Figure 3-9 Photomicrographs of sham and HIV-1 treated MDM.

MDM were inoculated with sham treatment (left) or HIV-1_{BAL} (right) overnight and then media replaced and incubated for 7 days. Cells were washed, permeabilised and fixed and wells then incubated with anti-p24. A β -galactosidase conjugated secondary antibody was used to show p24 containing cells as blue (top panels, 200 x magnification, black scale bar 100 μ m).

Alternatively, cells were incubated with an Alexa488 conjugated secondary antibody and examined by confocal fluorescence microscopy (bottom panel, p24 positive cells show as green, red scale bar 100 μ m).

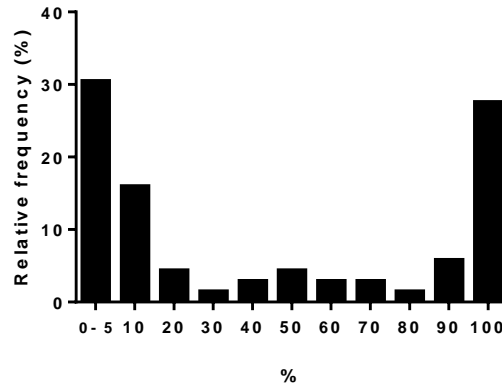


Figure 3-10 HIV-1 infection of monocyte-derived macrophages is variable and donor dependent

MDM were inoculated with HIV-1_{BAL} or sham virus overnight then incubated for 7 days. Cells were washed, permeabilised and fixed in wells then treated with anti-p24 and a β -galactosidase secondary in the presence of 5-bromo-4-chloro-3-indolyl—galactopyranoside (Xgal). The rate of p24 positive (HIV-1-infected) cells was calculated as the number of blue cells/total number of cells. Numbers on the x axis refer to the centre of the bin on the histogram.

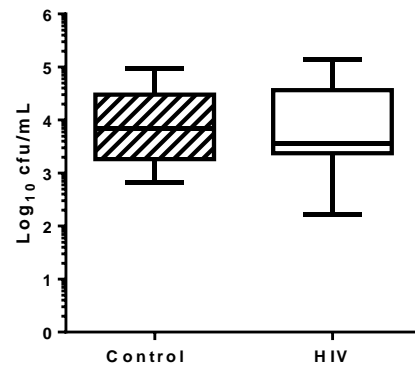


Figure 3-11 HIV-1 infection is not associated with any alteration in internalisation of *Streptococcus pneumoniae* at 4 hours

7 days after inoculation with HIV-1 or sham-infection (Control), monocyte-derived macrophages (MDM) were challenged with *S. pneumoniae* (D39). After four hours viable internalized bacteria were counted using a gentamicin protection assay. n=26, p=0.36, Wilcoxon matched-pairs signed rank test. Data show median with IQR (box) and range (whiskers) CFU = colony forming units.

3.2.9 HIV-1 infection of MDM is associated with altered induction of apoptosis

Healthy donor MDM were either infected with HIV-1_{BAL} (HIV-1 MDM) or sham-infected (control MDM) for 7 days and then challenged with *S. pneumoniae* or mock-infected. 20 hours following challenge significantly fewer HIV-1 MDM (20.86±4.2%) had morphological changes of apoptosis compared with control MDM (34.07±5.8%, n=14, p=0.0023 by Wilcoxon matched-pairs signed rank test) (Figure 3-12). By contrast, more apoptosis was seen in the 20 hour mock-infected HIV-1 MDM (3.25±0.51%) than control MDM (2.25±0.46%, p<0.05)(Figure 3-12). When the difference in apoptosis between 20 hour mock-infected and D39 challenged MDM was compared, the induction of apoptosis was significantly smaller in HIV-1 MDM (+15.64 ±4.1%) than control MDM (+31.57±5.6%, n=14, p=0.0001 Wilcoxon matched-pairs signed rank test). For 4 donors, additional experiments were performed to determine the level of apoptosis at 16 hours and 20 hours. These demonstrated that significant divergence in *S. pneumoniae* associated apoptosis between HIV-1 MDM and control MDM occurred between 16 and 20 hours (p=0.0301, 2 way ANOVA with Bonferroni's posttest)(Figure 3-13).

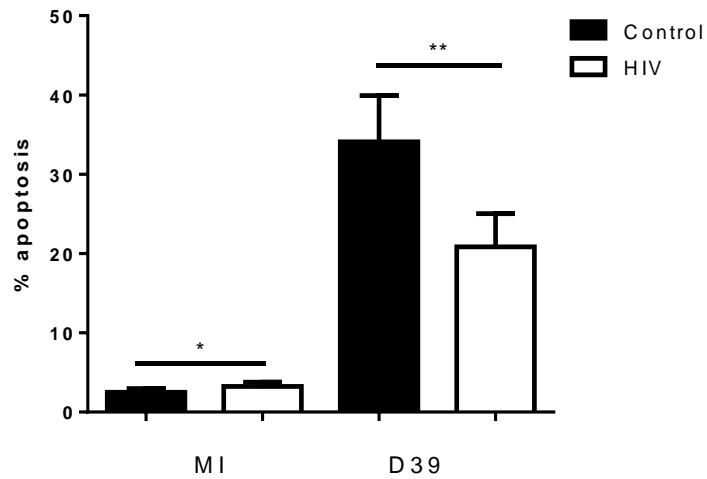


Figure 3-12 HIV-1 infection is associated with reduced induction of apoptosis by MDM after *Streptococcus pneumoniae* challenge.

Monocyte-derived macrophages (MDM) were challenged with HIV-1_{BAL} (HIV) or sham-infected (Control) for 7 days and then challenged with *S. pneumoniae* (D39) or mock-infection (MI). At 20 hours fixed cells were stained with DAPI and examined for nuclear morphological changes of apoptosis. n=14, *p<0.05, **p<0.005 Wilcoxon matched-pairs signed rank test.

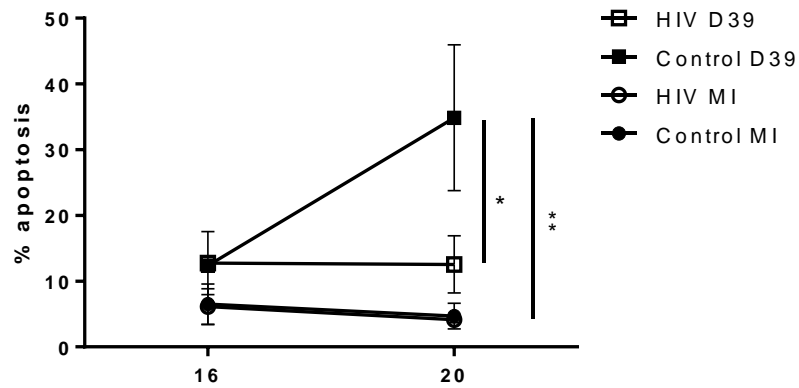


Figure 3-13 HIV-1 infection is associated with reduced induction of MDM apoptosis between 16 and 20 hours following *Streptococcus pneumoniae* challenge.

Monocyte-derived macrophages (MDM) were challenged with HIV-1_{BAL} (HIV) or sham-infected (Control) for 7 days and then challenged with *S. pneumoniae* (D39) or mock-infection (MI). At 16 and 20 hours fixed cells were stained with DAPI and examined for nuclear morphological changes of apoptosis. n=4, ** p<0.005, * p<0.05, 2 way ANOVA with Bonferroni's posttest.

3.2.10 HIV-1 infection is associated with reduced induction of caspase 3/7 activity following *Streptococcus pneumoniae* infection.

Healthy donor MDM were either infected with HIV-1_{BAL} (HIV-1 MDM) or sham-infected (control MDM) for 7 days and then challenged with *S. pneumoniae* (D39) or mock-infected. Caspase 3/7 activity was measured at 16 hour post infection. To normalize for variability in the cell numbers between donors results were normalised for numbers of cells and fold induction in caspase 3/7 activity from mock-infection to *S. pneumoniae* challenge was calculated for each donor's paired MDM. The fold increase in caspase 3/7 activity was significantly smaller in HIV-1-infected MDM (1.64 fold \pm 0.14) than control (2.16 fold \pm 0.27, n=11, p=0.0386, Wilcoxon matched-pairs signed rank test) (Figure 3-14).

3.2.11 HIV-1 infection of MDM is associated with reductions in late bacterial killing

Bacterial killing at 20 hours post infection was compared between HIV-1 MDM and control MDM. At 4 hours post bacterial challenge cells were incubated with gentamicin for 30 minutes to kill extracellular bacteria then incubated in fresh medium in the presence of vancomycin until 20 hours when and the number of viable intracellular bacteria was measured. At 20 hours, greater numbers of viable pneumococci were recovered from HIV-1 MDM (1299 \pm 563.2 cfumL⁻¹) than control MDM (467.9 \pm 238.9 cfumL⁻¹, n=25, p=0.0017, Wilcoxon matched-pairs signed rank test)(Figure 3-15).

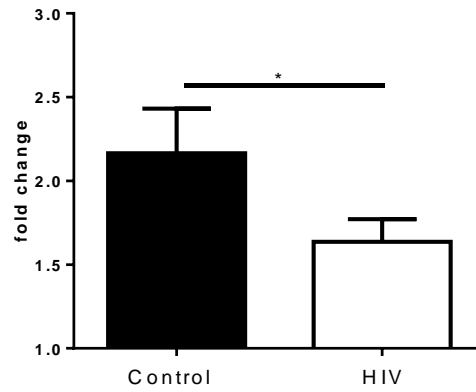


Figure 3-14 HIV-1 infection is associated with reduced induction of caspase 3/7 activity by MDM following *Streptococcus pneumoniae* challenge.

Monocyte-derived macrophages (MDM) were challenged with HIV-1_{BAL} (HIV) or sham-infected (Control) for 7 days and then challenged with *S. pneumoniae* or mock-infection. At 16 hours post infection with *S. pneumoniae* caspase 3/7 activity was measured using a luciferase based assay. Caspase 3/7 activity is expressed as fold change from mock-infection. n=11, *p<0.05, Wilcoxon matched-pairs signed rank test.

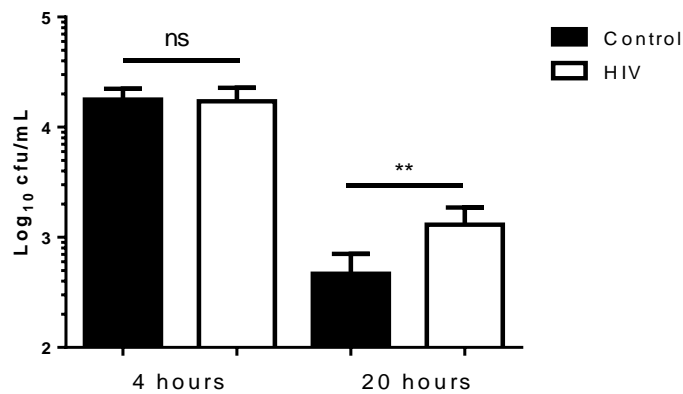


Figure 3-15 HIV-1 infection is associated with reduced killing of internalised *Streptococcus pneumoniae* in MDM at 20 hours post infection.

Monocyte-derived macrophages (MDM) were challenged with HIV-1_{BAL} (HIV) or sham-infected (Control) for 7 days and then challenged with *S. pneumoniae* or mock-infection. The numbers of viable intracellular *S. pneumoniae* in MDM were measured at 4 hours after bacterial challenge using a gentamicin protection assay and at 20 hours after bacterial challenge in cultures incubated from 4-20 hours in the presence of vancomycin. cfu colony forming units, n=25 ** p<0.01, Wilcoxon matched-pairs signed rank test. Data show mean and standard error of the mean. 4 hour data are from the same experiments as shown in Figure 3-11

3.3 Discussion

In this chapter I have demonstrated that challenge with serotype 2 *S. pneumoniae* is associated with apoptotic cell death in differentiated U937 and U1 cells. I confirmed that PMA was able to induce both cell lines to differentiate in a broadly similar fashion towards a macrophage-like phenotype, with increases in size, autofluorescence and granularity, which are surrogate measures of the increased numbers of mitochondria and lysosomes that are seen when macrophages differentiate (Daigneault, Preston et al. 2010). Apoptosis-associated DNA fragmentation was demonstrated using the hypodiploid peak assay which identifies cells containing less than the diploid complement of DNA. Nuclear fragmentation, the classical morphological feature of apoptosis, was seen using fluorescence microscopy. Caspase 3/7 activity, a critical component of the *S. pneumoniae* associated apoptosis programme in macrophages, also increased following exposure to *S. pneumoniae*. These findings are consistent with those described in differentiated THP-1 cells following *S. pneumoniae* infection (Bewley, Marriott et al. 2011).

For each of these measures, the *S. pneumoniae* associated apoptosis was significantly less in U1 than U937 cells, implying that the HIV-1 infection of U1 cells reduces the apoptotic response to *S. pneumoniae*. This finding is consistent with work showing reduced U1 apoptosis compared with U937 in other settings (Pinti, Biswas et al. 2003; Patel, Zhu et al. 2007; Fernandez Larrosa, Croci et al. 2008). However, the experiments in these other studies had used U1 and U937 cells that had been allowed to differentiate for no more than 48 hours. I have shown that the HIV-associated effect on apoptosis was consistent whether cells were studied after 4, 5 or 8 days of differentiation with 50-100nM PMA and resting, therefore demonstrating they were also a feature of a more differentiated macrophage phenotype. Although the degree to which a monocytic cell has differentiated toward a macrophage phenotype will affect the magnitude of the apoptotic response to *S.pneumoniae* (Ali, Lee et al.

2003), and I cannot absolutely exclude the possibility of subtle differences in the differentiation of U1 and U937 cells, I found that for cells with a broadly similar level of differentiation there was an association between HIV-1 infection and resistance to apoptosis during *S. pneumoniae* infection.

The use of the U937 / U1 cell lines has the advantage that they are easily cultured, the U1 cell line is already infected with inducible HIV, preventing the need for more complex *in vitro* infection protocols, and being clonal the cell lines behave with uniformity. Additionally they have been widely used to study the effect of HIV-1 on monocytes/macrophages. However, as models of HIV-1 infection of macrophages they present a number of deficiencies. Originally derived from the pleural effusion of a man with hystiocytic lymphoma, U937 are promonocytic cells, less differentiated than the monocytic cell lines THP-1, and express RNA for the c-myc oncogene (reviewed in (Cassol, Alfano et al. 2006). They are also subject to potential clonal variability. Thus, even with PMA treatment they will not share all of the characteristics of differentiated tissue macrophages and represent at best a partially differentiated macrophage phenotype. The nature of HIV-1 infection of U1 is also different from that in macrophages. The U1 were obtained from U937 cells acutely infected with the X4 HIV-1_{LAI/IIIIB} strain, which is not a macrophage tropic strain *in vivo*. Furthermore, they require PMA stimulation to produce TNF α in order to up-regulate their HIV-1 expression, which is otherwise latent due to a defective Tat interaction with the trans-activating response element (TAR) of HIV-1 RNA, and may support only low levels of HIV-1 replication (Cassol, Alfano et al. 2006). For these reasons, I established a second model of HIV-1 infection of macrophages by differentiating primary human monocytes into macrophages and then infecting them with a macrophage tropic strain of HIV-1, HIV-1_{BAL}. Although it is possible to achieve high levels of uniform and productive MDM infection by HIV-1 (Tsang, Chain et al. 2009), I was able to achieve a range of infection rates by using a smaller virus inoculum, to reflect the fact that only a proportion of tissue macrophages are HIV-1-infected *in vivo*. However, there was considerable donor variability in the rate of

HIV-1 infection and it was difficult to accurately count the rate of p24 antigen positive cells due to multinucleated giant cell formation among HIV-1-infected cells, both recognized consequences of MDM infection with HIV-1 (Novak, Holzer et al. 1990).

I have shown that *S. pneumoniae* infection is associated with MDM apoptosis as previously described (Dockrell, Lee et al. 2001; Ali, Lee et al. 2003). While at 4 hours following infection the phagocytosis and killing of *S. pneumoniae* by MDM was not affected, HIV-1 infection was associated with a significantly smaller degree of apoptosis induction; both the induction of caspase 3/7 activity at 16 hours and the number of cells with morphological features of apoptosis at 20 hours were lower. These findings are consistent with those from the U937 and U1 cell lines. I have also demonstrated that at 20 hours following *S. pneumoniae* infection the HIV-1-infected MDM are more likely to contain viable bacteria, and at higher rates, even though at four hours the balance of phagocytosis and killing is no different. This implies an HIV-1 associated impairment in MDM killing of *S. pneumoniae* after the initial phase of phagolysosomal killing. This is the same period when reduced apoptosis was observed. When macrophage apoptosis is prevented by inhibiting caspase activation, similar impairments in pneumococcal killing are observed (Dockrell, Marriott et al. 2003) .

These experiments and their interpretation are subject to some limitations. Firstly, *S. pneumoniae* serotype 2 (D39) was used as it is a well characterized laboratory isolate. This serotype is less commonly associated with invasive pneumococcal disease (IPD) or carriage in HIV-1-seropositive individuals and may be less relevant to clinical disease (Rodriguez-Barradas, Tharapel et al. 1997). However, *S. pneumoniae* associated apoptosis in macrophages is also observed with other serotypes known to cause IPD, such as type 1 and type 4 (Dockrell, Lee et al. 2001; Daigneault, De Silva et al. 2012) making it unlikely that the HIV-1 associated effects demonstrated are limited to this strain. A second limitation is that some assays of apoptosis could not be used with HIV-1-infected macrophages. All cell culture work with HIV-1 is carried

out in a containment level three laboratory (CL3) and material must be fixed in paraformaldehyde before removal from the CL3, limiting the use of assays that require unfixed cells. It therefore proved not possible to measure lysosomal membrane permeabilisation, dissipation of the inner mitochondrial transmembrane potential ($\Delta\Psi_m$) and the cell surface expression of phosphatidylserine using flowcytometry based techniques to describe stages in the *S. pneumoniae* associated apoptotic cascade preceding nuclear fragmentation (Bewley, Marriott et al. 2011). Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was used to corroborate the nuclear morphological changes of apoptosis seen with DAPI in MDM. However, when MDM *S. pneumoniae* infections were performed in the CL3 laboratories, with and without HIV-1 infection, and subsequently fixed and transferred to standard laboratories, the performance of the TUNEL assay was very inconsistent and it was not possible to use this additional assay of apoptosis. One additional, albeit indirect, assay of apoptosis that would be unlikely to be influenced by the constraints of the CL3 working conditions is the measurement of cytochrome *c* translocation. As a consequence of changes in mitochondrial outer membrane permeability cytochrome *c* is released into the cytosol, a change that can be measured by SDS page and western blot of separated cytosolic and mitochondrial compartments of the cell (Bewley, Marriott et al. 2011).

In conclusion, the results of these experiments suggest that there is an HIV-1 associated reduction in macrophage apoptosis following *S. pneumoniae* challenge which is linked to impaired clearance of viable *S. pneumoniae* by HIV-1-infected macrophages.

Chapter 4. HIV-1 infection is associated with persistent alterations in bronchoalveolar fluid T lymphocytes and alveolar macrophage phenotype despite antiretroviral therapy

4.1 Introduction

The evidence from cell lines and primary monocyte-derived macrophages (MDM) presented so far demonstrates that HIV-1 infection is associated with a defect in the macrophage's host-mediated apoptotic response following challenge with *S.pneumoniae*, which is associated with reduced killing of internalized bacteria.

To investigate whether this defect is pertinent to the increased risk of invasive pneumococcal disease (IPD) in those living with HIV-1 infection it is essential to establish whether these observations reflect the responses of differentiated macrophages from HIV-1-seropositive individuals, when challenged with *S. pneumoniae ex vivo*. As it is necessary to use live and virulent *S. pneumoniae* in these experiments, *in vivo* work with human volunteers is not safe or possible. The closest animal models to HIV-1 infection in humans are either primates, such as SIV infected rhesus macaques, or 'humanized mice', mice that have been reconstituted with a human immune system then infected with HIV-1 (Hatzioannou and Evans 2012). These animals could be used in experiments to model pulmonary *S. pneumoniae* infection as has been done elsewhere without HIV-1 (Marriott, Hellewell et al. 2006). However, these animal HIV models are beyond the technical and financial constraints of this PhD. One option is to work with differentiated primary tissue macrophages from human volunteers *ex vivo*. An accessible source of differentiated macrophages which is particularly relevant to pneumococcal disease is the pool of alveolar macrophages (AM) in the lung. These can be retrieved and isolated using fibre optic bronchoscopy with no harm to the individual. The advantage of working with *ex vivo* AM is that they will have differentiated over a longer time period, with exposure to virus and in an environment that is not well replicated by the *in vitro* models; AM are very long lived, differentiated cells. Their maturation is influenced by their

local environment and they may derive from either peripheral blood monocytes or interstitial macrophages depending on inflammation (Jenkins, Ruckerl et al. 2011; Dockrell, Collini et al. 2012). By comparison, the promonocytic U1 and U937 cells and MDM have been differentiated in isolation and for no more than 8 or 21 days respectively. HIV-1 virus exposure is likely to be very different given the clinical observation that the increased risk of IPD in HIV-1-seropositive individuals pertains even when they have been treated with antiretroviral therapy (ART) for prolonged periods and have undetectable plasma viral loads (Jordano, Falco et al. 2004; Grau, Ardanuy et al. 2009). If the alveolar environment has similarly low or undetectable levels of HIV-1 then the AM's exposure to the virus will have been substantially different from the *in vitro* models where anywhere up to 100% of the cells will be infected with replication competent virus. Additional to this point, macrophages in the *in vitro* models are responding to a new, acute infection with a laboratory propagated virus while any viral exposure for the AM will have been more prolonged and with a strain adapted to that host. The third important difference is that the alveolar microenvironment is not replicated *in vitro*; AM are in contact with other cells, principally T lymphocytes and epithelial cells and they are also exposed to soluble factors such as cytokines and chemokines. Each of these may be altered in HIV-1-seropositive individuals, who have detectable levels of free HIV-1 proteins such as gp120 in the lung microenvironment (Klasse and Moore 2004). The alveolar microenvironment can influence the differentiation and activation/polarisation phenotype of the AM, as shown for example in smokers (Shaykhiev, Krause et al. 2009) and thus could be relevant to any observed differences in apoptotic responses.

For these reasons human alveolar macrophages were studied soon after retrieval from the lungs by bronchoalveolar lavage (BAL) from HIV-1-seropositive individuals and HIV-seronegative, matched controls. Another advantage of this approach was that it enabled additional analyses of the BAL fluid to describe the alveolar microenvironment. The cellular constituents of the BAL fluid from each volunteer were analysed and the AM separated for

characterisation of their phenotype and response to *ex vivo* *S. pneumoniae* challenge, 48 hours after isolation. Volunteers were recruited to allow comparison between HIV-1-seropositive individuals on protease inhibitor (PI) based or non nucleoside reverse transcriptase inhibitor (NNRTI) based ART or naive to ART treatment and HIV-seronegative controls.

4.2 Results

4.2.1 Patient demographics

Ethical approval for BAL of HIV-1-seropositive and control volunteers had been granted volunteer to review of the cell line and primary MDM model data presented in the previous chapter. These data were reviewed by a data review committee in June 2012 which consisted senior academics from the University of Sheffield medical school who were independent of this study. They considered that the *in vitro* model results justified further work involving clinical volunteers. Recruitment commenced in July 2012 and BAL were performed between July 2012 and September 2013. Over 500 sets of notes from the STH HIV clinics were screened for inclusion criteria and 20% of these patients were approached regarding the study. In total, 31 individuals were recruited and 29 individuals underwent BAL (Table 4-1). 22 HIV-1-seropositive individuals attended a full screening appointment and 19 went on to have BAL. With agreement from the ethics committee each volunteer was paid £150 for their participation. One volunteer declined to take part, one failed screening as he was subsequently discovered to be a smoker and a third failed as her forced expiratory volume in 1 second (FEV1) was <70%. 11 HIV-seronegative control volunteers were recruited, screened and enrolled in parallel to match the age, sex and ethnicity of the HIV-1-seropositive volunteers. No control volunteer screened positive for HIV, hepatitis B virus (HBV) or hepatitis C virus (HCV). 10 of these went on to have BAL. No control volunteers failed screening but one declined BAL following successful screening. Only 3 of a planned 7 ART naïve HIV-1-seropositive (ART naïve) volunteers were recruited, due in most part to the small number of such individuals attending clinic who were not already involved in other clinical trials. Additional volunteers were recruited to the HIV-1-seropositive on ART (HIV ART) and control arms to increase numbers after some assays had failed to achieve a result for technical reasons. There were no statistically significant differences detected between HIV ART and control donors for their age (unpaired t-test), sex and ethnicity (Fischer's exact test). Following

bronchoscopy three HIV-1-seropositive volunteers were excluded from further analyses. One (white female, age 38) was found to have a suspicious endobronchial lesion during the bronchoscopy and subsequently diagnosed as having immune reconstitution inflammatory syndrome (IRIS) due to pulmonary tuberculosis. One (black male, age 52) was seen to have multiple black inclusion bodies within the isolated alveolar macrophages which, although he was a never smoker, were similar in appearance to those seen in smokers' AM. A third (white male, age 39) had been in receipt of both NNRTI and PI based ART.

All but 6 volunteers tolerated the instillation of the maximum 200 mL of saline. The median volume returned was 96 mL (48%) and 6.8×10^6 alveolar macrophages per volunteer. When compared by donor group, there were no differences in the volume returned or yield of alveolar macrophages (Figure 4-1).

4.2.2 Alveolar macrophages from a minority HIV-1-seropositive volunteers on fully suppressive ART are infected with HIV-1

The alveolar macrophages from 11 HIV ART and 2 ART naïve donors were tested for the presence of HIV-1 p24 antigen detected by immunohistochemistry with anti p24 and a β -galactosidase conjugated secondary antibody. 3 of 11 (27%) HIV ART donors had detectable p24 in 1.1%, 4.3% and 51.6% of the AM (Figure 4-2). Both of the 2 ART naïve donors tested had detectable p24 in 7.6% and 21.3% of their AM.

	HIV ART	ART Naive	Control
N	16	3	10
male (%)	9 (56%)	3 (100%)	7 (70%)
age (years)			
median (IQR)	40.3 (37.5-49.0)	46.2 (38.7 - 47.0)	40.4 (34.2 - 46.8)
mean	42.7	41.7	41.7
ethnicity			
White	10 (62.5%)	3 (100%)	8 (80%)
Black	5 (31.3%)	0	2 (20%)
other	1 (6.2%)	0	0
ART type			
NNRTI	8	-	-
PI	7	-	-
Mixed	1	-	-
Naive	-	3	-
duration of ART (weeks)			
(median, IQR)	325 (207 - 454)	-	-
range	65 -884	-	-
mean	373	-	-
log₁₀ VL (median, range)	all undetectable	4.39 (4.22 - 4.61)	-
CD4			
(median, IQR)	633.5 (500.3 - 730.0)	500 (495 -762.5)	-
range	257.0 - 1112.0	490 – 1025	-
CD4% (median, IQR)	30.5 (27.8 - 35.8)	31 (30.0 - 31.5)	-
range	11.0 - 39.0	29.0 - 32.0	-
Nadir CD4 (median, IQR)	211.0 (130.5 - 200.6)	-	-
CD8 (median, IQR)	831.5 (635.3-1104.8)	760 (747.5 - 1160)	-
range	400.0 - 1501.0	735 – 1560	-
CD8% (median, IQR)	40.0 (34.8 - 47.0)	46 (45.0 - 47.0)	-
range	27.0 - 56.0	44.0 - 48.0	-
CD4:CD8 (median, IQR)	0.81 (0.62 - 0.98)	0.66 (0.66 - 0.66)	-
range	0.24 - 1.31	0.66-0.67	-
Interval between BAL and blood test (days)			
Median (IQR)	24 (17.5 – 44)	26 (25 – 46)	-

Table 4-1 Volunteers undergoing bronchoalveolar lavage

Peripheral blood results were recorded from clinic notes at the visit closest to the BAL (14 prior, 5 after). ART = antiretroviral therapy, VL = plasma viral load, BAL = bronchoalveolar lavage, IQR inter-quartile range.

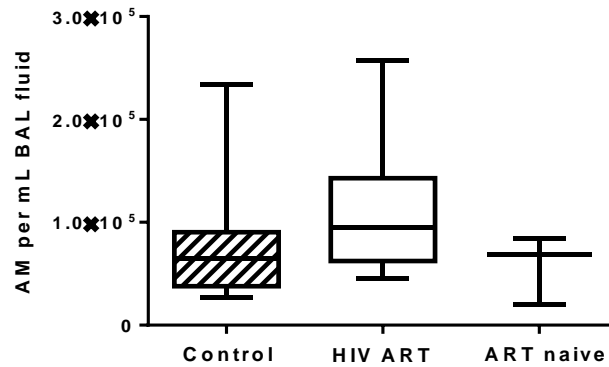


Figure 4-1 Alveolar macrophage yields from BAL

Bronchoalveolar lavage (BAL) fluid was collected from HIV-1-seropositive volunteers and controls, and cells filtered and resuspended in growth medium. Alveolar macrophages were identified by their morphology and counted with a haemocytometer at 100 times magnification. Data show as median \pm IQR and range of live alveolar macrophages per mL of BAL fluid.

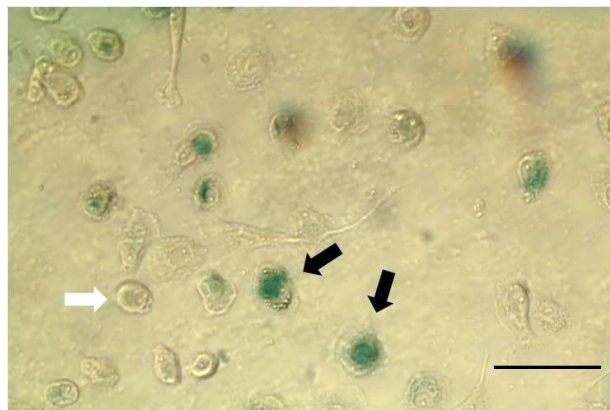


Figure 4-2 Representative photomicrograph showing alveolar macrophages from an HIV-1 seropositive donor on fully suppressive ART staining positive for p24

Bronchoalveolar lavage fluid was collected from HIV-1-seropositive volunteers and controls, filtered and cells resuspended in growth medium. After 48 hours adherent alveolar macrophages (AM) were washed, permeabilised and fixed and then incubated with anti-p24 antibody and a β -galactosidase conjugated secondary antibody. Bright-field microscopy at x 400 magnification. AM with p24 stain as blue (black arrow) without are clear (white arrow), scale bar 50 μ m.

4.2.3 HIV-1-seropositive individuals have a greater proportion of lymphocytes in their BAL fluid than HIV-seronegative controls.

Immediately following bronchoscopy, bronchoalveolar cells were isolated from BAL fluid by centrifugation and resuspended in 10 mL medium. 100 μ L of this cell suspension was diluted with 20% heat inactivated foetal calf serum (HIFCS) and fixed in 0.5% PFA and added to a cytopsin chamber, centrifuged, fixed with methanol and stained with Diff-Quik™. A differential white cell count was performed at 100 times magnification using bright field microscopy. Results were available for 24 of the 29 individuals that underwent bronchoscopy: 2 control donors' cell cytopsin failed to adhere to the slide and 3 HIV-1-seropositive cases were excluded after bronchoscopy as above. The age and sex and ethnicity of these 3 groups remained similar despite these exclusions. Epithelial cells and erythrocytes were excluded from cell counts. For all donors the predominant leukocytes were alveolar macrophages ($\geq 65\%$), followed by lymphocytes and then neutrophils ($\leq 2.5\%$). Only 2 of 25 donors (both HIV ART) had any eosinophils identified so these were not included in the counts. The HIV ART group had significantly greater proportions of lymphocytes ($12.91\pm 2.07\%$, $n=13$) than control volunteers ($7.66\pm 1.27\%$, $n=9$, $p=0.0313$ Mann Whitney test)(Figure 4-3 A). ART naïve donors had even higher proportions of lymphocytes ($21.06\pm 6.03\%$, $n=3$). When all three groups were compared there was a significant association between HIV-1 with and without ART and BAL lymphocytosis ($p=0.0348$, Kruskal-Wallis test)(Figure 4-3 B). The proportion of alveolar macrophages was correspondingly lower for both HIV ART ($86.24\pm 2.05\%$) and ART naïve donors ($78.19\pm 5.99\%$) than controls ($91.14\pm 1.39\%$). There were no differences in the proportions of neutrophils across the groups, which were measured as $0.75\pm 0.17\%$, $0.79\pm 0.40\%$ and $1.32\pm 0.27\%$ respectively.

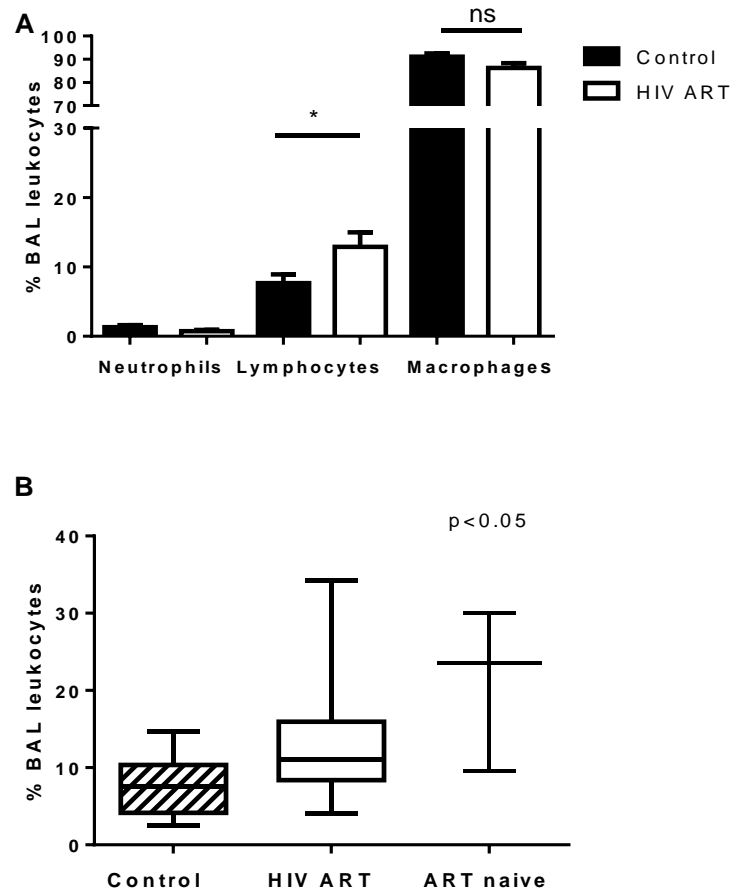


Figure 4-3 Bronchoalveolar lavage fluid lymphocyte proportions are elevated in HIV-1-seropositive volunteers.

Bronchoalveolar (BAL) fluid was collected from HIV-1-seropositive volunteers and controls by bronchoalveolar lavage. Cells were centrifuged in a cytospin chamber and slides fixed and stained with Diff-Quik™ for differential white cell count measurement. **(A)** Leukocytes from 13 HIV-1 ART treated (HIV ART) and 9 control donors expressed as percentage of total BAL leukocytes (eosinophils not shown) * $p < 0.05$ Mann Whitney test. **(B)** Lymphocytes from 9 control, 13 HIV ART and 3 HIV ART naive donors (HIV naive) expressed as percentage of total BAL leukocytes, Kruskal-Wallis test. Data shown as median with IQR and range.

4.2.4 The HIV-1 associated BAL lymphocytosis is predominantly from CD8⁺ T cells.

The HIV-1 associated BAL lymphocytosis became apparent after the first 5 volunteers had been studied. To further characterize the nature of the lymphocytes, subset analyses were performed on subsequent donors' BAL. Following overnight culture in plastic tissue culture plates, medium was removed and centrifuged for 10 minutes at 400 g to isolate non-adherent cells. These were washed and stained with fluorophore conjugated antibodies against CD3, CD4, CD8 (or isotype control) to identify CD3⁺/CD4⁺ and CD3⁺/CD8⁺ T lymphocyte subsets by flowcytometry. Dead cells were identified with a viability dye and excluded from analysis. A further group of larger, CD3⁻/CD4⁺ cells were identified as non adherent alveolar macrophages and also excluded from analysis. Complete data were available for 6 control and 11 HIV ART volunteers. There were no significant differences between these groups with respect to age, sex and ethnicity. Compared with controls, HIV ART donor BAL contained a significantly smaller percentage of CD4⁺ T cells ($46.53 \pm 3.68\%$ vs. $70.05 \pm 3.95\%$, $p=0.0006$, Mann Whitney) but significantly more CD8⁺ T cells ($45.01 \pm 3.88\%$ vs. $22.33 \pm 4.01\%$, $p=0.0031$, Mann Whitney) (Figure 4-4). This alteration in the relative proportions of CD4⁺ and CD8⁺ T lymphocytes in HIV ART volunteers was also reflected in a much reduced BAL lymphocyte CD4:CD8 ratio (1.16 ± 0.15 vs. 3.79 ± 0.76 , $p=0.0019$, Mann Whitney)(Figure 4-5). To determine if the greater proportion of CD8⁺ T cells might contain greater numbers of activated lymphocytes, the cells were also incubated with fluorescein isothiocyanate (FITC) conjugated anti-CD38 or isotype control. Enhanced CD38 expression on CD8 cells is well described characteristic of immune activation associated with untreated HIV (Barry, Johnson et al. 2003). The geometric mean fluorescence intensity (MFI) of FITC was compared to isotype control to calculate a geometric mean fluorescence intensity ratio (GMR) for each donor. CD38 was only weakly expressed and no difference in GMR was seen between 9 HIV ART and 5 control volunteers (1.275 ± 0.12 vs. 1.324 ± 0.30 , ns, Mann Whitney). In addition, it was not possible to consistently identify a

distinct CD38⁺ or CD38^{bright} population in the event plots and histograms of the CD3⁺
/CD8⁺ subset that was different for HIV ART compared with control donors (Figure 4-6).

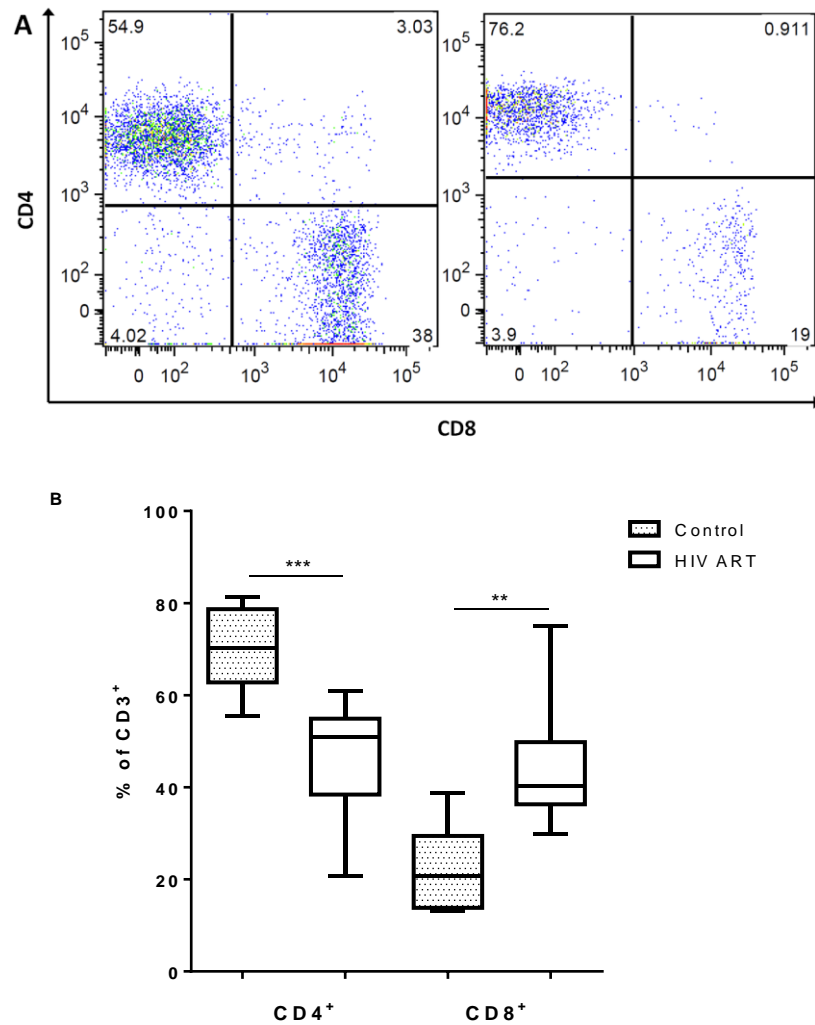


Figure 4-4 There are fewer CD4⁺ and more CD8⁺ T-cells in the bronchoalveolar lavage fluid of HIV-1-seropositive individuals on fully suppressive antiretroviral therapy

BAL fluid was collected from HIV-1-seropositive volunteers on ART (HIV ART) and controls by bronchoalveolar lavage. At 24 hours cells were labelled with fluorophore conjugated antibodies against CD3, CD4 and CD8 and analysed by flow cytometry. **(A)** Representative event plots showing the CD3⁺ lymphocyte gate with CD4 (APC, y axis) against CD8 (Brilliant violet, x axis) for HIV ART (left) and control (right) donor BAL. **(B)** median ± IQR and range CD3⁺/CD4⁺/CD8⁻ (CD4) and CD3⁺/CD4⁻/CD8⁺ (CD8) cells expressed as a percentage of CD3⁺ BAL cells for 6 control and 11 HIV ART donors *** p<0.001, **p<0.005, Mann Whitney test.

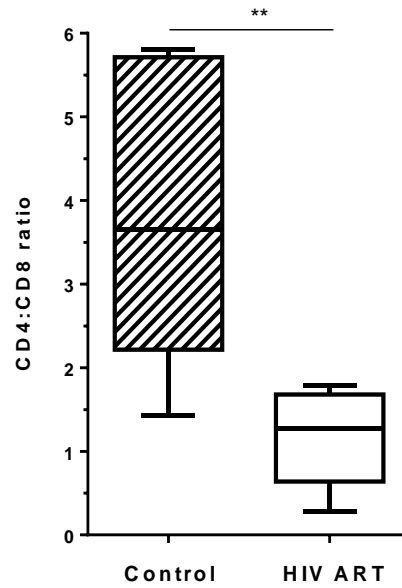


Figure 4-5 The BAL T lymphocyte CD4:CD8 ratio is reduced in HIV-1-seropositive individuals on fully suppressive antiretroviral therapy .

BAL fluid was collected from 11 HIV-1-seropositive volunteers on ART (HIV ART) and 6 controls by bronchoalveolar lavage. At 24 hours cells were labelled with fluorophore conjugated antibodies against CD3, CD4 and CD8 and analysed by flow cytometry. CD3⁺/CD4⁺/CD8⁻ (CD4) and CD3⁺/CD4⁻/CD8⁺ (CD8) are expressed as a ratio ** p<0.01, Mann Whitney Data shown as median ± IQR and range.

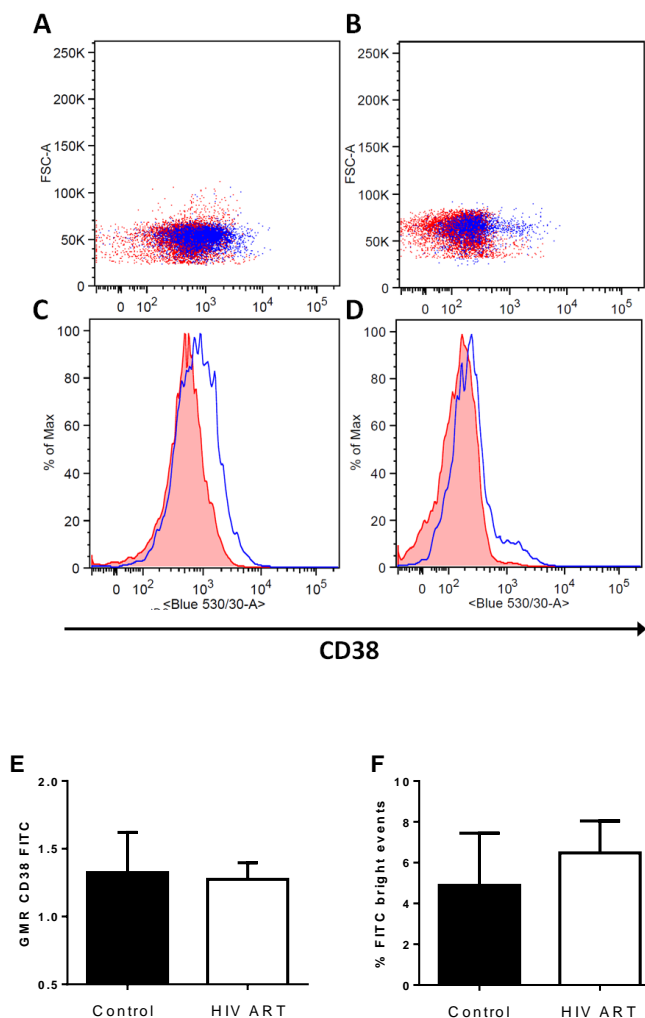


Figure 4-6 There is similar low level expression of CD38 on CD8 T lymphocyte from BAL of HIV-1-seropositive individuals on fully suppressive antiretroviral therapy and controls

BAL fluid was collected from 9 HIV-1-seropositive volunteers on ART (HIV ART) and 5 controls by bronchoalveolar lavage. At 24 hours cells were labelled with fluorophore conjugated antibodies against CD3, CD4 and CD8 and CD38 then analysed by flow cytometry. Geometric mean fluorescence intensity of FITC (CD38) expression was measured in the CD3⁺/CD4⁻/CD8⁺ subset (blue) and compared with isotype control (red). Representative event plots and histograms for control (A, C) and HIV ART (B,D). The ratio of intensity of FITC fluorescence between isotype and stained cells was calculated (GMR, E). CD38^{bright} cells were identified as events to the right of histograms (F). FSC-A forward scatter - area.

4.2.5 Alterations in T lymphocyte subset proportions in the lung are distinct from those in the peripheral blood of HIV-1-seropositive individuals

For the HIV-1-seropositive donors, BAL lymphocyte counts were compared with peripheral blood lymphocyte subsets, treatment history and age. Peripheral blood lymphocyte counts were not collected for the control subjects. There was a non significant trend for a positive correlation between the peripheral blood CD4% and nadir CD4 count as well as a negative correlation between duration of ART and peripheral blood CD8%. When the peripheral blood CD4 and CD8 T-cell subsets were compared with those recovered from the BAL, no correlation was found between blood and BAL CD4% ($r=0.29$, $p=0.34$), CD8% ($r=0.27$ $p=0.37$) or CD4:CD8 ratio ($r=0.11$ $p=0.72$, $n=13$, Pearson's correlation)(Figure 4-7).

4.2.6 Alveolar macrophage cell surface expression of polarisation markers is similar in HIV-1-seropositive individuals and controls

The polarisation status of alveolar macrophages was studied. BAL cells were cultured overnight before the adherent AM were isolated and incubated with fluorophore conjugated anti-CD206, CD80, CD163 and CD200r or isotype control and then measuring surface expression of these proteins by flowcytometry. AM were studied after overnight resting rather than immediately following BAL in keeping with established protocols with HIV-1 seropositive donors (Gordon, Molyneux et al. 2001; Gordon, Jarman et al. 2005) and as the extraction procedure may transiently cause a pro- inflammatory phenotype in AM (Tomlinson, Booth et al. 2012). The geometric mean fluorescence intensity was measured for each antibody and compared with isotype control as the geometric mean ratio (GMR). CD206 was strongly expressed on all AM from both groups. CD80 and CD163 were moderately expressed but CD200r expression was no different from isotype control. HIV ART donors' AM showed no significant differences in the GMR of any of the four surface proteins (Figure 4-8).

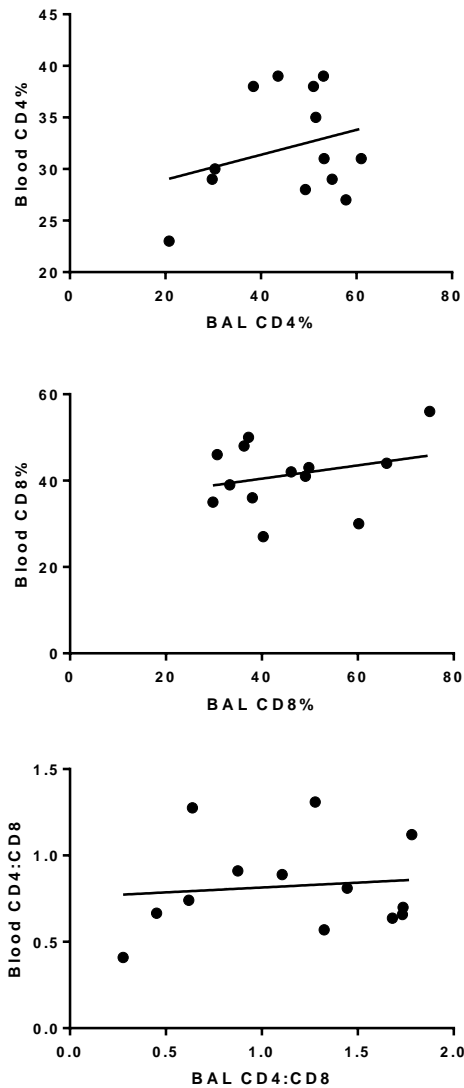


Figure 4-7 T lymphocyte subsets and ratios in the blood and bronchoalveolar lavage fluid of HIV-1-seropositive individuals on fully suppressive antiretroviral therapy show no association

CD3⁺/CD4⁺/CD8⁻ and CD3⁺/CD4⁻/CD8⁺ lymphocyte subsets were measured by flow cytometry in the BAL fluid of 13 HIV-1-seropositive individuals on fully suppressive antiretroviral therapy within 24 hours of bronchoscopy and compared with paired peripheral blood CD4⁺ and CD8⁺ lymphocyte percentages.

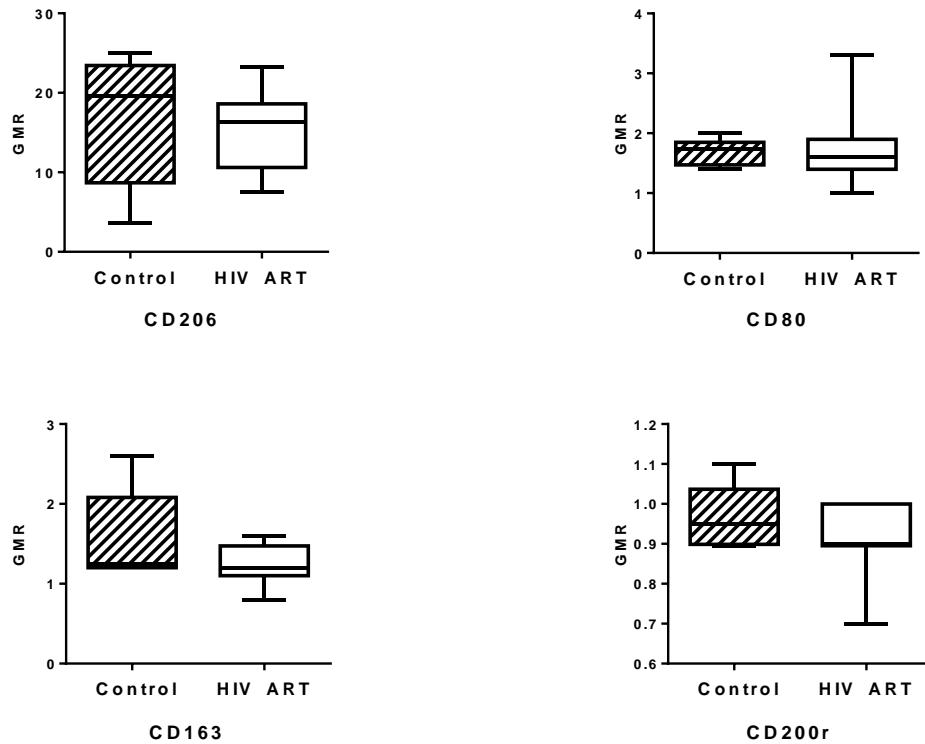


Figure 4-8 Cell surface expression of polarisation markers on AM from HIV-1-seropositive individuals on fully suppressive antiretroviral therapy is similar to controls

AM were extracted from BAL fluid of 11 HIV-1-seropositive volunteers on ART (HIV ART) and 6 controls. After 24 hours the expression of cell surface markers associated with macrophage polarisation was measured by flow cytometry. Data shown are median \pm IQR and range of the ratio of geometric mean intensity of antibody to isotype control (GMR) for each of CD206, CD80, CD163 and CD200r.

4.2.7 Alveolar macrophages from HIV-1-seropositive and seronegative individuals exhibit similar rates of *Streptococcus pneumoniae* phagocytosis.

48 hours after BAL and isolation AM were challenged with opsonised serotype 2 *S.*

pneumoniae (D39) at an MOI of 10. To compare bacterial internalization and early killing

between AM from HIV ART and control donors, at 4 hours post infection viable internalized

bacteria were extracted and counted using a gentamicin protection assay. To correct for

variation in the density of AM between donors at the start of each infection, the CFU/mL was

adjusted according to the average number of AM per high power field (x 1000 magnification)

on a coverslip from an uninfected well for each donor. Of the eligible volunteers who

underwent BAL, not all donors yielded sufficient AM from the BAL and in some cases the assay

failed (4 control and 1 HIV-1ART), leaving only 5 control (3 male, 3 white) and 13 HIV ART (8

male 9 white) donor AM available for full analysis. The ages of two groups were not different

($p=0.43$, Man Whitney). There was no difference in the number of viable internalized *S.*

pneumoniae at four hours between HIV ART and control donors ($p=0.9958$, Mann Whitney)

(Figure 4-9).

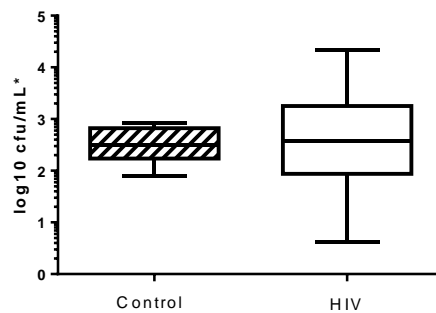


Figure 4-9 Rates of *Streptococcus pneumoniae* phagocytosis by alveolar macrophages from HIV-1-seropositive on fully suppressive antiretroviral therapy and control volunteers are similar 4 hours after bacterial challenge.

48 hours after BAL, AM from 13 HIV-1 seropositive individuals on ART (HIV ART) and 5 controls were challenged with *S. pneumoniae* for four hours. Viable internalized bacteria were counted using a gentamicin protection assay. the density of cells on an uninfected coverslip was counted and the measured CFU normalized for cell numbers (*cfu/mL). Data are shown as median with IQR and range.

4.2.8 Alveolar macrophages from HIV-1-seropositive individuals on ART undergo less apoptosis than those from control individuals following challenge with *Streptococcus pneumoniae*.

48 hours following BAL AM were challenged with *S. pneumoniae* at an MOI of 10 or mock-infected for 20 hours. Adherent cells were then washed, fixed in PFA, stained with DAPI and examined by fluorescence microscopy. Compared with mock-infection, *S. pneumoniae* challenge was associated with a significant reduction in the number of cells remaining adherent to coverslips for control (from 20.1 ± 3.4 to 10.9 ± 3.4 cells/HPF, $n=7$, $p=0.0313$ Wilcoxon matched-pairs signed rank test) but not for HIV ART donors (from 25.4 ± 3.4 to 22.0 ± 4.4 cells/HPF, $n=14$, ns). When compared together with 2 way ANOVA there was a significant interaction for *S. pneumoniae* for control alone (Figure 4-10).

When fluorescence microscopy was used to count the number of cells with condensed or fragmented nuclei 20 hours after challenge with *S. pneumoniae* a smaller proportion of AM from HIV ART donors had undergone apoptosis ($11.92 \pm 3.09\%$, $n=14$) than from control (29.83 ± 5.8 , $n=7$, $p=0.031$ Wilcoxon matched-pairs signed rank test). Conversely, AM from HIV ART donors exposed to 20 hours of mock-infection exhibited greater levels of apoptosis than controls ($0.36 \pm 0.11\%$ vs. $0.05 \pm 0.03\%$, $p=0.018$ Mann Whitney), although absolute levels remained low for both. Consequently, the *S. pneumoniae* related induction of apoptosis (the difference between mock-infection and *S. pneumoniae*) was smaller in the HIV ART donor AM (11.57 ± 3.1) compared with control AM ($27.75 \pm 6.9\%$, $p=0.022$, Mann Whitney). When compared together, HIV was associated with a significant reduction in *S.pneumoniae* associated apoptosis, $p<0.001$, 2 way ANOVA (Figure 4-11).

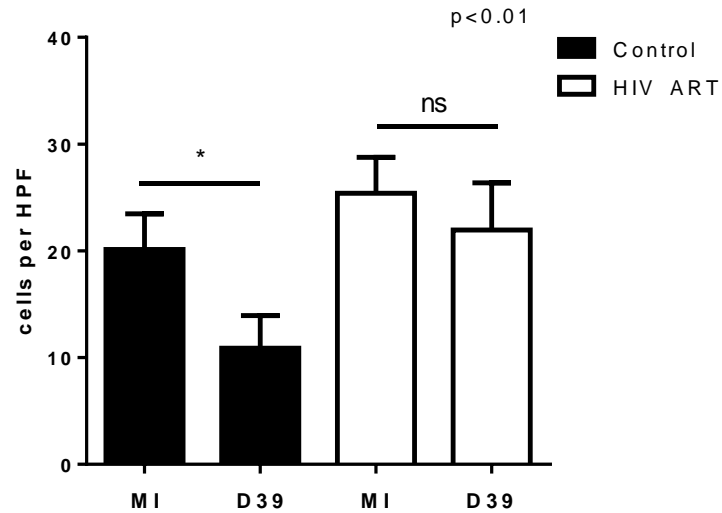


Figure 4-10 There is significant loss of AM from control donors but not HIV-1-seropositive volunteers on fully suppressive ART 20 hours following challenge with *Streptococcus pneumoniae*.

Alveolar macrophages were isolated from BAL fluid of 14 HIV-1-seropositive individuals on ART (HIV ART) and 7 controls then rested for 48 hours before being challenged with *S. pneumoniae* (D39) or mock-infected. After 20 hours cells were fixed and stained with DAPI and the number of adherent cells per high power field (HPF) were counted microscopically. * $p < 0.05$, ns = non significant, 2 way ANOVA with Bonferroni's post test.

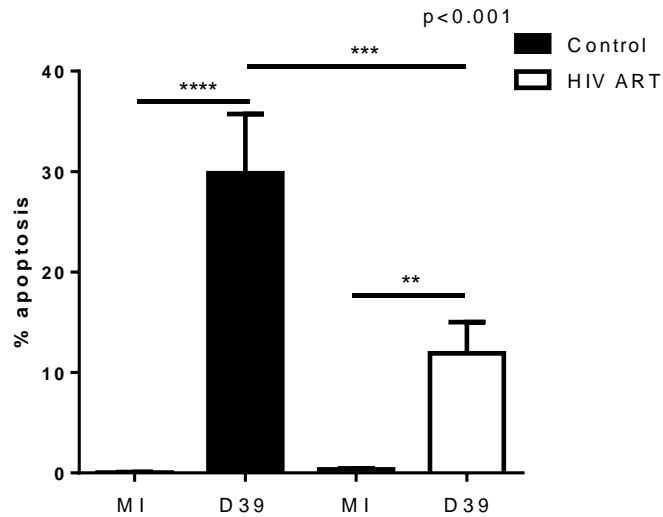


Figure 4-11 AM from HIV-1-seropositive volunteers on fully suppressive ART show reduced levels of apoptosis following *Streptococcus pneumoniae* challenge when compared with AM from controls.

Alveolar macrophages were isolated from BAL fluid of 14 HIV-1-seropositive individuals on ART (HIV ART) and 7 controls then rested for 48 hours before being challenged with *S. pneumoniae* (D39) or mock-infected (MI). After 20 hours cells were fixed and stained with DAPI and examined microscopically for nuclear morphological changes of apoptosis. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ 2 way ANOVA with Bonferroni's post test.

4.2.9 The induction of caspase 3/7 activity by *Streptococcus pneumoniae* infection is reduced in AM from HIV-1-seropositive volunteers on fully suppressive ART compared to controls

AM caspase 3/7 activity was measured at 16 hours following *S. pneumoniae* challenge or mock-infection in the two groups. To normalize for variability in the cell numbers between donors the fold induction in caspase 3/7 activity between mock-infected and *S. pneumoniae* exposed AM was calculated. The fold increase in caspase 3/7 activity was significantly smaller in AM from the 11 HIV ART donors (1.76 ± 0.17) than 5 controls (4.8 ± 1.91 , $p=0.0483$ Mann Whitney)(Figure 4-12)

4.2.10 Killing of internalised *Streptococcus pneumoniae* may be reduced in AM from HIV-1-seropositive volunteers on fully suppressive ART at 20 hours post challenge.

48 hours after BAL AM were challenged with *S. pneumoniae* at an MOI of 10. To compare bacterial killing at 20 hours after challenge between AM from HIV-1ART and control donors a modified gentamicin protection assay was used. The CFU/mL was adjusted to correct for variation in AM density at the start of infection as before (see Alveolar macrophages from HIV-1-seropositive and seronegative individuals exhibit similar rates of *Streptococcus pneumoniae* phagocytosis.4.2.7). The same donors were available as for the 4 hour internalization assay (see 4.2.7) except for 1 from each group where the 20 hour assay failed to give an interpretable result. Although only 1 of 4 control AM failed to kill all bacteria by 20 hours, viable bacteria survived in 5 of 12 HIV ART AM. This difference was not statistically significant (Fischer's exact test). The average yield of viable bacteria was also greater for HIV ART than control AM at 20 hours (25.2 ± 10.8 vs. 9.7 ± 9.7 adjusted CFU/mL) but this difference was not statistically significant (Mann Whitney)(Figure 4-13).

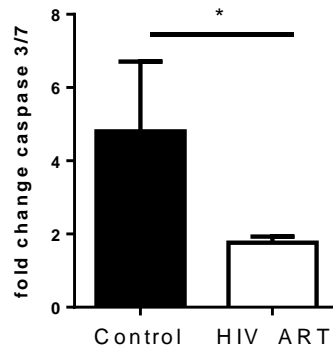


Figure 4-12 AM from HIV-1-seropositive volunteers on fully suppressive ART demonstrate less induction of caspase 3/7 activity following *Streptococcus pneumoniae* challenge.

Alveolar macrophages were isolated from BAL fluid of 11 HIV-1-seropositive on ART (HIV ART) and 5 control volunteers then rested for 48 hours before being challenged with *S. pneumoniae* or mock-infected. At 16 hours caspase 3/7 activity was measured by a luminogenic assay. Induction of caspase 3/7 activity is presented as the fold change from mock infection. * $p < 0.05$ Mann Whitney test.

4.3 Discussion

These experiments demonstrate that AM from HIV-1-seropositive donors on ART are less likely to undergo apoptotic cell death in response to challenge with *S. pneumoniae*. Both the induction of caspase 3/7 activity, a key event in the apoptotic pathway during the macrophage response to *S. pneumoniae* (Dockrell, Lee et al. 2001; Bewley, Marriott et al. 2011), and the proportion of cells showing nuclear chromatin condensation and nuclear fragmentation were at lower levels. The relative survival of HIV ART AM was reflected in the smaller reduction in cell loss overall during bacterial challenge.

As the induction of macrophage apoptosis by *S. pneumoniae* requires internalization and killing of the bacteria (Dockrell, Lee et al. 2001; Ali, Lee et al. 2003) it is important that in these experiments no difference in the early phagocytosis and killing of *S. pneumoniae* was detected in the AM from HIV ART and control donors. This implies that the observed difference in proportions of apoptosis is the result of an HIV-1 related alteration in the apoptosis programme rather than simply a consequence of different levels of initial phagocytosis and early killing. These results are consistent with the literature, as discussed in chapter 1, that demonstrates no defect in macrophage phagocytosis and early killing of *S. pneumoniae* in HIV-1 infection (Gordon, Molyneux et al. 2001). There was a trend towards an increased frequency and number of bacteria surviving through to 20 hours after pneumococcal challenge in the AM of the HIV-1-seropositive donors, suggesting a defect in late bacterial killing that could be related to the relative failure of apoptosis. However, the difference did not reach statistical significance. This may be due to insufficient power resulting from the small number of donors used and the variability of the modified gentamicin protection assay. These results are consistent with those from the models of HIV-1 infected macrophages described in the previous chapter. With HIV-1 infection, primary monocyte-derived macrophages (MDM) and cell lines have been demonstrated to produce similarly altered apoptotic responses to *ex vivo*

alveolar macrophages with exposure to agents including *Mycobacterium tuberculosis*, TLR ligands, staurosporine and cadmium chloride (Patel, Zhu et al. 2007; Nicol, Mathys et al. 2008; Giri, Nebozyhn et al. 2009; Kalsdorf, Scriba et al. 2009).

As discussed already, the MDM and U1 models may not adequately represent the polarisation or activation status of tissue macrophages in human HIV-1 seropositive volunteers as exposure to virus and the specific microenvironment are different. Levels of HIV-1 infection were lower in HIV-1 donor AM than in U1 cells, and while most HIV-1_{BAL} inoculated MDM cultures had some level of productive infection only a minority of donors who were receiving antiretrovirals had detectable intracellular HIV-1 p24, suggesting that the remainder of donor AM were either not productively infected or not infected with HIV-1 at all. Despite the fact that donors on antiretrovirals were receiving suppressive levels of antiretroviral therapy it is noteworthy that as many as 3 of 11 ART treated HIV-1-seropositive donors had detectable HIV-1 p24. Although these volunteers had undetectable peripheral blood viral loads before and after BAL, because they were not tested on the same day as the bronchoscopy, the possibility of a brief plasma viral load elevation (blip) at this time cannot be excluded. However, it would seem more likely that the results suggest many donors may have on-going viral replication in the lung. Although alveolar macrophages from untreated HIV-1-seropositive individuals are known to be infected with HIV-1 (Chayt, Harper et al. 1986; Jeffrey, Israel-Biet et al. 1991; Jambo, Banda et al. 2014), detectable virus is thought to be lost from the bronchoalveolar compartment with ART, in parallel to reductions seen in the peripheral blood (Twigg, Weiden et al. 2008; Twigg, Schnizlein-Bick et al. 2010). It is not clear how frequently and at what rates AM from HIV-1-seropositive individuals established on ART are infected with HIV.

To determine whether the cellular environment of the alveolar macrophage may be altered in the HIV-1-seropositive volunteers a differential white cell count from the BAL was compared with control donors. For the 3 ART naïve donors the average lymphocyte count was higher

than controls. This finding is consistent with the well described lymphocytic alveolitis associated with untreated HIV-1 infection (Guillon, Autran et al. 1988; Twigg, Soliman et al. 1999). Importantly, the differential BAL leukocyte count in the controls in this study is similar to that described elsewhere in healthy populations (De Brauwer, Jacobs et al. 2002; Heron, Grutters et al. 2012). However, an unexpected finding was that a BAL lymphocytosis persisted in the ART treated group of HIV-1-seropositive volunteers (HIV ART), albeit at a lower level. This was despite the fact that these volunteers had been on treatment for many years, all had undetectable HIV-1 viral loads and had reconstituted the CD4 count in their peripheral blood to normal or near normal range. Only one previous study has reported on the fate of the BAL lymphocyte count with ART (Twigg, Weiden et al. 2008). This compared the same group of volunteers before and up to 24 weeks following the initiation of ART and demonstrated that on average the BAL lymphocyte counts reduced towards a level similar to that of control volunteers. However, when smokers, who typically have low BAL lymphocyte counts (Heron, Grutters et al. 2012), are removed from this analysis, the average BAL lymphocyte count at 24 weeks remains elevated at 22% (Twigg, Weiden et al. 2008). My data extends on these findings by studying a group with a much longer duration of ART.

Further subtype analysis of the BAL lymphocytosis demonstrated that the HIV-1-seropositive volunteers had a predominance of CD8⁺ T cells in their BAL, and a reduced CD4:CD8 ratio. HIV-1 infection is known to be associated with both a peripheral blood and pulmonary CD8⁺ T cell expansion relative to the CD4⁺ T cell subset which also results in a reduced or reversed CD4:CD8 ratio in both compartments (Agostini, Poletti et al. 1988; Twigg, Soliman et al. 1999; Robbins, Spritzler et al. 2009). With prolonged ART the peripheral blood CD8 T cell level reduces, but for most cases it does not return back to the level seen in HIV-seronegative individuals (Robbins, Spritzler et al. 2009; Ronsholt, Ullum et al. 2012; Emu, Moretto et al. 2014). Only a single study has reported the fate of the CD8 lymphocytosis in the lung with ART and has shown that it is a fall in the CD8⁺ lymphocytes that exclusively contributes to the

reduction in overall lymphocyte counts (Twigg, Weiden et al. 2008). The present work was not designed to assess the actual numbers of CD8⁺ and CD4⁺ T cells in the BAL, as no correction for the dilution of BAL fluid was possible. Thus it is not possible to definitively determine if the difference in the ratio represents a surfeit of CD8⁺ T cells rather than a loss of CD4⁺ T cells (or both) in the lungs of these HIV-1-seropositive volunteers. However, from haemocytometer counts of BAL cells and estimates of percentages it appears that for the HIV ART donors there was approximately a three-fold increase in numbers of CD8⁺ T cells with only a modest reduction in CD4⁺ T cells.

Despite the persistence of a CD8⁺ T cell lymphocytosis in the HIV ART donor group, no evidence was found of any increase in expression of CD38 either in general or in a subpopulation of the CD8 cells of HIV ART donors compared with controls. CD8⁺ T cells from the lungs and blood of untreated HIV-1 seropositive individuals have higher levels of CD38 expression compared with HIV-seronegative individuals and these also correlate with viral load and the immune activation of HIV (Barry, Johnson et al. 2003). In addition other states of increased immune activation such as the tuberculosis immune reconstitution syndrome (TB IRIS) that can follow ART initiation in those with TB and HIV-1 co-infection are associated with transient increases in CD38 expressing CD8⁺ T cells (Espinosa, Romero-Rodriguez et al. 2013). CD38 is expressed on activated T cells and signalling induces secretion of IL-6, IFN- γ and granulocyte-macrophage colony-stimulating factor (GM-CSF)(Ausiello, la Sala et al. 1996) whereas it is not found on resting memory cells (Savarino, Bottarel et al. 2000). With antiretroviral treatment the proportion of CD38⁺ CD8⁺ T cells in the blood is known to decline (Tilling, Kinloch et al. 2002; Landay, da Silva et al. 2007) but changes in the lung compartment have not been studied. It is therefore possible that the low level expression of CD38 on CD8⁺ T cells from the BAL in this study is a consequence of the suppressive ART and the fact that these volunteers all had no active lung disease. However, it may also be the case that the reconstitution of normal immune cells in lung compartment of HIV-1 seropositive individuals with ART does not exactly

mirror the changes in the blood. That no found no correlation between BAL and blood T cells was observed in these donors may reflect that the bronchoalveolar space is a compartment in which T cell responses to HIV-1 are distinct from those of the blood, as has been described by others (Twigg, Spain et al. 1999). An important question that cannot be answered here is that if not activated CD8⁺ T cells, what subtype or subtypes of CD8⁺ T cell are contributing to excess numbers that have been observed.

The activation status of the macrophages was directly measured using four cell surface markers known to be altered in their expression in different polarisation states. As discussed in chapter 1, a fundamental property of macrophages is that they are plastic, responding to local and exogenous stimuli. They can become differentially activated or polarized and exhibit phenotypes on a spectrum from highly inflammatory to tissue repair (Mosser and Edwards 2008). In one classification macrophages have been labelled as M0, M1 and M2a,b,c each being associated with different gene transcription programmes which lead to altered cytokine production and cell surface protein expression (Mantovani, Sica et al. 2004). For example, priming of MDM with IFN- γ , and LPS or TNF α induces the cell to become more inflammatory or M1 and upregulate cell surface CD80 and CD86. Alternatively treatment with IL-4 increases expression of CD206 and induces the M2a phenotype (Ambarus, Krausz et al. 2012). Alveolar macrophages show similar patterns of polarisation and altered polarisation has been detected in those who smoke or have chronic obstructive pulmonary disease (COPD) (Shaykhiev, Krause et al. 2009). In this study, no significant differences in the expression of CD80, CD163, CD200r or CD206 were found between HIV-1 and control volunteers. AM from viraemic, untreated HIV-1-seropositive volunteers were found by one group to express 10 of 35 genes associated with classical activation ≥ 2 fold greater than AM from seronegative controls (Gordon, Jagoe et al. 2013) but there are no reports for AM from virus suppressed ART treated patients. The lack of a detectable difference in this study may be because there is no difference in the activation status of AM in HIV-1-seropositive volunteers once established on treatment, particularly

when they have no recent history of any pulmonary disease or infection. Alternatively, as the activation of macrophages is a dynamic process it is possible intrinsic differences in polarisation could be lost with the isolation and culture process (Agostini, Zambello et al. 1999; Tomlinson, Booth et al. 2012) and a refinement would be to repeat these studies both immediately post BAL and following overnight culture. However, it is quite likely that this limited range of markers was insufficiently sensitive at detecting changes in macrophage polarisation and analysis of a broader range of markers is required. Numerous markers can be differentially expressed by macrophages depending on their polarisation status (Shaykhiev, Krause et al. 2009; Cassol, Cassetta et al. 2010; Ambarus, Krausz et al. 2012). Thus the lack of any difference expression of CD206, CD80 CD163 and CD200r in these experiments does not completely exclude the possibility that HIV-1 infection is associated with an effect on AM polarisation.

In conclusion, findings in this chapter support those from chapter 3 of this thesis that HIV-1 inhibits macrophage apoptosis following exposure to pneumococci. These findings are discussed in more depth in my concluding chapter. Overall there are persistent changes in the cellular environment of the lung despite longstanding suppressive ART. In particular I found sustained increases in CD8⁺ T cells and evidence that at least in some donors there was on-going HIV-1 replication in some AM. Although these differences did not alter AM polarisation, as assessed in my preliminary screen, phagocytosis or early bacterial killing they were associated with defects in AM apoptosis and potentially with delayed bacterial killing.

Chapter 5. Investigating the mechanism of HIV-1 associated changes in the apoptotic response of macrophages to *Streptococcus pneumoniae*

5.1 Introduction

The experimental data presented in the previous chapters demonstrate a change in the apoptotic behaviour of macrophages in the context of HIV-1 infection. HIV-1 alters diverse cellular functions and, as discussed in chapter 1, can alter transcription (Vazquez, Greenwell-Wild et al. 2005), intracellular signal transduction (Giri, Nebozyhn et al. 2009) and surface receptor expression (Zhu, Shi et al. 2011), each being associated with the regulation of apoptosis in macrophages. Thus a number of potential mechanisms could underlie the observations so far presented. In choosing candidates for further investigation, a key consideration is whether the effect is mediated directly within HIV-1 infected macrophages or indirectly on bystander cells. While in the U1 macrophage model there is universal HIV-1 infection, available evidence shows that fewer than 0.1% of alveolar macrophages (AM) from HIV-1-seropositive individuals are productively infected (Chayt, Harper et al. 1986; Lewin, Kiriara et al. 1998). In this study HIV-1 was only detected in the AM of 3 out of 11 donors receiving antiretroviral therapy who were tested, and in the monocyte-derived macrophage (MDM) HIV_{BAL} infection model infection rates ranged from <1% to 100%. The size of the HIV-1 associated difference in the induction of apoptosis in these latter two models cannot be explained by altered behaviour of the directly HIV-1 infected macrophages alone.

In this chapter I set out to investigate whether the observed effects are likely to be the consequence of direct HIV-1 infection or an alternative indirect effect on uninfected macrophages. I have studied the HIV-1 env gene product gp120 as a candidate indirect mediator. Given the central role of Mcl-1 as a regulator of mitochondrial outer membrane permeabilisation and macrophage survival I also look at Mcl-1 expression in the HIV-1 MDM model. I next investigate the role of superoxide, a reactive oxygen species generated in the

mitochondria, which has been linked to both HIV-1 (Lassiter, Fan et al. 2009) (Vilhardt, Plastre et al. 2002) and mitochondrial stress responses including apoptosis (Madesh and Hajnoczky 2001; Ricci, Pastukh et al. 2008). Finally I have addressed whether there may be other features of the alveolar environment which are associated with the altered apoptosis, including the antiretroviral therapy compounds the alveolar macrophages of the donors are exposed to *in vivo*.

5.2 Results

5.2.1 There is no association between the level of MDM HIV-1 infection and *Streptococcus pneumoniae* associated apoptosis

The relationship between HIV-1 infection of human MDM and the cells' apoptotic response to *S. pneumoniae* was examined. The proportions of MDM staining positive for p24 were calculated to give a percentage of infected cells for each donor HIV-1 preparation. The increment in macrophage apoptosis between mock-infection and serotype 2 *S. pneumoniae* (D39) exposure for 20 hours was then determined as a function of the level of HIV-infection in the culture. The calculation was based not on the increment in apoptosis in the HIV-infected cultures alone but on the value obtained when the value in the HIV-infected culture was subtracted from the sham-infected culture to correct for any variability in rates of apoptosis between donors. It was calculated as [(sham-infected MDM D39 apoptosis – MI apoptosis) – (HIV-1 MDM D39 apoptosis – MI apoptosis)]. Across a full range of HIV-1 infection levels (<1% to 100%) there was no correlation between the direct HIV-infection of MDM and induction of apoptosis, n=13, r=0.13, p=0.66, Pearson's correlation (Figure 5-1).

5.2.2 Exposure to HIV-1 gp120 is not associated with any altered early phagocytosis of *Streptococcus pneumoniae* by MDM.

In view of the lack of a relationship between direct infection of macrophages with HIV-1 and levels of apoptosis I reasoned that release of an HIV-1 protein might be responsible for the observed reduction in apoptosis through effects on uninfected bystander cells. Since gp120 is easily shed from the HIV-1 virion (Oh, Cruikshank et al. 1992; Klasse and Moore 2004) and can mediate effects on uninfected macrophages (Conti, Fantuzzi et al. 2004), I first examined whether it was sufficient to inhibit apoptosis.

14 day old MDM were treated with recombinant gp120 at concentrations of 10 ngmL⁻¹ or 100 ngmL⁻¹ from 1 hour prior to and then during exposure to *S. pneumoniae*. After 4 hours viable

internalized bacteria were extracted using a gentamicin protection assay and counted. There was no significant difference in the number of internalized viable *S. pneumoniae* between the conditions (Kruskal-Wallis)(Figure 5-2).

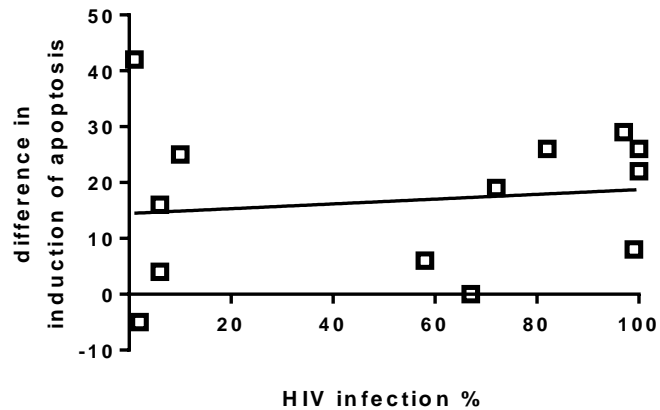


Figure 5-1 The level of direct MDM infection with HIV-1 does not correlate with the difference in level of induction of apoptosis following *Streptococcus pneumoniae* challenge.

HIV-1 or sham-infected monocyte-derived macrophages (MDM) were challenged with *S. pneumoniae* or mock-infected. Apoptosis was measured at 20 hours by counting the percentage of 4',6-diamidino-2-phenylindole (DAPI) stained cells with nuclear fragmentation. The difference in induction of apoptosis in *S. pneumoniae* challenged as compared to mock-infected MDM was compared between sham-infected and HIV-1 infected MDM. Data show the correlation between the difference in induction of apoptosis for each donor's paired MDM (y axis) against the observed rate of HIV-1 infection (x axis).

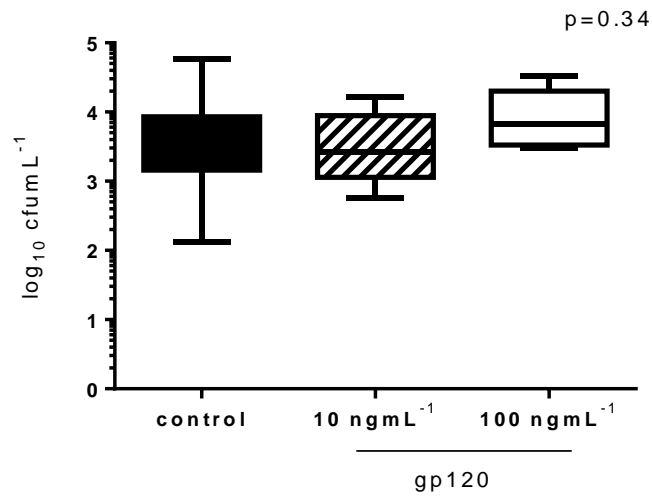


Figure 5-2 Rates of viable internalised *Streptococcus pneumoniae* in MDM four hours after infection are similar irrespective of gp120 exposure

Monocyte-derived macrophages (MDM) were challenged with *S. pneumoniae* for four hours in the presence of gp120 at 10 ngmL⁻¹ or 100 ngmL⁻¹ and medium alone (control). Viable internalized bacteria were counted using a gentamicin protection assay. Data are shown as median, IQR and range, $n \geq 5$, cfu colony forming units and compared with Kruskal-Wallis.

5.2.3 Exposure to HIV-1 gp120 reduces the degree of apoptosis in MDM following *Streptococcus pneumoniae* challenge.

14 day old MDM were treated with gp120 at concentrations of 10 ngmL⁻¹ or 100 ngmL⁻¹ from 1 hour prior to and for 20 hours *S. pneumoniae* challenge or mock-infection. Recombinant gp120 from a baculovirus expression system was used to avoid any potential contamination of the cultures with lipopolysaccharide (LPS) from bacterial systems. Adherent cells were then washed, fixed in paraformaldehyde (PFA), stained with 4',6-diamidino-2-phenylindole (DAPI) and examined by fluorescence microscopy for morphological changes of apoptosis. There was no difference in the rate of apoptosis following mock-infection between gp120 10 ngmL⁻¹ treated MDM (3.6±0.7%) and control (2.4±0.6%). 20 hours post infection the number of apoptotic cells was smaller in gp120 10 ngmL⁻¹ exposed MDM (19.2±5.4%) than control (36.2±7.4%), resulting in a significant difference in *S. pneumoniae* associated apoptosis (n=7, p=0.0313 Wilcoxon matched pairs signed rank test). When compared together the same pattern of significance was seen using 2 way ANOVA (Figure 5-3 A and B). The same pattern was observed when MDM were exposed to gp120 at 100 ngmL⁻¹; after 20 hours mock-infection gp120 and control MDM showed similar low rates of apoptosis (1.1±0.3% vs. 0.9±0.3%), but there was a significantly lower level of apoptosis counted after *S. pneumoniae* infection in gp120 than control (18.2±3.6% vs. 34.0±4.2%, n=6, p=0.0313 Wilcoxon matched pairs signed rank test), which represented a significantly smaller *S. pneumoniae* associated induction of MDM apoptosis with gp120 exposure (Figure 5-3 C and D).

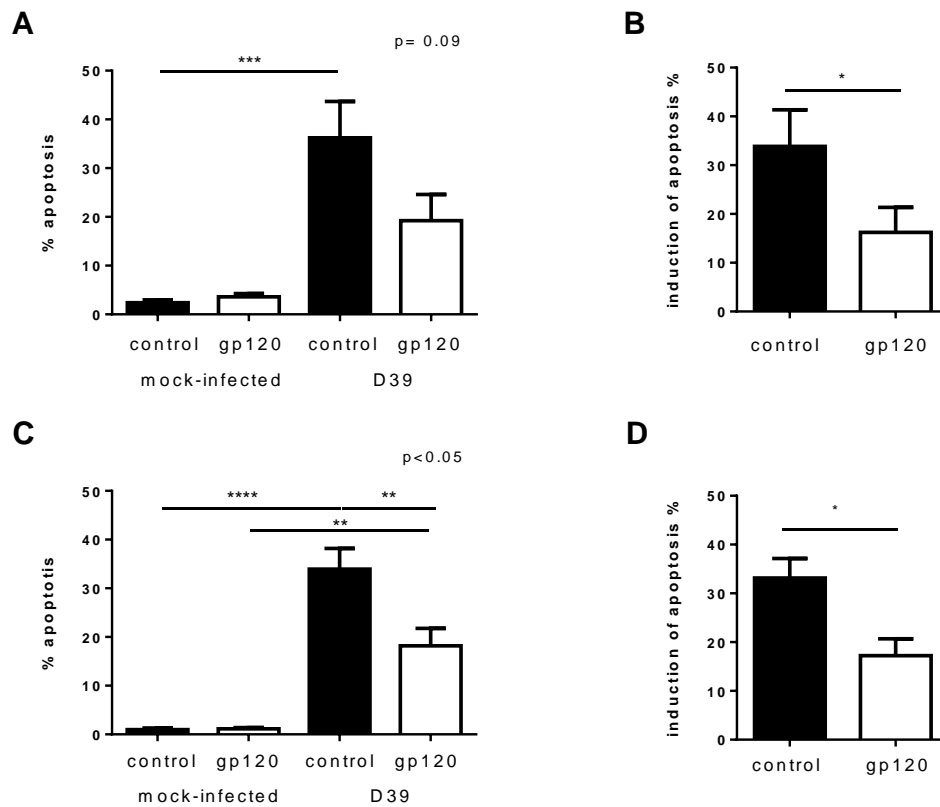


Figure 5-3 gp120 exposure reduces the induction of MDM apoptosis following *Streptococcus pneumoniae* challenge.

Monocyte-derived macrophages (MDM) were treated with medium alone (control) or gp120 10 ngmL⁻¹ (A+B) or 100 ngmL⁻¹ (C+D) then challenged with *S. pneumoniae* (D39) or mock-infection. At 20 hours cells were fixed and stained with DAPI to measure nuclear morphological changes of apoptosis (A,C) and the increment in apoptosis from mock-infection to 20 hours post bacterial challenge (B,D). n = 6-7, * p<0.05, Wilcoxon matched pairs signed rank test. **p<0.01, ***p<0.001, ****p<0.0001, 2 way ANOVA with Bonferroni's post test.

5.2.4 Exposure to HIV-1 gp120 reduces the degree of *Streptococcus pneumoniae* associated caspase 3/7 activation in MDM.

To measure caspase 3/7 activity, 14 day old MDM were exposed to gp120 10 ngmL⁻¹ or medium alone for one hour before and during challenge with *S. pneumoniae* or mock-infection. After 16 hours cells were lysed and caspase 3/7 measured using a fluorescent assay. The protein concentration was also quantified in each lysate as a measure of cell density and fluorescence readings were adjusted accordingly to normalize for variability in the cell numbers between donors. The level of caspase 3/7 activity was significantly smaller in gp120 exposed MDM after both 16 hours mock-infection (0.7±0.1 vs. 1.1±0.1 RFU, p<0.0156) and *S. pneumoniae* (3.0±0.5 vs. 4.7±0.6 RFU, p<0.0313 Wilcoxon matched-pairs signed rank test) challenge compared with control MDM. When compared together with 2 way ANOVA there was a significant interaction for gp120 after *S.pneumoniae* challenge (Figure 5-4).

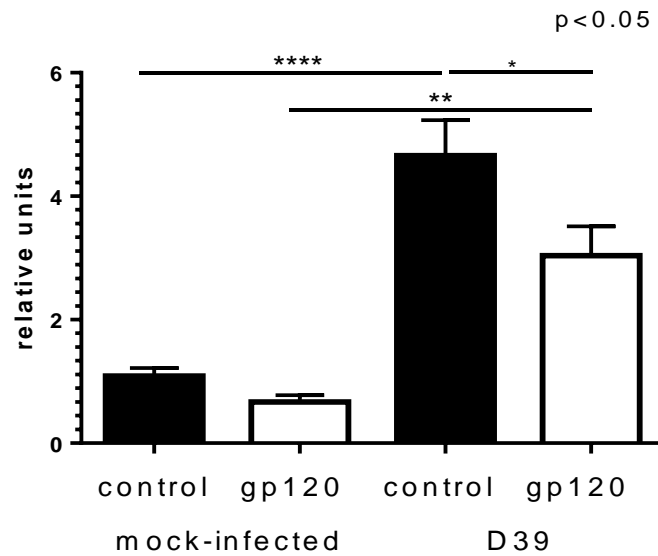


Figure 5-4 gp120 treatment is associated with reductions in MDM caspase 3/7 activity with and without *Streptococcus pneumoniae* challenge.

Monocyte-derived macrophages (MDM) were treated with medium alone (control) or gp120 10 ngmL⁻¹ then challenged with *S. pneumoniae* (D39) or mock-infection. Caspase 3/7 activity was measured using a fluorescent assay. Data show relative fluorescent units normalized to protein concentration. n=7, * p<0.05, **p<0.01, ****p<0.001 2 way ANOVA with Bonferroni's post test.

5.2.5 Exposure to gp120 is associated with increased bacterial survival in MDM at 20 hours post *Streptococcus pneumoniae* challenge, despite similar levels of early killing

14 day old MDM were again exposed to gp120 10 ngmL⁻¹, gp120 100 ngmL⁻¹ or medium alone (control) for one hour and during challenge with *S. pneumoniae*. Using a modified gentamicin protection assay viable internalized bacteria were measured at 4 and 6 hours, as measures of early phagocytosis and killing and at 20 hours after infection and compared with those counted at four hours. Similar reductions in the number of viable bacteria were seen between four and six hours for all conditions (mean difference 1.07, 1.26 and 1.31 log₁₀ cfumL⁻¹ for gp120 10 ngmL⁻¹, gp120 100 ngmL⁻¹ and control respectively). At 20 hours post infection the number of viable bacteria in control and gp120 10 ngmL⁻¹ exposed MDM had continued to fall. However, this was not seen in MDM that had been treated with the higher concentration of 100 ngmL⁻¹ gp120; there was a significant difference between the viable counts in gp120 100 ngmL⁻¹ (2.87± log₁₀ cfumL⁻¹) vs. control MDM (0.83±0.83 log₁₀ cfumL⁻¹, p=0.0403, Mann Whitney) at 20 hours post infection (Figure 5-5). These data suggest that while initial killing of *S. pneumoniae* by MDM is not affected by exposure to gp120, gp120 at 100 ngmL⁻¹ results in a reduction of later phase killing of internalized *S. pneumoniae* by MDM.

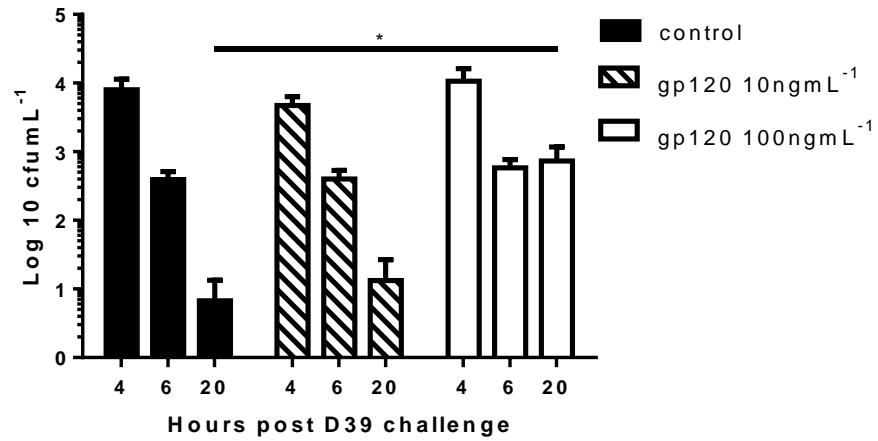


Figure 5-5 Exposure to gp120 is associated with reduction in late phase killing of *Streptococcus pneumoniae* by MDM.

Monocyte-derived macrophages (MDM) were treated with medium alone or gp120 10 ngmL⁻¹ or 100 ngmL⁻¹ then challenged with *S. pneumoniae* (D39). At the indicated time points viable intracellular bacteria were counted using a modified gentamicin protection assay. n=5-10, * p<0.05, gp120 100ngmL⁻¹ vs. control at 20 hours Mann Whitney test. 4 hour data are those shown also in Figure 5-2.

5.2.6 HIV-1 infection of MDM is associated with persistent expression of Mcl-1 during *Streptococcus pneumoniae* challenge

These observations that gp120 reduces *S. pneumoniae* associated macrophage apoptosis are in keeping with the findings of Swingler et al. that gp120 protects macrophages from the apoptotic effects of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) (Swingler, Mann et al. 2007). This group also reported that the anti-apoptotic effect of gp120 was associated with up regulation of the anti-apoptotic B cell lymphoma (Bcl)-2 family member Mcl-1. Given that dynamic expression of Mcl-1 plays a key role regulating macrophage apoptosis following *S. pneumoniae* challenge, I next questioned whether HIV-1 infection modified the expression of Mcl-1 (Marriott, Bingle et al. 2005). HIV-1 and sham infected MDM were challenged with *S. pneumoniae* or mock-infected as before. After 20 hours cells were lysed and the expression of Mcl-1 was measured in MDM by western blot. To give a semi quantitative estimate of any change in the levels of Mcl-1 expression the blots from 5 separate experiments were analysed using densitometry. The ratio of the density of the Mcl-1 bands to the loading controls were calculated and compared with the sham virus treated (control), mock-infected result. As expected, Mcl-1 was expressed in the control macrophages but the level fell significantly following challenge with *S. pneumoniae* ($p = 0.0293$, 2 way ANOVA with Fisher's post test). Mcl-1 expression was similar in HIV-1 infected MDM after mock-infection but there was no reduction following *S. pneumoniae* challenge (Figure 5-6).

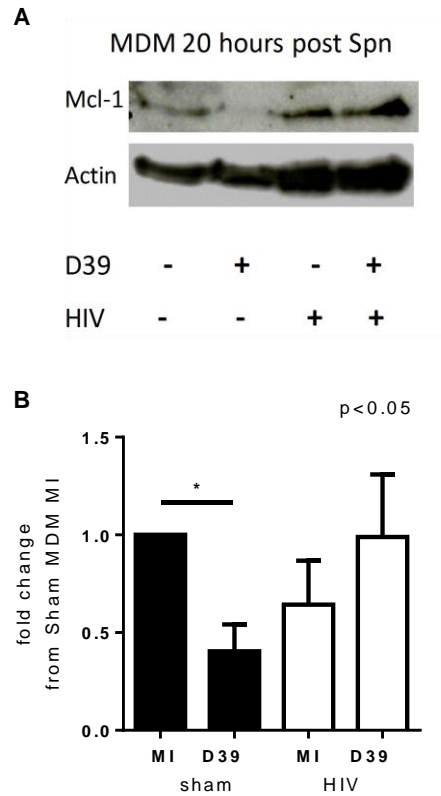


Figure 5-6 HIV-1 infection is associated with persisting expression of Mcl-1 in MDM at 20 hours following *Streptococcus pneumoniae* challenge.

Monocyte-derived macrophages (MDM) were infected with HIV-1 or sham infected (sham) for 7 days and then challenged with serotype 2 *S. pneumoniae* (D39) or mock-infected (MI). Cells were lysed and Mcl-1 expression measured by western blot. **(A)** Representative western blot **(B)** densitometry bar chart. Data shown as fold change in band density compared with mock-infected, sham MDM after adjustment for any fold change in actin. $n = 5$ * $p < 0.05$, 2 way ANOVA with Fisher's post test

5.2.7 Mitochondrial superoxide is increased by HIV-1 and by *Streptococcus pneumoniae* challenge

Mitochondrial superoxide contributes to intracellular bacterial killing (West, Brodsky et al. 2011) and its release is enhanced during induction of apoptosis (Ricci, Pastukh et al. 2008). As HIV-1 has also been linked with alteration in both reactive oxygen species and antioxidants I therefore investigated whether *S. pneumoniae* and HIV-1 infection influenced mitochondrial superoxide (O_2^-) in macrophages. Generation of mitochondrial O_2^- was measured using MitoSOX Red™. MDM were differentiated as before and then inoculated with HIV-1_{BAL} (HIV-1 MDM) or sham infected (control) in 96 well tissues culture plates. They were then challenged with *S.pneumoniae* or mock-infected. Generation of fluorescent signal was detected in unfixed cells using a plate reader. Initial experiments at 8, 12 and 16 hours established that changes in O_2^- were best detected at 16 hours post *S. pneumoniae* challenge. To ensure that any differences in mitochondrial O_2^- were not due to alteration in the number of mitochondria the density of mitochondria was measured on the plate reader using Mitotracker Green FM™, a green-fluorescent mitochondrial stain. No difference in the mass of mitochondria was detected between MDM irrespective of HIV-1_{BAL} or *S. pneumoniae* infection (Figure 5-7 A). When control MDM were challenged with *S. pneumoniae*, the level of O_2^- increased (to 0.09 ± 0.01 RFU, $p=0.043$, Friedman test with Dunn's post test), consistent with previous results from my host group. There were significantly greater levels of mitochondrial O_2^- in HIV-1 MDM (0.08 ± 0.01 RFU) than control MDM (0.05 ± 0.01 RFU, $p= 0.043$, Friedman test with Dunn's post test) 16 hours after mock-infection. Of note, however, the level of superoxide in HIV-1 MDM did not increase further with *S. pneumoniae* infection (0.07 ± 0.02 RFU, ns)(Figure 5-7 B). These data show that while both HIV-1 infection leads to increased generation of mitochondrial superoxide, and HIV-1 MDM are unable to generate an additional increment in mitochondrial O_2^- in response to *S. pneumoniae*.

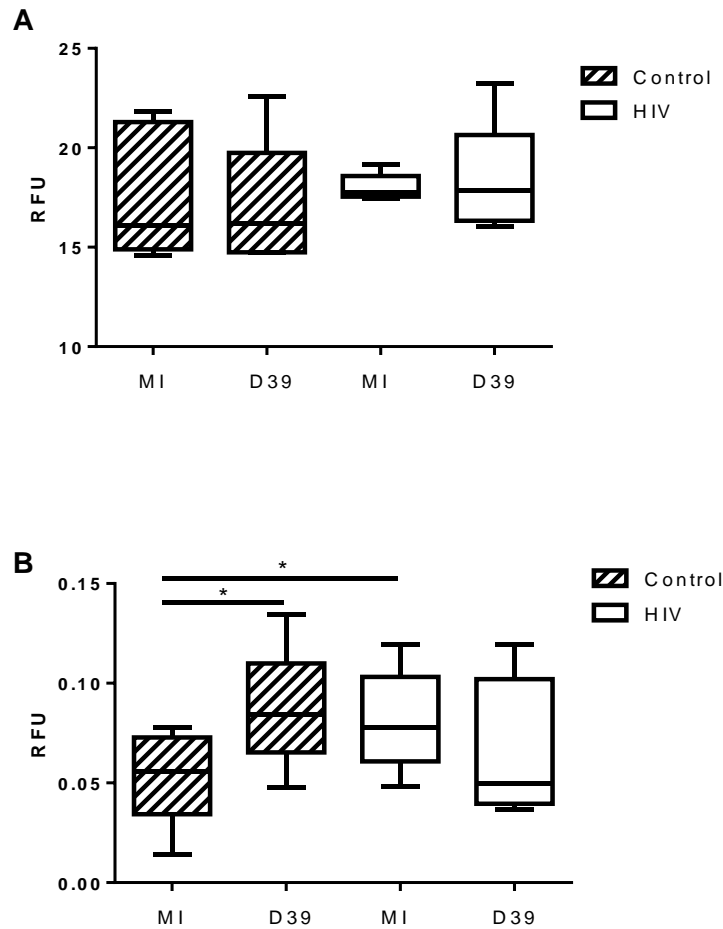


Figure 5-7 In MDM HIV-1 infection and *Streptococcus pneumoniae* infection are associated with elevated mitochondrial superoxide levels without any alteration in mitochondrial density.

(A) Monocyte-derived macrophages (MDM) were mock-infected (MI) or exposed to *S. pneumoniae* (D39) for 20 hours in sham-infected (Control) or HIV-1_{BAL} infected (HIV) MDM and then the mitochondrial density measured in MDM using Mitotracker Green™. The RFU define was measured on a fluorescence plate reader (B) Production of mitochondrial superoxide was also measured by MitoSOX Red™ in parallel wells from the same experiments. n=5, * p<0.05, Friedman test with Dunn's post test. Data are shown as median with IQR and range

5.2.8 The level of induction of AM apoptosis following *Streptococcus pneumoniae* challenge shows a relationship to the proportion of CD4⁺ and CD8⁺ T cells in the bronchoalveolar lavage fluid.

I next addressed whether the immunological environment and more specifically the persistent alterations in pulmonary T-cells also contributed to apoptosis resistance of the AM. To investigate whether there was a relationship between the observed differences in *S. pneumoniae* associated AM apoptosis between HIV-1 and control donors and the proportion of CD4⁺ and CD8⁺ T cells counted in their bronchoalveolar lavage fluid (BAL) fluid these two parameters were compared using Pearson correlation. Paired data were available from 11 HIV-1-seropositive and 4 controls donors. Positive correlations were seen between both the CD4:CD8 ratio ($r=0.69$, $p=0.005$) and the CD4% ($r=0.59$, $p=0.024$, $n=15$, Pearson's correlation) in the BAL and the induction of apoptosis (Figure 5-8). There was however, no significant association between total lymphocyte % and apoptosis.

I also analysed whether AM apoptosis had a relationship with more general donor factors that can impact on immune responses, namely duration of ART, nadir CD4 count before commencement of ART and volunteer age. No significant correlation was found between any of these and the level of AM apoptosis after *S. pneumoniae* challenge. Additionally, in keeping with the lack of any detectable influence of HIV-1 on AM surface polarisation markers, no association was found between apoptosis induction and expression of CD80, CD163, CD200r or CD206 AM for these subjects.

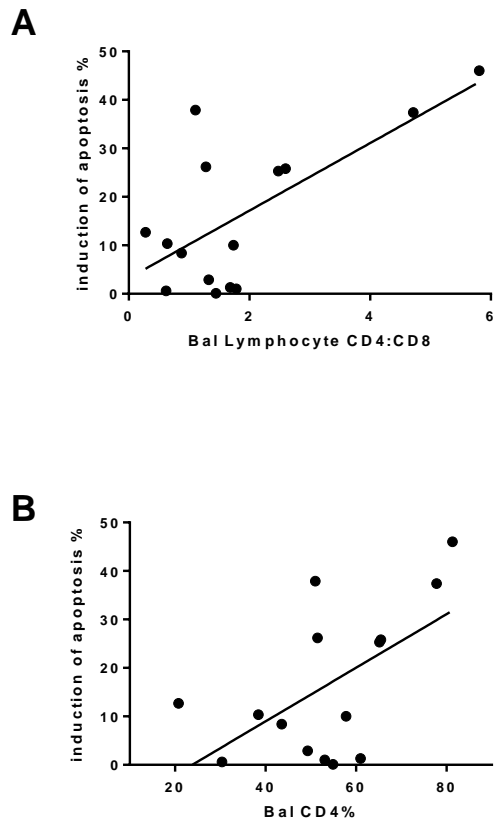


Figure 5-8 Induction of AM apoptosis following *Streptococcus pneumoniae* challenge correlates with the bronchoalveolar lavage CD4:CD8 ratio and CD4%.

Induction of apoptosis following *S. pneumoniae* challenge was measured in AM from HIV-1-seropositive and control donors and compared with (A) the ratio of CD4⁺:CD8⁺ CD3⁺ cells, and (B) the proportion of CD3⁺/CD4⁺ T-cells in paired BAL samples. Induction of apoptosis is calculated as the % of cells with fragmented nuclei 20 hours after *S. pneumoniae* challenge minus the value from mock-infected cells at 20 hours.

5.2.9 Receipt of a non nucleoside reverse transcriptase inhibitor or protease inhibitor as a third agent in an ART regimen is not associated with any difference in the apoptosis response of AM to *Streptococcus pneumoniae* challenge.

Antiretroviral protease inhibitors can inhibit apoptosis (Weaver, Tarze et al. 2005; Vlahakis, Bennett et al. 2007) and unpublished data from my host group has shown inhibition of alveolar macrophage apoptosis in mice during pneumococcal infection (Marriott 2010). I therefore explored whether protease inhibitors (PI) influenced the apoptotic responses to *S. pneumoniae* observed in the AM from HIV-1-seropositive donors. The HIV ART group was divided into those in receipt of PI vs. non nucleoside reverse transcriptase inhibitor (NNRTI) based ART. To avoid the possibility that past exposure might have an ongoing influence, individuals were enrolled who had exclusively received drugs from only one of these classes during their total treatment history. Of 12 available HIV-1 donors 7 (4 male, 4 white) had been on NNRTI (efavirenz, nevirapine, rilpivirine) and 5 (4 male, 3 white) on PI based HAART (nelfinavir, saquinavir, atazanavir, darunavir). The rates of *S. pneumoniae* associated apoptosis induction by AM at 20 hours were $17.09 \pm 4.015\%$ for PI donors and $10.36 \pm 5.22\%$ for NNRTI donors. While both groups demonstrated lower rates of apoptosis than control donors ($29.83 \pm 5.90\%$), they did not differ significantly from each other ($p = 0.20$, Mann Whitney, Figure 5-9). In view of the relationship between the CD4 % and CD4:CD8 ratio in the BAL and levels of AM apoptosis described above I also assessed whether the different ART regimens had an influence on these BAL T cell subsets. There was no difference in the CD4 ($41.56 \pm 7.07\%$ vs. $48.53 \pm 5.45\%$, $p = 0.32$) and CD8 ($51.26 \pm 7.34\%$ vs. $39.78 \pm 3.84\%$, $p = 0.25$, Mann Whitney) T cell populations in BAL fluid between the PI and NNRTI groups respectively, although only 5 and 6 donors respectively were available for these analyses (Figure 5-10). Taken together these data suggest that the altered rates of apoptosis observed in the AM from HIV-1 compared with control donors is unlikely to be related to any effect from their antiretroviral drugs.

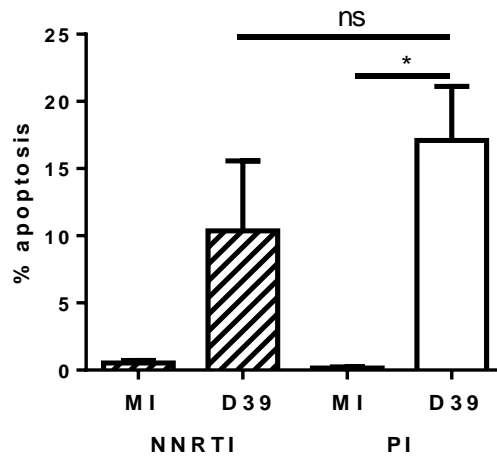


Figure 5-9 Receipt of protease inhibitor or non-nucleoside reverse transcriptase inhibitor therapy is not associated with any difference in *Streptococcus pneumoniae* associated AM apoptosis.

AM isolated from HIV-1-seropositive individuals on fully suppressive ART were mock-infected or challenged with *S. pneumoniae* (D39). At 20 hours fixed cells were stained with 4',6-diamidino-2-phenylindole (DAPI) and examined for nuclear morphological changes of apoptosis. n= 5 protease inhibitor (PI) and n=7 on non nucleoside reverse transcriptase (NNRTI) based therapy. ns = non significant, Mann Whitney test.

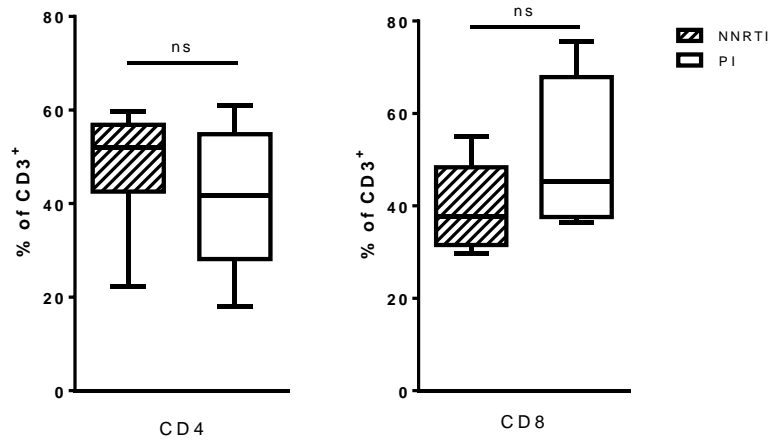


Figure 5-10 Receipt of protease inhibitor or non-nucleoside reverse transcriptase inhibitor therapy is not associated with any difference in the proportions of CD4⁺ and CD8⁺ lymphocytes in the BAL.

Bal fluid was collected from 5 protease inhibitor (PI) and 6 non nucleoside reverse transcriptase inhibitor (NNRTI) therapy treated HIV-1 seropositive individuals. At 24 hours cells were labelled with fluorophore conjugated antibodies against CD3, CD4 and CD8 and analysed by flow cytometry. ns = non significant, Mann Whitney test, data shown as median \pm IQR and range.

5.3 Discussion

The work described in this chapter addresses the mechanisms and associations of *S. pneumoniae* associated apoptosis in macrophages and its reduction by HIV-1. First I focused on whether the HIV-mediated effects were influenced directly or indirectly. No correlation was found between the rate of MDM infection with HIV-1 and the induction of apoptosis following bacterial challenge in the MDM model. It follows that, as well as directly infected MDM, uninfected macrophages are also being influenced by the effects of HIV-1 in these models. Furthermore, it is also unlikely that this effect requires direct contact of uninfected with infected cells as demonstrated by the four donors where even though fewer than 10% of MDM were infected, making it unlikely they were in contact with many of the uninfected cells, rates of apoptosis were nevertheless reduced. A refinement of these experiments would be to label p24 antigen containing cells with a fluorescent secondary antibody and then simultaneously observe whether apoptosis occurred in p24 expressing MDM, p24 negative MDM or both. However, the lack of correlation between HIV-1 infection and apoptosis level and the low levels of infection in many cultures despite reduced apoptosis make a direct effect of HIV-1 less likely. These observations lead to the hypothesis that a soluble factor, released by HIV-1 infected macrophages mediates an alteration in apoptosis of the uninfected cells.

HIV-1 proteins, and in particular HIV-1 env gp120, can mediate indirect effects of HIV-1 on uninfected macrophages. Free gp120 is detectable in the serum and lung of HIV-1-seropositive individuals (Oh, Cruikshank et al. 1992; Klasse and Moore 2004). Purified or recombinant gp120 is sufficient to alter the phagocytosis and intracellular killing by macrophages of *Cryptococcus neoformans* and *Mycobacterium avium* (Wagner, Levitz et al. 1992; Denis and Ghadirian 1994) and recombinant gp120 alone mimics the action of replication competent HIV-1 in MDM cultures, inducing the secretion of IL-10 and macrophage inflammatory protein 1 α (MIP-1 α), MIP-1 β , RANTES, and tumour necrosis factor alpha (Borghi, Fantuzzi et al. 1995;

Choe, Volsky et al. 2001). Furthermore, gp120 is a relevant candidate as it is a modulator of apoptotic responses (Herbein, Mahlknecht et al. 1998; Swingler, Mann et al. 2007). I therefore investigated whether exposure to the HIV-1 env gene product, gp120 was associated with an alteration in MDM apoptosis. I found that 10-100 ngmL⁻¹ of gp120 was not associated with any change in the number of viable bacteria contained within MDM at 4 and 6 hours following *S. pneumoniae* exposure. This implies that up to this time, the balance of phagocytosis and killing of *S. pneumoniae* is not affected by gp120, which is in keeping with the lack of any impairment of macrophage phagocytosis of pneumococci in HIV-1 infection in general (Gordon, Molyneux et al. 2001). However, there was significantly less apoptosis in gp120 exposed MDM following challenge with *S. pneumoniae*, which was further associated with reductions in bacterial killing between 6 and 20 hours where MDM were exposed to 100ngmL⁻¹ gp120. In addition, although the rate of MDM apoptosis in mock-infected cultures was low, gp120 exposure reduced this background rate of apoptosis further. Taken together these results suggest that gp120 affects the apoptotic programme of macrophages in a manner that includes, but is not limited to, the *S. pneumoniae* associated apoptotic response in MDM. These results are consistent with the evidence that gp120 can reduce apoptosis in macrophages, as discussed in chapter 1 (Swingler, Mann et al. 2007). A potential limitation to this interpretation is that the concentration of free gp120 in HAART treated HIV-1-seropositive individuals is not well defined. 10 ngmL⁻¹ to 100 ngmL⁻¹ equates to the concentration of free and antibody bound gp120 detected in the serum of HAART naïve individuals (Oh, Cruikshank et al. 1992) but these levels may not be the same in other compartments (Klasse and Moore 2004). Further experiments would need to measure the level of gp120 in the MDM HIV-1 infection model and use env deficient HIV-1. I would also measure the gp120 concentration in the BAL fluid of HIV-1 donors on HAART and establish both to what extent the BAL gp120 is bound to immunoglobulin and whether such bound gp120 has the same effect on MDM apoptosis regulation.

The reduction in apoptosis attributed to gp120 by Swingler et al. was associated with changes in the expression of Mcl-1 (Swingler, Mann et al. 2007). I also measured the expression of Mcl-1 in MDM following *S. pneumoniae* infection and found that there was a significant reduction in Mcl-1 expression in sham infected MDM which was not seen in MDM treated with HIV, despite similar expression without *S. pneumoniae*. Increased Mcl-1 expression is a feature of differentiated macrophages (Liu, Perlman et al. 2001). Changes in Mcl-1 expression have also been demonstrated during *S. pneumoniae* infection of macrophages with a reduction in Mcl-1 expression between 16 and 20 hours post infection regulating the induction of apoptosis (Marriott, Bingle et al. 2005). Over expression of Mcl-1 in transgenic mice is associated with both delayed alveolar macrophage apoptosis and delayed clearance of *S. pneumoniae* from mouse lungs (Marriott, Bingle et al. 2005). My results suggest that one mechanism of HIV-1 associated reduction in macrophage apoptosis following *S. pneumoniae* infection could involve the increased or persistent expression of Mcl-1, and this may be mediated by soluble gp120 produced by HIV-1 infected MDM. Thus adding gp120 alone to healthy MDM would be predicted to have the same effect on Mcl-1 expression following pneumococcal challenge. This hypotheses could be tested further as it predicts that apoptosis would be restored by blocking the CD4/CCR5 receptor to prevent gp120 binding, or by inhibiting Mcl-1 transcription using siRNA.

Pneumococcal killing associated with macrophage apoptosis involves the generation of reactive oxygen and nitrogen species (ROS/RNS); In MDM, *S. pneumoniae* challenge induces Mcl-1 down regulation, translocation of cytochrome C and caspase activation, all of which can be abrogated by inhibition of inducible nitric oxide synthase (iNOS) (Marriott, Ali et al. 2004). Concurrent with a fall in Mcl-1, there is a burst of mitochondrial ROS and an associated cathepsin D dependent upregulation of the antioxidant superoxide dismutase (SOD)(Bewley, Pham et al. 2011). In addition to its role in stabilising the mitochondrial outer membrane, and so preventing apoptosis, Mcl-1 is required for optimum oxidative phosphorylation (Perciavalle,

Stewart et al. 2012). In the absence of a 36 kD isoform of Mcl-1, that localises to the mitochondrial matrix, impaired function of the electron transport chain results in increased mitochondrial superoxide (O_2^-) (Perciavalle, Stewart et al. 2012). Mitochondrial O_2^- is increased during apoptosis and plays a role in intracellular bacterial killing (Ricci, Pastukh et al. 2008; West, Brodsky et al. 2011). My host research group has shown that blocking mitochondrial O_2^- leads to increased bacterial survival at later time points suggesting that overall, mitochondrial ROS enables bacterial killing downstream of the induction of a mitochondrial pathway of apoptosis (Bewley, Pham et al. 2011; Bewley 2013). HIV-1 is also associated with increased ROS and increased antioxidant production at baseline (Suresh, Annam et al. 2009) and in alveolar macrophages in particular from HIV-1 seropositive donors, there is increased SOD (Gordon, Jagoe et al. 2013). I therefore investigated whether levels of mitochondrial O_2^- were altered in HIV-1 infected MDM and how this changed after challenge with *S. pneumoniae*. As expected, *S. pneumoniae* infection led to increased O_2^- in control MDM as did HIV-1 infection of MDM alone. These findings were not an artefact of altered mitochondrial numbers as the total mitochondrial mass was not altered by either pneumococcal challenge or HIV-1, in keeping with previous work showing no effect of HIV-1 infection on macrophage mitochondrial DNA content (Azzam, Lal et al. 2006). However, there was no additional rise in mitochondrial O_2^- in HIV-1 infected MDM subsequently challenged with *S. pneumoniae*. One explanation may be that MDM exposed to HIV-1 and/or soluble elements from other HIV-1 infected MDM respond with not only increased ROS production but also with parallel upregulation of antioxidants, which then serve to rein in and neutralize any further increment in superoxide provided by the mitochondria as the cell undergoes apoptosis. This hypothesis could be further tested by measuring SOD and the consequences of its inhibition in these conditions.

In the alveolar environment, factors other than gp120 could be playing a role in influencing the apoptotic and bacterial killing behaviour of alveolar macrophages in HIV-1 seropositive individuals on ART. Potential candidates include other cells (e.g. epithelial cells and

lymphocytes), soluble host factors (immunoglobulin, complement, and cytokines) and antiretroviral compounds. Since I had observed that there was a moderate but significant CD8 T lymphocytosis in the BAL of the HIV-1-seropositive donors in this study, I compared both the total lymphocyte and CD4/CD8 lymphocyte percentages in the BAL fluid with the apoptotic responses of the AM. There was a significant correlation between the induction of apoptosis following *S. pneumoniae* infection and the percentage of CD4⁺ and ratio of CD4:CD8 T lymphocytes in the BAL, but not with the percentage of lymphocytes as a proportion of all leukocytes. This association may have arisen simply because the HIV-1 donors had lower CD4:CD8 ratios than the control donors through an HIV-1 mediated effect that was independent of that on the macrophages. That no correlation was observed when control donors were excluded might support this interpretation, although it is also possible that too few donors were included to adequately power for a detectable effect. Alternatively this finding could mean that there is a relationship between CD4⁺ T helper cells or cytotoxic T cells and alveolar macrophages that influences macrophage responses to *S. pneumoniae*, as has been demonstrated for the immune regulation of Mycobacterium tuberculosis (MTB) infection in untreated HIV-1-seropositive individuals (Kalsdorf, Scriba et al. 2009). Ultimately an association between pulmonary lymphocytes and the apoptotic response of alveolar macrophages needs to be tested by functional experiments with cell cultures that provide evidence of the cells required to modulate macrophage responses and define the molecular basis of this effect.

Antiretroviral compounds inhibit the replication of HIV-1 by action on virus specific targets such as the HIV-1 protease and reverse transcriptase enzymes. However, these drugs can also have off target effects and the HIV-1 protease inhibitors have been shown to inhibit apoptosis by blocking the mitochondrial adenine nucleotide translocator and thus maintain mitochondrial integrity (Weaver, Tarze et al. 2005; Vlahakis, Bennett et al. 2007). Additionally, protease inhibitors are associated with inhibition of alveolar macrophage apoptosis in mice

during pneumococcal infection (Marriott 2010). The majority of individuals on highly active antiretroviral therapy receive a three drug combination derived from two classes; 2 nucleoside reverse transcriptase inhibitors (NRTI) plus a third drug which is either a protease inhibitor (PI) or a non nucleoside reverse transcriptase inhibitor (NNRTI). Thus it is plausible that the altered apoptotic responses of the AM were a consequence of the HAART these donors were receiving and not HIV-1 per se. To investigate the possibility that the altered apoptotic responses of the AM were a consequence of their HAART, HIV-1 donors were recruited specifically with regard to their HAART treatment history and grouped into those who had continuously received PI based therapy or non PI (NNRTI) based therapy. When the results from the *S. pneumoniae* infections were compared, both PI and NNRTI treated donors showed similarly reduced AM apoptotic responses compared with control. The effect was also seen for AM from HIV-1 donors who were antiretroviral treatment naïve. These data indicate that the altered apoptosis following challenge with *S. pneumoniae* that is observed in the context of HIV-1 infection is unlikely to be a consequence of specific antiretroviral drug therapy.

In conclusion I have shown in this section that HIV-1 mediates its effects on apoptosis through indirect mechanisms. gp120 mediates some of these effects and there is potential relationship with persistent abnormalities in lung T cell subsets, although whether this is causal remains to be determined. Ultimately this leads to inhibition of the molecular switch for apoptosis provided by Mcl-1 downregulation and a failure to induce mitochondrial superoxide. The net effect of this change when combined with the likely effects of chronic adaptation to oxidative stress are discussed more in the next section.

Chapter 6. Discussion

6.1 HIV-1 infection results in decreased macrophage apoptosis following challenge with *Streptococcus pneumoniae* which is linked to impaired bacterial killing.

In this thesis I set out to investigate why individuals living with HIV-1 infection continue to be at increased risk of developing invasive pneumococcal disease (IPD), compared with the general population, even when they were established on effective antiretroviral therapy (ART). I focused on the role of the macrophage, the first immune cell responsible for defence against pneumococcal infection. I used three models of macrophage HIV-1 infection, an HIV-1 infected cell line, healthy monocyte-derived macrophages (MDM) infected with HIV-1 *in vitro* and alveolar macrophages (AM) retrieved from the lungs of HIV-1-seropositive individuals. I have shown that HIV-1 infection is associated with reduced macrophage apoptosis in response to pneumococcal challenge, and that this is linked to impairment in late phase killing of phagocytosed pneumococci.

This apoptosis-associated killing function of the macrophage in pneumococcal disease has not been studied before in the context of HIV-1 infection and my data suggest that it could be a key determinant of the increased risk of IPD for HIV-1-seropositive individuals. Individuals receiving antiretroviral therapy (ART) continue to be at increased risk from IPD and pneumococcal pneumonia. This is despite the fact that they have reconstituted T cell immunity and that, as discussed in the introduction to this thesis, those features of untreated HIV-1 that contribute to increased IPD risk such as *S. pneumoniae* pharyngeal colonization and impaired humoral immunity are also less prominent. Importantly I observed the defect in apoptosis following pneumococcal challenge in AM from HIV-1-seropositive individuals receiving fully suppressive ART who had reconstituted their peripheral blood CD4 count, demonstrating that

this phenomenon is also likely to be relevant to the ongoing IPD risk in the era of highly active antiretroviral therapy.

Reduced apoptotic responses to pneumococci are part of a more general alteration in macrophage apoptotic reprogramming; a feature of HIV-1 infection which supports HIV latency in the macrophage pool by promoting the cells' longevity (Lum and Badley 2003). The survival of a directly HIV-1 infected macrophage, or from the point of view of host defence, its failure to die, fits this paradigm. I have also shown that uninfected bystander macrophages exhibit the same survival phenotype, and that the HIV-1 viral envelope glycoprotein gp120 is sufficient to mediate this effect. Whether this indirect inhibition of the apoptotic response in an uninfected macrophage, before virus entry, gains additional advantage for HIV-1 is not clear.

Nevertheless, this phenomenon is important because changes in the behaviour of uninfected macrophages would be required for there to be any significant effect on apoptosis-associated pneumococcal killing as the best estimates state that only very few macrophages are directly infected by HIV-1 *in vivo*, even in untreated, viraemic individuals. In fact change in the behaviour of uninfected or bystander cells is a well established phenomenon in HIV-1 infection in general, with examples including loss of uninfected CD4⁺ T cells through activation induced cell death (Patki, Zielske et al. 2000) or neuronal tissue death (Aquaro, Calio et al. 2002). A limitation of this work is that I did not measure the level of gp120 in the bronchoalveolar lavage (BAL) fluid of these individuals. gp120 is detectable in the blood and BAL fluid of untreated, viraemic HIV-1 seropositive individuals (Klasse and Moore 2004). These concentrations might be expected to be lower in aviraemic, ART treated individuals, but in the small number of subjects where it has been measured, gp120 is detectable both in the plasma and lungs of individuals on ART, even with suppressed viral loads (Rychert, Strick et al. 2010; Gundavarapu, Mishra et al. 2013). However, another important finding in this study was that HIV-1 p24 antigen, a marker of replication, was detected in alveolar macrophages from donors that had undetectable virus in the plasma. As discussed in chapter 4 and below, ongoing

replication of HIV-1 has been described in compartments other than the blood in those on ART (Yukl, Gianella et al. 2010). I predict that any ongoing HIV-1 replication in the lung alveolar macrophages of these volunteers would be a source of gp120 that could mediate the observed phenotype in bystander AM in the same manner as demonstrated by *in vitro* treatment of MDM with gp120.

It is important to recognise that these single cell type *in vitro* and *ex vivo* experiments are only crude models for the host pathogen interactions that occur during the pathogenesis of IPD; the contribution of T lymphocytes and epithelial cells to AM responses, either directly or by paracrine cytokine and chemokine release is not modelled; the complement and immunoglobulin added to cultures was from the serum of healthy volunteers and will differ quantitatively and qualitatively from that in the alveolar microenvironment of HIV-1-seropositive individuals. Furthermore IPD can arise by invasion of the upper respiratory tract without the need for pulmonary infection and may involve a significantly different role for the macrophage (Mook-Kanamori, Geldhoff et al. 2011). Nevertheless, results from these experimental systems have been demonstrated to mirror those from *in vivo* mouse models of invasive pneumococcal disease and so can provide valid insights into the mechanisms underlying the pathogenesis of IPD in the lung (Dockrell, Marriott et al. 2003; Marriott, Hellewell et al. 2006).

6.2 HIV-1 is associated with persistence of Mcl-1 expression and failure to augment mitochondrial superoxide by macrophages following *Streptococcus pneumoniae* challenge

My host research group has established that dynamic regulation of myeloid cell leukemia protein-1 (Mcl-1) is a key determinant of apoptosis following pneumococcal challenge (Marriott, Bingle et al. 2005). I have also observed this association between apoptosis and Mcl-1 expression in MDM and found that HIV-1 infection of MDM is associated with the persistent

expression of Mcl-1 after pneumococcal challenge. Although the mechanism is not yet fully elucidated, there is a link between the down regulation of Mcl-1, increased mitochondrial reactive oxygen species (ROS) and killing of pneumococci by the macrophage that is distinct from phagolysosomal killing (Bewley, Pham et al. 2011; Perciavalle, Stewart et al. 2012).

HIV-1 infection is associated with increased oxidative stress (Gil, Martinez et al. 2003); as examples, HIV-1-seropositive individuals have increased malondialdehyde (MDA), an index of lipid peroxidation, in their serum (Nakamura, De Rosa et al. 1996) and hydrogen peroxide (H_2O_2) production is higher in HIV-1 volunteers' monocytes (Elbim, Pillet et al. 2001).

Concurrently, levels of some antioxidants are decreased such as has been described for glutathione in BAL fluid and plasma from HIV-1-seropositive compared with seronegative donors (Staal, Ela et al. 1992). Other antioxidants, such as thioredoxin (Nakamura, De Rosa et al. 1996) and superoxide dismutase (SOD2) (in alveolar macrophages) (Gordon, Jagoe et al. 2013) are increased. Differences in antioxidants measured in studies depend on which fluid or cell type is analysed; macrophages over express the SOD2 gene in response to HIV-1 infection (Raoul, Le Naour et al. 1998), unlike T cells where SOD2 is reduced (Westendorp, Frank et al. 1995). *In vitro* studies also demonstrate that HIV-1 infection of primary human macrophages increase MDA (Aquaro, Muscoli et al. 2007) and exposure of macrophages to gp120 (Pietraforte, Tritarelli et al. 1994) and nef (Olivetta, Pietraforte et al. 2005) are each sufficient to induce increased ROS. The findings of increased SOD2 in HIV-1 may represent a chronic adaptation to oxidative stress which could limit the impact of further increases in superoxide (O_2^-) levels following infection. I observed that O_2^- was increased in the mitochondria of MDM with HIV-1 infection but did not increase further following *S. pneumoniae* challenge. Taking these together it my data suggest that with HIV-1 infection, both directly infected and uninfected bystander macrophages lack the capacity to further escalate mitochondrial ROS production because of chronic elevation of antioxidant mechanisms and that this leads to a

consequent deficit in bacterial killing capacity. Combined with the observation that HIV-1 is associated with persistent Mcl-1 expression, and therefore reduced apoptosis, I hypothesise that there may be a 'double hit' in HIV-1; a reduction in the capability for Mcl-1 downregulation, with the consequence of impaired increases in mitochondrial ROS during pneumococcal challenge and, with chronic elevation of antioxidants, associated impairment in the ROS mediated microbicidal activity of macrophages in HIV-1 infection.

6.3 HIV-1-seropositive individuals on fully suppressive ART continue to have a CD8⁺ T lymphocytosis with a reversed CD4:CD8 ratio in the BAL

An unexpected finding from this PhD was that HIV-1-seropositive individuals on fully suppressive ART had a BAL lymphocytosis. This was driven by higher numbers of CD8⁺ T cells with a consequently reduced CD4:CD8 ratio. These observations reflect those in reports from untreated, viraemic HIV-1 infected individuals with lymphocytic BAL fluid (Twigg, Soliman et al. 1999). There are, however, no previous reports describing the lymphocyte populations in the BAL of long term ART treated HIV-1-seropositive individuals and this finding is new.

Furthermore, lower CD4:CD8 ratios correlated with lower levels of pneumococci associated AM apoptosis. These findings raise two issues. First, whether the persistence of a BAL CD8 lymphocytosis may have long term consequences for the lung and second, whether there is an interaction between pulmonary lymphocytes and alveolar macrophages that contributes to the HIV-1 associated impairment in apoptosis.

In addition to the increased risk of pneumococcal pneumonia HIV-1-seropositive individuals experience more non-infective respiratory illnesses which have been characterized as diseases of the aging lung (Crothers, Huang et al. 2011). Clinical changes occur in other systems in people with HIV-1 which are similar to those seen in the elderly, HIV-1 seronegative population including osteoporosis (Stone, Dockrell et al. 2010), neurocognitive decline (Ikezu 2009) and

atherosclerosis (Kaplan, Kingsley et al. 2007). Both populations demonstrate comparable immunologic alterations, in particular reduced T cell renewal and a change from naive to predominantly differentiated T cells with associated restriction in T cell diversity; common to both is an increased population of antigen experienced cells with increased CD57 and absent expression of CD28, the T cell co-stimulatory molecule that binds to CD80 and CD86 on APCs, and this CD8⁺/CD28⁻/CD57⁺ population also has reduced proliferative capacity (Merino, Martinez-Gonzalez et al. 1998; Brenchley, Karandikar et al. 2003). Importantly, ART treated individuals with viral suppression and good CD4 cell count reconstitution continue to have higher numbers of CD57⁺/CD28⁻ and lower numbers of CD45RA⁺/CCR7⁺ (naive) CD8 T cells in the blood compared with age matched seronegative controls (Desai SR, Usuga X et al.). Indeed, the changes in this group of HIV-1-seropositive individuals in their 50s (median age 56) were comparable to those seen in an elderly (median age 88) HIV-1-seronegative control group. Thus, even with suppressive ART, there is an overall picture of a reduced CD4:CD8 ratio, driven by an expanded CD8 cell population which has a differentiated effector phenotype (Emu, Moretto et al. 2014). The changes are believed to be driven by the immune activation associated with HIV-1 and in both HIV-1 and older age, by dysregulation of T cell homeostasis, for example through thymic atrophy. In HIV-1-seropositive individuals on treatment, and despite undetectable peripheral blood virus, ongoing low level viral replication in tissue reservoirs, for example in the gut (Yukl, Gianella et al. 2010), may be responsible for the ongoing antigenic stimulation (Buzon, Massanella et al. 2010).

In my study p24 antigen was detected in AM from some of the ART treated individuals indicating that HIV-1 replication continued in the lungs of these individuals despite there being no detectable HIV-1 RNA in their blood. This same finding was very recently reported elsewhere (Cribbs, Guidot et al. 2014). HIV-1 infected AM in the ART treated HIV-1-seropositive individuals from my study could therefore be influencing the lung T cell population. Direct interactions between HIV-1 infected AM and CD4⁺ T cells are seen to occur

in untreated HIV-1 and lead to CD4⁺ T cell loss from the lung (Twigg, Lipscomb et al. 1989). Additionally in untreated HIV-1, AM secrete greater amounts of macrophage inflammatory protein-1 α (MIP-1 α) which is chemo attractant for CD8⁺ T cells (Denis and Ghadirian 1994) and AM from HIV-1 seropositivie volunteers with lymphocytic alveolitis also produce more interleukin (IL)-1 β , IL-6 and IL-15 (Twigg, Iwamoto et al. 1992; Agostini, Zambello et al. 1999), some of which may by induced by free gp120 (Choe, Volsky et al. 2001). AM from HIV-1 (but not control) donors express more CD80 and CD86 and in *ex vivo* mitogen assays promote the proliferation of T cells by engagement with CD28 (Agostini, Zambello et al. 1999), which could, through repeated stimulation, drive the CD8 cells toward the differentiated and exhausted phenotype described above. These responses may not be as marked in ART treated HIV-1 volunteers if the overall number of HIV-1 infected AM is much lower and, as such, may explain why in my work there was no difference in AM CD80 expression compared with seronegative controls. However, it remains possible that any altered CD80 expression may have reversed during overnight culture, as discussed in chapter 4 (Agostini, Zambello et al. 1999; Tomlinson, Booth et al. 2012).

Chronic obstructive pulmonary disease (COPD) is an example of a non infective lung disease associated with aging that is also more common in HIV (Crothers, Huang et al. 2011). COPD patients have been characterised as having an accelerated aging phenotype in the lung (Sharma, Hanania et al. 2009) and, like the elderly, also have more CD8⁺ T cells (Hogg, Chu et al. 2004), an inverted CD4:CD8 (Hodge, Nairn et al. 2007) and reduced CD8⁺/CD28⁺ (Barcelo, Pons et al. 2008; Hodge, Mukaro et al. 2011) T cell populations in their blood and BAL fluid. These changes may in part be caused by cigarette smoking, as demonstrated by mouse models of cigarette exposure (Hodge, Mukaro et al. 2011), but are not limited to COPD patients who smoke and may promote ongoing tissue damage as CD8⁺ T cells levels are high in the lungs of those with more severe disease (Hogg, Chu et al. 2004). As stated, COPD is more common in HIV-1-seropositive cohorts, even after adjusting for smoking (Crothers, Huang et al. 2011;

Hirani, Cavallazzi et al. 2011; Madeddu, Fois et al. 2013), raising the possibility that the CD8⁺/CD28⁻ lymphocytosis found in the peripheral blood of HIV-1-seropositive individuals could drive the inflammatory lung damage that contributes to COPD and lung disease in these individuals in general. My finding of a BAL CD8 lymphocytosis and reduced CD4:CD8 ratio further advances this intriguing possibility, and suggests that ART is insufficient to abrogate this process. More detail regarding the nature of the expanded CD8 population in these individuals is needed. As discussed in chapter 4, ART naive HIV-1-seropositive individuals also have higher levels of CD38⁺ CD8⁺ T cells which decline in number with ART (Tilling, Kinloch et al. 2002; Landay, da Silva et al. 2007). However, I did not detect any signal for increased CD38 on the CD8 cells from the BAL of the ART treated individuals in this study. Future work will need to measure CD45RA, CD28, CD57, CCR7, Ki67 and antigenic responsiveness of the cells.

The second issue to address is whether the altered lymphocyte milieu in the lung could contribute to the HIV-1 associated impairment in apoptosis. Although I found evidence of a correlation between lower BAL CD4:CD8 lymphocyte ratios and reduced *S. pneumoniae* associated AM apoptosis, my experiments have not tested whether this is a causal relationship. It has been demonstrated that CD8 T cells in the lung of untreated HIV-1-seropositive individuals develop specific responses against HIV-1 infected AM and, at least until late stages of HIV-1 disease, produce an interferon (IFN)- γ mediated inflammatory response that can activate other, uninfected, AM (Plata, Autran et al. 1987; Twigg, Spain et al. 1999). These activated AM have increased expression of major histocompatibility complex (MHC) class II and O₂⁻ (Buhl, Jaffe et al. 1993), which can be generated by IFN- γ stimulation of the phagocyte oxidase system in macrophages (Cassatella, Bazzoni et al. 1990). Although I did observe increased mitochondrial O₂⁻ among *in vitro* HIV-1 infected MDM, these markers were not measured in AM in this study. However, persistence of HIV-1 infected AM in ART treated HIV-1-seropositive individuals, as seen in this study, might continue to induce a CD8 T cell response that, in turn contributes to ongoing AM activation. The nature of any activation of

AM, which persists *ex vivo* for the 48-72 hour period following BAL until when I performed pneumococcal challenge experiments, and leads to the altered apoptosis phenotype I observed, needs further study. However, it remains plausible that CD8⁺ T cells produced in response to HIV-1 infection of AM in lung could in turn lead to activation and increased oxidative stress of AM, for instance through IFN- γ induction of ROS (Cassatella, Bazzoni et al. 1990; Casbon, Long et al. 2012), thus inhibiting their capacity for apoptosis-associated bacterial killing and so linking the pulmonary T cell abnormalities to the impairment of host defence against *S. pneumoniae*.

6.4 Future work

The observations in this PhD have raised additional questions about the nature of both alveolar macrophages and T lymphocytes in the lung in ART treated HIV-1-seropositive individuals.

I found evidence that HIV-1 replication persists in alveolar macrophages of HIV-1 seropositive individuals on ART. After recruiting additional volunteers for BAL, I would systematically test all donors' AM for the presence of p24 and use RT-PCR to detect HIV-1 RNA and PCR to detect proviral DNA. These tests would also be extended to looking at cell free BAL fluid for HIV-1 RNA and the lymphocytes for RNA and proviral DNA. In view of my finding that gp120 mediated the altered AM apoptosis, I would determine its concentration in BAL fluid of these volunteers using an ELISA, and adjust the results to the concentration of urea in the BAL fluid, which equates to the volume of lung epithelial lining fluid, and so normalise the gp120 results for the variation in volumes of saline instilled and recovered from donor to donor.

I have demonstrated that the behaviour of AM was altered, but did not detect any difference in the expression of a limited set of macrophage surface molecules associated with different polarisation states. More detailed phenotyping of BAL AM would provide insights into which receptors and signalling pathways are differentially expressed and activated in these cells

compared with those from control seronegative donors. In the first instance I would use a PCR array to investigate if there is differential gene expression in the intrinsic/mitochondrial pathway of apoptosis, the generation of antioxidant molecules and factors associated with macrophage polarisation. Any differentially expressed genes I detected would then inform multicolour flow cytometry to measure the surface expression and western blots of intracellular proteins in confirmatory tests. These tests could be extended to compare phenotypes of AM immediately following extraction and after 24-48 hours of *ex vivo* culture.

I also discovered that there was a CD8 T cell predominant BAL lymphocytosis in the HIV-1-seropositive donors which correlated with AM apoptotic responses. I would seek to investigate whether there was an interaction between macrophages and lymphocytes. Using the *in vitro* MDM *S. pneumoniae* infection model I would measure whether the addition of autologous lymphocytes near and during the pneumococcal infection alters apoptotic responses. These experiments could be extended to investigate the role of specific lymphocyte subsets using FACS sorting by positive or negative selection.

To examine the significance of the persistent Mcl-1 expression in HIV-1 infected MDM I would measure the expression of Mcl-1 in donor AM during the same pneumococcal challenge experiments. I would then seek to control the level of Mcl-1 in the *in vitro* MDM HIV-1 infection model by inhibiting its transcription via siRNA and measure how apoptosis was affected, as discussed in chapter 5. To better understand the role of gp120 in preventing apoptosis in the HIV-1 MDM model I would confirm the presence of gp120 in the supernatants of these cultures using ELISA and then measure whether the rates of pneumococci associated apoptosis were reduced in the presence of anti gp120, CD4/CCR5 receptor blocking antibodies or an env deficient strain of HIV-1.

Finally, the inclusion of additional HIV-1-seropositive and control donors would provide a sufficiently large sample population to test whether there was significant impairment of apoptosis-associated killing of *S. pneumoniae* by AM from the HIV-1-seropositive group.

6.5 Conclusion

In summary, the data presented in this thesis demonstrate that there is impairment of the macrophage's apoptotic response to *S. pneumoniae* in HIV-1 infection which leads to reduced bacterial killing. These effects are associated with HIV-1 driven increases in anti-apoptotic Mcl-1 and mitochondrial superoxide in the macrophage. Direct infection with HIV-1 is not required and the soluble HIV-1 protein gp120 may be sufficient to mediate this altered phenotype. Importantly, the same impairment in apoptosis was observed in alveolar macrophages from HIV-1-seropositive individuals on fully suppressive antiretroviral therapy, demonstrating that, in so much that these experiments can model the pathogenesis of IPD, a failure of macrophage apoptosis-associated killing of pneumococci could play an important role underlying the increased risk invasive pneumococcal disease in ART treated HIV-1-seropositive individuals. I also found that there was a CD8 T cell driven lymphocytosis in the lungs of HIV-1-seropositive individuals on suppressive ART and suggest that this could both contribute to the altered behaviour of alveolar macrophages and promote chronic, inflammation mediated lung damage in this population.

Chapter 7. References

- Abdullahi, O., A. Karani, et al. (2012). "The prevalence and risk factors for pneumococcal colonization of the nasopharynx among children in Kilifi District, Kenya." *PLoS One* **7**(2): e30787.
- Agostini, C., V. Poletti, et al. (1988). "Phenotypical and functional analysis of bronchoalveolar lavage lymphocytes in patients with HIV infection." *Am Rev Respir Dis* **138**(6): 1609-1615.
- Agostini, C., R. Zambello, et al. (1999). "CD8 T-cell infiltration in extravascular tissues of patients with human immunodeficiency virus infection. Interleukin-15 upmodulates costimulatory pathways involved in the antigen-presenting cells-T-cell interaction." *Blood* **93**(4): 1277-1286.
- Aillet, F., H. Masutani, et al. (1998). "Human immunodeficiency virus induces a dual regulation of Bcl-2, resulting in persistent infection of CD4(+) T- or monocytic cell lines." *J Virol* **72**(12): 9698-9705.
- Akarid, K., D. Arnoult, et al. (2004). "Leishmania major-mediated prevention of programmed cell death induction in infected macrophages is associated with the repression of mitochondrial release of cytochrome c." *J Leukoc Biol* **76**(1): 95-103.
- Akhter, A., M. A. Gavrilin, et al. (2009). "Caspase-7 activation by the Nlr4/Ipaf inflammasome restricts Legionella pneumophila infection." *PLoS Pathog* **5**(4): e1000361.
- Alberts, B. (2002). *Molecular biology of the cell*. New York, Garland Science.
- Albiger, B., S. Dahlberg, et al. (2007). "Toll-like receptor 9 acts at an early stage in host defence against pneumococcal infection." *Cell Microbiol* **9**(3): 633-644.
- Alcabes, P., A. Munoz, et al. (1993). "Incubation period of human immunodeficiency virus." *Epidemiol Rev* **15**(2): 303-318.
- Ali, F., M. E. Lee, et al. (2003). "Streptococcus pneumoniae-associated human macrophage apoptosis after bacterial internalization via complement and Fcγ receptors correlates with intracellular bacterial load." *J Infect Dis* **188**(8): 1119-1131.
- Almirante, B., M. Saballs, et al. (1998). "Favorable prognosis of purulent meningitis in patients infected with human immunodeficiency virus." *Clin Infect Dis* **27**(1): 176-180.
- Ambarus, C. A., S. Krausz, et al. (2012). "Systematic validation of specific phenotypic markers for in vitro polarized human macrophages." *J Immunol Methods* **375**(1-2): 196-206.
- Antonini, J. M., N. J. Lawryk, et al. (1999). "Effect of welding fume solubility on lung macrophage viability and function in vitro." *J Toxicol Environ Health A* **58**(6): 343-363.
- Antonini, J. M., S. S. Leonard, et al. (2005). "Effect of stainless steel manual metal arc welding fume on free radical production, DNA damage, and apoptosis induction." *Mol Cell Biochem* **279**(1-2): 17-23.
- Appay, V. and D. Sauce (2008). "Immune activation and inflammation in HIV-1 infection: causes and consequences." *J Pathol* **214**(2): 231-241.
- Aquaro, S., R. Calio, et al. (2002). "Macrophages and HIV infection: therapeutical approaches toward this strategic virus reservoir." *Antiviral Res* **55**(2): 209-225.
- Aquaro, S., C. Muscoli, et al. (2007). "The contribution of peroxynitrite generation in HIV replication in human primary macrophages." *Retrovirology* **4**: 76.
- Arredouani, M., Z. Yang, et al. (2004). "The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles." *J Exp Med* **200**(2): 267-272.
- Arredouani, M. S., Z. Yang, et al. (2006). "The macrophage scavenger receptor SR-AI/II and lung defense against pneumococci and particles." *Am J Respir Cell Mol Biol* **35**(4): 474-478.
- Ashida, H., H. Mimuro, et al. (2011). "Cell death and infection: a double-edged sword for host and pathogen survival." *J Cell Biol* **195**(6): 931-942.

- Attali, C., C. Durmort, et al. (2008). "The interaction of *Streptococcus pneumoniae* with plasmin mediates transmigration across endothelial and epithelial monolayers by intercellular junction cleavage." *Infect Immun* **76**(11): 5350-5356.
- Ausiello, C. M., A. la Sala, et al. (1996). "Secretion of IFN-gamma, IL-6, granulocyte-macrophage colony-stimulating factor and IL-10 cytokines after activation of human purified T lymphocytes upon CD38 ligation." *Cell Immunol* **173**(2): 192-197.
- Austrian, R. (1984). Pneumococcal infections. *Bacterial vaccines*. R. Germanier. Orlando, Academic Press: 257–288.
- Austrian, R. (1986). "Some aspects of the pneumococcal carrier state." *J Antimicrob Chemother* **18 Suppl A**: 35-45.
- Azzam, R., K. Kedzierska, et al. (2006). "Impaired complement-mediated phagocytosis by HIV type-1-infected human monocyte-derived macrophages involves a cAMP-dependent mechanism." *AIDS Res Hum Retroviruses* **22**(7): 619-629.
- Azzam, R., L. Lal, et al. (2006). "Adverse effects of antiretroviral drugs on HIV-1-infected and -uninfected human monocyte-derived macrophages." *J Acquir Immune Defic Syndr* **42**(1): 19-28.
- Badley, A. D., D. Dockrell, et al. (1997). "Macrophage-dependent apoptosis of CD4+ T lymphocytes from HIV-infected individuals is mediated by FasL and tumor necrosis factor." *J Exp Med* **185**(1): 55-64.
- Baghai, M., D. R. Osmon, et al. (2001). "Fatal sepsis in a patient with rheumatoid arthritis treated with etanercept." *Mayo Clin Proc* **76**(6): 653-656.
- Baldwin, G. C., J. Fleischmann, et al. (1990). "Human immunodeficiency virus causes mononuclear phagocyte dysfunction." *Proc Natl Acad Sci U S A* **87**(10): 3933-3937.
- Baldwin, G. C., J. C. Gasson, et al. (1988). "Granulocyte-macrophage colony-stimulating factor enhances neutrophil function in acquired immunodeficiency syndrome patients." *Proc Natl Acad Sci U S A* **85**(8): 2763-2766.
- Bandres, J. C., J. Trial, et al. (1993). "Increased phagocytosis and generation of reactive oxygen products by neutrophils and monocytes of men with stage 1 human immunodeficiency virus infection." *J Infect Dis* **168**(1): 75-83.
- Banga, S., P. Gao, et al. (2007). "Legionella pneumophila inhibits macrophage apoptosis by targeting pro-death members of the Bcl2 protein family." *Proc Natl Acad Sci U S A* **104**(12): 5121-5126.
- Barcelo, B., J. Pons, et al. (2008). "Phenotypic characterisation of T-lymphocytes in COPD: abnormal CD4+CD25+ regulatory T-lymphocyte response to tobacco smoking." *Eur Respir J* **31**(3): 555-562.
- Barry, P. M., N. Zetola, et al. (2006). "Invasive pneumococcal disease in a cohort of HIV-infected adults: incidence and risk factors, 1990-2003." *AIDS* **20**(3): 437-444.
- Barry, S. M., M. A. Johnson, et al. (2003). "Increased proportions of activated and proliferating memory CD8+ T lymphocytes in both blood and lung are associated with blood HIV viral load." *J Acquir Immune Defic Syndr* **34**(4): 351-357.
- Basset, A., C. M. Thompson, et al. (2007). "Antibody-independent, CD4+ T-cell-dependent protection against pneumococcal colonization elicited by intranasal immunization with purified pneumococcal proteins." *Infect Immun* **75**(11): 5460-5464.
- Behar, S. M., M. Divangahi, et al. (2010). "Evasion of innate immunity by *Mycobacterium tuberculosis*: is death an exit strategy?" *Nat Rev Microbiol* **8**(9): 668-674.
- Behar, S. M., C. J. Martin, et al. (2011). "Apoptosis is an innate defense function of macrophages against *Mycobacterium tuberculosis*." *Mucosal Immunol* **4**(3): 279-287.
- Bergeron, Y., N. Ouellet, et al. (1998). "Cytokine kinetics and other host factors in response to pneumococcal pulmonary infection in mice." *Infect Immun* **66**(3): 912-922.
- Berke, G. (1994). "The binding and lysis of target cells by cytotoxic lymphocytes: molecular and cellular aspects." *Annu Rev Immunol* **12**: 735-773.

- Bewley, M. (2013). Personal Communication.
- Bewley, M. A., H. M. Marriott, et al. (2011). "A cardinal role for cathepsin d in co-ordinating the host-mediated apoptosis of macrophages and killing of pneumococci." PLoS Pathog **7**(1): e1001262.
- Bewley, M. A., T. K. Pham, et al. (2011). "Proteomic evaluation and validation of cathepsin D regulated proteins in macrophages exposed to *Streptococcus pneumoniae*." Mol Cell Proteomics **10**(6): M111 008193.
- Bieniasz, P. D. (2012). "An overview of intracellular interactions between immunodeficiency viruses and their hosts." AIDS **26**(10): 1243-1254.
- Biggs, B. A., M. Hewish, et al. (1995). "HIV-1 infection of human macrophages impairs phagocytosis and killing of *Toxoplasma gondii*." J Immunol **154**(11): 6132-6139.
- Blasi, F., M. Mantero, et al. (2012). "Understanding the burden of pneumococcal disease in adults." Clin Microbiol Infect **18 Suppl 5**: 7-14.
- Borghesi, P., L. Fantuzzi, et al. (1995). "Induction of interleukin-10 by human immunodeficiency virus type 1 and its gp120 protein in human monocytes/macrophages." J Virol **69**(2): 1284-1287.
- Born, W. K., C. L. Reardon, et al. (2006). "The function of gammadelta T cells in innate immunity." Curr Opin Immunol **18**(1): 31-38.
- Breiman, R. F., D. W. Keller, et al. (2000). "Evaluation of effectiveness of the 23-valent pneumococcal capsular polysaccharide vaccine for HIV-infected patients." Arch Intern Med **160**(17): 2633-2638.
- Brenchley, J. M., N. J. Karandikar, et al. (2003). "Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells." Blood **101**(7): 2711-2720.
- Brettell, R. P. (1997). "Bacterial infections in HIV: the extent and nature of the problem." Int J STD AIDS **8**(1): 5-15.
- Brichacek, B., S. Swindells, et al. (1996). "Increased plasma human immunodeficiency virus type 1 burden following antigenic challenge with pneumococcal vaccine." J Infect Dis **174**(6): 1191-1199.
- Brown, J. N., J. J. Kohler, et al. (2008). "HIV-1 activates macrophages independent of Toll-like receptors." PLoS ONE **3**(12): e3664.
- Brown, J. S., T. Hussell, et al. (2002). "The classical pathway is the dominant complement pathway required for innate immunity to *Streptococcus pneumoniae* infection in mice." Proc Natl Acad Sci U S A **99**(26): 16969-16974.
- Brundage, J. F. (2006). "Interactions between influenza and bacterial respiratory pathogens: implications for pandemic preparedness." Lancet Infect Dis **6**(5): 303-312.
- Bucy, R. P., R. D. Hockett, et al. (1999). "Initial increase in blood CD4(+) lymphocytes after HIV antiretroviral therapy reflects redistribution from lymphoid tissues." J Clin Invest **103**(10): 1391-1398.
- Buhl, R., H. A. Jaffe, et al. (1993). "Activation of alveolar macrophages in asymptomatic HIV-infected individuals." J Immunol **150**(3): 1019-1028.
- Burton, D. R., R. C. Desrosiers, et al. (2004). "HIV vaccine design and the neutralizing antibody problem." Nat Immunol **5**(3): 233-236.
- Busca, A., M. Saxena, et al. (2012). "Critical role for antiapoptotic Bcl-xL and Mcl-1 in human macrophage survival and cellular IAP1/2 (cIAP1/2) in resistance to HIV-Vpr-induced apoptosis." J Biol Chem **287**(18): 15118-15133.
- Buzon, M. J., M. Massanella, et al. (2010). "HIV-1 replication and immune dynamics are affected by raltegravir intensification of HAART-suppressed subjects." Nat Med **16**(4): 460-465.
- Cadogan, M. and A. G. Dalgleish (2008). "HIV immunopathogenesis and strategies for intervention." Lancet Infect Dis **8**(11): 675-684.

- Calbo, E. and J. Garau (2010). "Of mice and men: innate immunity in pneumococcal pneumonia." *Int J Antimicrob Agents* **35**(2): 107-113.
- Caldwell, R. L., B. S. Egan, et al. (2000). "HIV-1 Tat represses transcription from the mannose receptor promoter." *J Immunol* **165**(12): 7035-7041.
- Cameron, M. L., D. L. Granger, et al. (1994). "Human immunodeficiency virus (HIV)-infected human blood monocytes and peritoneal macrophages have reduced anticytotoxic activity whereas HIV-infected alveolar macrophages retain normal activity." *J Infect Dis* **170**(1): 60-67.
- Capsoni, F., F. Minonzi, et al. (1994). "Fc receptors expression and function in mononuclear phagocytes from AIDS patients: modulation by IFN-gamma." *Scand J Immunol* **39**(1): 45-50.
- Carotenuto, P., D. Looij, et al. (1998). "Neutralizing antibodies are positively associated with CD4+ T-cell counts and T-cell function in long-term AIDS-free infection." *AIDS* **12**(13): 1591-1600.
- Carson, P. J., R. L. Schut, et al. (1995). "Antibody class and subclass responses to pneumococcal polysaccharides following immunization of human immunodeficiency virus-infected patients." *J Infect Dis* **172**(2): 340-345.
- Casbon, A. J., M. E. Long, et al. (2012). "Effects of IFN-gamma on intracellular trafficking and activity of macrophage NADPH oxidase flavocytochrome b558." *J Leukoc Biol* **92**(4): 869-882.
- Cassatella, M. A., F. Bazzoni, et al. (1990). "Molecular basis of interferon-gamma and lipopolysaccharide enhancement of phagocyte respiratory burst capability. Studies on the gene expression of several NADPH oxidase components." *J Biol Chem* **265**(33): 20241-20246.
- Cassol, E., M. Alfano, et al. (2006). "Monocyte-derived macrophages and myeloid cell lines as targets of HIV-1 replication and persistence." *J Leukoc Biol* **80**(5): 1018-1030.
- Cassol, E., L. Cassetta, et al. "Macrophage polarization and HIV-1 infection." *J Leukoc Biol* **87**(4): 599-608.
- Cassol, E., L. Cassetta, et al. (2010). "Macrophage polarization and HIV-1 infection." *J Leukoc Biol* **87**(4): 599-608.
- Chan, G., M. T. Nogalski, et al. (2010). "PI3K-dependent upregulation of Mcl-1 by human cytomegalovirus is mediated by epidermal growth factor receptor and inhibits apoptosis in short-lived monocytes." *J Immunol* **184**(6): 3213-3222.
- Chaturvedi, S., P. Frame, et al. (1995). "Macrophages from human immunodeficiency virus-positive persons are defective in host defense against *Histoplasma capsulatum*." *J Infect Dis* **171**(2): 320-327.
- Chayt, K. J., M. E. Harper, et al. (1986). "Detection of HTLV-III RNA in lungs of patients with AIDS and pulmonary involvement." *JAMA* **256**(17): 2356-2359.
- Cheng, E. H., M. C. Wei, et al. (2001). "BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis." *Mol Cell* **8**(3): 705-711.
- Choe, W., D. J. Volsky, et al. (2001). "Induction of rapid and extensive beta-chemokine synthesis in macrophages by human immunodeficiency virus type 1 and gp120, independently of their coreceptor phenotype." *J Virol* **75**(22): 10738-10745.
- Choi, H. J. and T. E. Smithgall (2004). "HIV-1 Nef promotes survival of TF-1 macrophages by inducing Bcl-XL expression in an extracellular signal-regulated kinase-dependent manner." *J Biol Chem* **279**(49): 51688-51696.
- Chugh, P., B. Bradel-Tretheway, et al. (2008). "Akt inhibitors as an HIV-1 infected macrophage-specific anti-viral therapy." *Retrovirology* **5**: 11.
- Chun, T. W., L. Carruth, et al. (1997). "Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection." *Nature* **387**(6629): 183-188.

- Chun, T. W., R. T. Davey, Jr., et al. (2000). "Relationship between pre-existing viral reservoirs and the re-emergence of plasma viremia after discontinuation of highly active anti-retroviral therapy." *Nat Med* **6**(7): 757-761.
- Chun, T. W., D. Engel, et al. (1998). "Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection." *Proc Natl Acad Sci U S A* **95**(15): 8869-8873.
- Clarke, J. R., A. J. Gates, et al. (1994). "HIV-1 proviral DNA copy number in peripheral blood leucocytes and bronchoalveolar lavage cells of AIDS patients." *Clin Exp Immunol* **96**(2): 182-186.
- Clarke, T. B., K. M. Davis, et al. (2010). "Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity." *Nat Med* **16**(2): 228-231.
- Clatworthy, M. R. and K. G. Smith (2004). "FcγRIIIb balances efficient pathogen clearance and the cytokine-mediated consequences of sepsis." *J Exp Med* **199**(5): 717-723.
- Coberley, C. R., J. J. Kohler, et al. (2004). "Impact on genetic networks in human macrophages by a CCR5 strain of human immunodeficiency virus type 1." *J Virol* **78**(21): 11477-11486.
- Cocchi, F., A. L. DeVico, et al. (1995). "Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells." *Science* **270**(5243): 1811-1815.
- Coggon, D., H. Inskip, et al. (1994). "Lobar pneumonia: an occupational disease in welders." *Lancet* **344**(8914): 41-43.
- Collins, A. M., S. E. Batrawy, et al. (2013). "Increased IgG but normal IgA anti-pneumococcal protein antibodies in lung of HIV-infected adults." *Vaccine*.
- Colombel, J. F., E. V. Loftus, Jr., et al. (2004). "The safety profile of infliximab in patients with Crohn's disease: the Mayo clinic experience in 500 patients." *Gastroenterology* **126**(1): 19-31.
- Conti, L., L. Fantuzzi, et al. (2004). "Immunomodulatory effects of the HIV-1 gp120 protein on antigen presenting cells: implications for AIDS pathogenesis." *Immunobiology* **209**(1-2): 99-115.
- Contreras, X., T. Lenasi, et al. (2006). "HIV latency: present knowledge, future directions." *Future Virol* **1**(6): 733-745.
- Cribbs, S. K., D. M. Guidot, et al. (2014). Healthy HIV-Infected Subjects Harbor HIV in Alveolar Macrophages, Which Can Impair Lung Function. *21st Conference on Retroviruses and Opportunistic Infection*. Boston **poster 410**
- Crothers, K., L. Huang, et al. (2011). "HIV infection and risk for incident pulmonary diseases in the combination antiretroviral therapy era." *Am J Respir Crit Care Med* **183**(3): 388-395.
- Crowe, S., T. Zhu, et al. (2003). "The contribution of monocyte infection and trafficking to viral persistence, and maintenance of the viral reservoir in HIV infection." *J Leukoc Biol* **74**(5): 635-641.
- Crowe, S. M. and S. Sonza (2000). "HIV-1 can be recovered from a variety of cells including peripheral blood monocytes of patients receiving highly active antiretroviral therapy: a further obstacle to eradication." *J Leukoc Biol* **68**(3): 345-350.
- Crowe, S. M., N. J. Vardaxis, et al. (1994). "HIV infection of monocyte-derived macrophages in vitro reduces phagocytosis of *Candida albicans*." *J Leukoc Biol* **56**(3): 318-327.
- Cui, M., Y. Huang, et al. (2008). "Transcription factor FOXO3a mediates apoptosis in HIV-1-infected macrophages." *J Immunol* **180**(2): 898-906.
- Cundell, D. R., N. P. Gerard, et al. (1995). "Streptococcus pneumoniae anchor to activated human cells by the receptor for platelet-activating factor." *Nature* **377**(6548): 435-438.

- Curran, A., V. Falco, et al. (2008). "Bacterial pneumonia in HIV-infected patients: use of the pneumonia severity index and impact of current management on incidence, aetiology and outcome." *HIV Med* **9**(8): 609-615.
- Daigneault, M., T. I. De Silva, et al. (2012). "Monocytes regulate the mechanism of T-cell death by inducing Fas-mediated apoptosis during bacterial infection." *PLoS Pathog* **8**(7): e1002814.
- Daigneault, M., J. A. Preston, et al. (2010). "The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages." *PLoS One* **5**(1): e8668.
- Dave, S., S. Carmicle, et al. (2004). "Dual roles of PspC, a surface protein of *Streptococcus pneumoniae*, in binding human secretory IgA and factor H." *J Immunol* **173**(1): 471-477.
- Davis, K. M., S. Nakamura, et al. (2011). "Nod2 sensing of lysozyme-digested peptidoglycan promotes macrophage recruitment and clearance of *S. pneumoniae* colonization in mice." *J Clin Invest* **121**(9): 3666-3676.
- Day, C. L., D. E. Kaufmann, et al. (2006). "PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression." *Nature* **443**(7109): 350-354.
- De Brauwier, E. I., J. A. Jacobs, et al. (2002). "Bronchoalveolar lavage fluid differential cell count. How many cells should be counted?" *Anal Quant Cytol Histol* **24**(6): 337-341.
- De Clercq, E. and D. Schols (2001). "Inhibition of HIV infection by CXCR4 and CCR5 chemokine receptor antagonists." *Antivir Chem Chemother* **12 Suppl 1**: 19-31.
- Dean, M., M. Carrington, et al. (1996). "Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the *CCR5* structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study." *Science* **273**(5283): 1856-1862.
- Denis, M. and E. Ghadirian (1994). "Alveolar macrophages from subjects infected with HIV-1 express macrophage inflammatory protein-1 alpha (MIP-1 alpha): contribution to the CD8+ alveolitis." *Clin Exp Immunol* **96**(2): 187-192.
- Denis, M. and E. Ghadirian (1994). "Dysregulation of interleukin 8, interleukin 10, and interleukin 12 release by alveolar macrophages from HIV type 1-infected subjects." *AIDS Res Hum Retroviruses* **10**(12): 1619-1627.
- Desai SR, Usuga X, et al. Immune senescence, activation and abnormal T cell homeostasis despite effective HAART, a hallmark of early aging in HIV. 16th Conference on Retroviruses and Opportunistic Infections. Montreal.
- Dimmock, N. J., A. J. Easton, et al. (2007). The biology of HIV infection. Introduction to modern virology. Oxford, Blackwell Science: 314.
- Dimmock, N. J., A. J. Easton, et al. (2007). Integration of retroviral DNA into cell DNA Introduction to modern virology. N. J. Dimmock, A. J. Easton and K. Leppard. Oxford, Blackwell Science: 121.
- Dimmock, N. J., A. J. Easton, et al. (2007). Molecular Biology of HIV-1. Introduction to modern virology. N. J. Dimmock, A. J. Easton and K. Leppard. Oxford, Blackwell Science: 315.
- Dobmeyer, T. S., B. Raffel, et al. (1995). "Decreased function of monocytes and granulocytes during HIV-1 infection correlates with CD4 cell counts." *Eur J Med Res* **1**(1): 9-15.
- Dockrell, D. H., P. Collini, et al. (2012). Alveolar Macrophages. Mucosal immunology of acute bacterial pneumonia. A. Prince. New York, Springer.
- Dockrell, D. H., M. Lee, et al. (2001). "Immune-mediated phagocytosis and killing of *Streptococcus pneumoniae* are associated with direct and bystander macrophage apoptosis." *J Infect Dis* **184**(6): 713-722.
- Dockrell, D. H., H. M. Marriott, et al. (2003). "Alveolar macrophage apoptosis contributes to pneumococcal clearance in a resolving model of pulmonary infection." *J Immunol* **171**(10): 5380-5388.

- Dockrell, D. H., M. K. Whyte, et al. (2012). "Pneumococcal pneumonia: mechanisms of infection and resolution." *Chest* **142**(2): 482-491.
- Domingo, P., I. Suarez-Lozano, et al. (2009). "Bacterial meningitis in HIV-1-infected patients in the era of highly active antiretroviral therapy." *J Acquir Immune Defic Syndr* **51**(5): 582-587.
- Douek, D. C. (2003). "Disrupting T-cell homeostasis: how HIV-1 infection causes disease." *AIDS Rev* **5**(3): 172-177.
- Dragic, T., V. Litwin, et al. (1996). "HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5." *Nature* **381**(6584): 667-673.
- Droemann, D., T. Goldmann, et al. (2003). "Toll-like receptor 2 is expressed by alveolar epithelial cells type II and macrophages in the human lung." *Histochem Cell Biol* **119**(2): 103-108.
- Dworkin, M. S., J. W. Ward, et al. (2001). "Pneumococcal disease among human immunodeficiency virus-infected persons: incidence, risk factors, and impact of vaccination." *Clin Infect Dis* **32**(5): 794-800.
- Eagan, R., H. L. Twigg, 3rd, et al. (2007). "Lung fluid immunoglobulin from HIV-infected subjects has impaired opsonic function against pneumococci." *Clin Infect Dis* **44**(12): 1632-1638.
- Eales, L. J., O. Moshtael, et al. (1987). "Microbicidal activity of monocyte derived macrophages in AIDS and related disorders." *Clin Exp Immunol* **67**(2): 227-235.
- Eckstein, D. A., M. P. Sherman, et al. (2001). "HIV-1 Vpr enhances viral burden by facilitating infection of tissue macrophages but not nondividing CD4+ T cells." *J Exp Med* **194**(10): 1407-1419.
- Elbim, C., V. Monceaux, et al. (2008). "Early divergence in neutrophil apoptosis between pathogenic and nonpathogenic simian immunodeficiency virus infections of nonhuman primates." *J Immunol* **181**(12): 8613-8623.
- Elbim, C., S. Pillet, et al. (2001). "The role of phagocytes in HIV-related oxidative stress." *J Clin Virol* **20**(3): 99-109.
- Elbim, C., S. Pillet, et al. (1999). "Redox and activation status of monocytes from human immunodeficiency virus-infected patients: relationship with viral load." *J Virol* **73**(6): 4561-4566.
- Emu, B., W. J. Moretto, et al. (2014). "Composition and Function of T Cell Subpopulations Are Slow to Change Despite Effective Antiretroviral Treatment of HIV Disease." *PLoS One* **9**(1): e85613.
- Endeman, H., M. C. Cornips, et al. (2009). "The Fcγ receptor IIA-R/R131 genotype is associated with severe sepsis in community-acquired pneumonia." *Clin Vaccine Immunol* **16**(7): 1087-1090.
- Espinosa, E., D. P. Romero-Rodriguez, et al. (2013). "Transient expansion of activated CD8+ T cells characterizes tuberculosis-associated immune reconstitution inflammatory syndrome in patients with HIV: a case control study." *J Inflamm (Lond)* **10**(1): 21.
- Etuwewe, O. M., N. Swann, et al. (2009). "Effect of recurrent invasive pneumococcal disease on serum anti-pneumolysin IgG titres in HIV infected adults." *Vaccine* **27**(29): 3881-3884.
- Fauci, A. S., G. Pantaleo, et al. (1996). "Immunopathogenic mechanisms of HIV infection." *Ann Intern Med* **124**(7): 654-663.
- Feikin, D. R., C. M. Elie, et al. (2001). "Randomized trial of the quantitative and functional antibody responses to a 7-valent pneumococcal conjugate vaccine and/or 23-valent polysaccharide vaccine among HIV-infected adults." *Vaccine* **20**(3-4): 545-553.
- Fernandez Larrosa, P. N., D. O. Croci, et al. (2008). "Apoptosis resistance in HIV-1 persistently-infected cells is independent of active viral replication and involves modulation of the apoptotic mitochondrial pathway." *Retrovirology* **5**: 19.

- Fine, M. J., M. A. Smith, et al. (1994). "Efficacy of pneumococcal vaccination in adults. A meta-analysis of randomized controlled trials." *Arch Intern Med* **154**(23): 2666-2677.
- Flasche, S., A. J. Van Hoek, et al. (2011). "Effect of pneumococcal conjugate vaccination on serotype-specific carriage and invasive disease in England: a cross-sectional study." *PLoS Med* **8**(4): e1001017.
- Folks, T. M., J. Justement, et al. (1987). "Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line." *Science* **238**(4828): 800-802.
- Folks, T. M., J. Justement, et al. (1988). "Characterization of a promonocyte clone chronically infected with HIV and inducible by 13-phorbol-12-myristate acetate." *J Immunol* **140**(4): 1117-1122.
- French, N., C. F. Gilks, et al. (1998). "Pneumococcal vaccination in HIV-1-infected adults in Uganda: humoral response and two vaccine failures." *AIDS* **12**(13): 1683-1689.
- French, N., S. B. Gordon, et al. (2010). "A trial of a 7-valent pneumococcal conjugate vaccine in HIV-infected adults." *N Engl J Med* **362**(9): 812-822.
- French, N., J. Nakiyingi, et al. (2000). "23-valent pneumococcal polysaccharide vaccine in HIV-1-infected Ugandan adults: double-blind, randomised and placebo controlled trial." *Lancet* **355**(9221): 2106-2111.
- Gantner, F., R. Kupferschmidt, et al. (1997). "In vitro differentiation of human monocytes to macrophages: change of PDE profile and its relationship to suppression of tumour necrosis factor-alpha release by PDE inhibitors." *Br J Pharmacol* **121**(2): 221-231.
- Geleziunas, R., W. Xu, et al. (2001). "HIV-1 Nef inhibits ASK1-dependent death signalling providing a potential mechanism for protecting the infected host cell." *Nature* **410**(6830): 834-838.
- Gendelman, H. E., O. Narayan, et al. (1986). "Tropism of sheep lentiviruses for monocytes: susceptibility to infection and virus gene expression increase during maturation of monocytes to macrophages." *J Virol* **58**(1): 67-74.
- Geretti, A. M., G. Brook, et al. (2008). "British HIV Association guidelines for immunization of HIV-infected adults 2008." *HIV Med* **9**(10): 795-848.
- Gil, L., G. Martinez, et al. (2003). "Contribution to characterization of oxidative stress in HIV/AIDS patients." *Pharmacol Res* **47**(3): 217-224.
- Gilks, C. F., S. A. Ojoo, et al. (1996). "Invasive pneumococcal disease in a cohort of predominantly HIV-1 infected female sex-workers in Nairobi, Kenya." *Lancet* **347**(9003): 718-723.
- Giorgi, J. V., Z. Liu, et al. (1993). "Elevated levels of CD38+ CD8+ T cells in HIV infection add to the prognostic value of low CD4+ T cell levels: results of 6 years of follow-up. The Los Angeles Center, Multicenter AIDS Cohort Study." *J Acquir Immune Defic Syndr* **6**(8): 904-912.
- Giri, M. S., M. Nebozyhn, et al. (2009). "Circulating monocytes in HIV-1-infected viremic subjects exhibit an antiapoptosis gene signature and virus- and host-mediated apoptosis resistance." *J Immunol* **182**(7): 4459-4470.
- Glennie, S. J., D. Banda, et al. (2013). "Defective pneumococcal-specific Th1 responses in HIV-infected adults precedes a loss of control of pneumococcal colonization." *Clin Infect Dis* **56**(2): 291-299.
- Glennie, S. J., E. Sepako, et al. (2011). "Impaired CD4 T cell memory response to *Streptococcus pneumoniae* precedes CD4 T cell depletion in HIV-infected Malawian adults." *PLoS One* **6**(9): e25610.
- Gordin, F. M., M. P. Roediger, et al. (2008). "Pneumonia in HIV-infected persons: increased risk with cigarette smoking and treatment interruption." *Am J Respir Crit Care Med* **178**(6): 630-636.

- Gordon, M. A., S. B. Gordon, et al. (2007). "Primary macrophages from HIV-infected adults show dysregulated cytokine responses to Salmonella, but normal internalization and killing." *AIDS* **21**(18): 2399-2408.
- Gordon, S. (2004). "Pneumococcal infections in HIV infected adults--clinical features, reasons behind the association and future hopes for prevention." *Trop Doct* **34**(4): 200-203.
- Gordon, S. B., G. R. Irving, et al. (2000). "Intracellular trafficking and killing of Streptococcus pneumoniae by human alveolar macrophages are influenced by opsonins." *Infect Immun* **68**(4): 2286-2293.
- Gordon, S. B., R. T. Jagoe, et al. (2013). "The alveolar microenvironment of patients infected with human immunodeficiency virus does not modify alveolar macrophage interactions with Streptococcus pneumoniae." *Clin Vaccine Immunol* **20**(6): 882-891.
- Gordon, S. B., E. R. Jarman, et al. (2005). "Reduced interleukin-8 response to Streptococcus pneumoniae by alveolar macrophages from adults with HIV/AIDS." *AIDS* **19**(11): 1197-1200.
- Gordon, S. B., H. Kayhty, et al. (2007). "Pneumococcal conjugate vaccine is immunogenic in lung fluid of HIV-infected and immunocompetent adults." *J Allergy Clin Immunol* **120**(1): 208-210.
- Gordon, S. B., M. E. Molyneux, et al. (2001). "Opsonic phagocytosis of Streptococcus pneumoniae by alveolar macrophages is not impaired in human immunodeficiency virus-infected Malawian adults." *J Infect Dis* **184**(10): 1345-1349.
- Gorry, P. R. and P. Ancuta (2011). "Coreceptors and HIV-1 pathogenesis." *Curr HIV/AIDS Rep* **8**(1): 45-53.
- Grau, I., C. Ardanuy, et al. (2009). "Trends in mortality and antibiotic resistance among HIV-infected patients with invasive pneumococcal disease." *HIV Med* **10**(8): 488-495.
- Grau, I., R. Pallares, et al. (2005). "Epidemiologic changes in bacteremic pneumococcal disease in patients with human immunodeficiency virus in the era of highly active antiretroviral therapy." *Arch Intern Med* **165**(13): 1533-1540.
- Green, D. R. and G. Kroemer (2004). "The pathophysiology of mitochondrial cell death." *Science* **305**(5684): 626-629.
- Groot, F., S. Welsch, et al. (2008). "Efficient HIV-1 transmission from macrophages to T cells across transient virological synapses." *Blood* **111**(9): 4660-4663.
- Guillemard, E., C. Jacquemot, et al. (2004). "Human immunodeficiency virus 1 favors the persistence of infection by activating macrophages through TNF." *Virology* **329**(2): 371-380.
- Guillon, J. M., B. Autran, et al. (1988). "Human immunodeficiency virus-related lymphocytic alveolitis." *Chest* **94**(6): 1264-1270.
- Gundavarapu, S., N. C. Mishra, et al. (2013). "HIV gp120 induces mucus formation in human bronchial epithelial cells through CXCR4/alpha7-nicotinic acetylcholine receptors." *PLoS One* **8**(10): e77160.
- Gurtler, L. (1996). "Difficulties and strategies of HIV diagnosis." *Lancet* **348**(9021): 176-179.
- Haase, A. T., K. Henry, et al. (1996). "Quantitative image analysis of HIV-1 infection in lymphoid tissue." *Science* **274**(5289): 985-989.
- Hanson, D. L., S. Y. Chu, et al. (1995). "Distribution of CD4+ T lymphocytes at diagnosis of acquired immunodeficiency syndrome--defining and other human immunodeficiency virus-related illnesses. The Adult and Adolescent Spectrum of HIV Disease Project Group." *Arch Intern Med* **155**(14): 1537-1542.
- Haorah, J., D. Heilman, et al. (2004). "Alcohol and HIV decrease proteasome and immunoproteasome function in macrophages: implications for impaired immune function during disease." *Cell Immunol* **229**(2): 139-148.
- Harboe, Z. B., T. L. Benfield, et al. (2010). "Temporal trends in invasive pneumococcal disease and pneumococcal serotypes over 7 decades." *Clin Infect Dis* **50**(3): 329-337.

- Harboe, Z. B., R. W. Thomsen, et al. (2009). "Pneumococcal serotypes and mortality following invasive pneumococcal disease: a population-based cohort study." *PLoS Med* **6**(5): e1000081.
- Harrer, T., E. Harrer, et al. (1996). "Cytotoxic T lymphocytes in asymptomatic long-term nonprogressing HIV-1 infection. Breadth and specificity of the response and relation to in vivo viral quasispecies in a person with prolonged infection and low viral load." *J Immunol* **156**(7): 2616-2623.
- Harris, P. and P. Ralph (1985). "Human leukemic models of myelomonocytic development: a review of the HL-60 and U937 cell lines." *J Leukoc Biol* **37**(4): 407-422.
- Hatzioannou, T. and D. T. Evans (2012). "Animal models for HIV/AIDS research." *Nat Rev Microbiol* **10**(12): 852-867.
- Heffernan, R. T., N. L. Barrett, et al. (2005). "Declining incidence of invasive Streptococcus pneumoniae infections among persons with AIDS in an era of highly active antiretroviral therapy, 1995-2000." *J Infect Dis* **191**(12): 2038-2045.
- Hendley, J. O., M. A. Sande, et al. (1975). "Spread of Streptococcus pneumoniae in families. I. Carriage rates and distribution of types." *J Infect Dis* **132**(1): 55-61.
- Herbein, G., U. Mahlknecht, et al. (1998). "Apoptosis of CD8+ T cells is mediated by macrophages through interaction of HIV gp120 with chemokine receptor CXCR4." *Nature* **395**(6698): 189-194.
- Heron, M., J. C. Grutters, et al. (2012). "Bronchoalveolar lavage cell pattern from healthy human lung." *Clin Exp Immunol* **167**(3): 523-531.
- Hill, P. C., A. Akisanya, et al. (2006). "Nasopharyngeal carriage of Streptococcus pneumoniae in Gambian villagers." *Clin Infect Dis* **43**(6): 673-679.
- Hirani, A., R. Cavallazzi, et al. (2011). "Prevalence of obstructive lung disease in HIV population: a cross sectional study." *Respir Med* **105**(11): 1655-1661.
- Hirschtick, R. E., J. Glassroth, et al. (1995). "Bacterial pneumonia in persons infected with the human immunodeficiency virus. Pulmonary Complications of HIV Infection Study Group." *N Engl J Med* **333**(13): 845-851.
- Hodge, G., V. Mukaro, et al. (2011). "Role of increased CD8/CD28(null) T cells and alternative co-stimulatory molecules in chronic obstructive pulmonary disease." *Clin Exp Immunol* **166**(1): 94-102.
- Hodge, G., J. Nairn, et al. (2007). "Increased intracellular T helper 1 proinflammatory cytokine production in peripheral blood, bronchoalveolar lavage and intraepithelial T cells of COPD subjects." *Clin Exp Immunol* **150**(1): 22-29.
- Hogg, J. C., F. Chu, et al. (2004). "The nature of small-airway obstruction in chronic obstructive pulmonary disease." *N Engl J Med* **350**(26): 2645-2653.
- Huang, Y., N. Erdmann, et al. (2006). "TRAIL-mediated apoptosis in HIV-1-infected macrophages is dependent on the inhibition of Akt-1 phosphorylation." *J Immunol* **177**(4): 2304-2313.
- Hung, C. C., S. Y. Chang, et al. "A 5-year longitudinal follow-up study of serological responses to 23-valent pneumococcal polysaccharide vaccination among patients with HIV infection who received highly active antiretroviral therapy." *HIV Med* **11**(1): 54-63.
- Hyams, C., J. Yuste, et al. (2010). "Streptococcus pneumoniae resistance to complement-mediated immunity is dependent on the capsular serotype." *Infect Immun* **78**(2): 716-725.
- leong, M. H., C. C. Reardon, et al. (2000). "Human immunodeficiency virus type 1 infection of alveolar macrophages impairs their innate fungicidal activity." *Am J Respir Crit Care Med* **162**(3 Pt 1): 966-970.
- Igarashi, T., C. R. Brown, et al. (2001). "Macrophage are the principal reservoir and sustain high virus loads in rhesus macaques after the depletion of CD4+ T cells by a highly

- pathogenic simian immunodeficiency virus/HIV type 1 chimera (SHIV): Implications for HIV-1 infections of humans." *Proc Natl Acad Sci U S A* **98**(2): 658-663.
- Ikezu, T. (2009). "The aging of human-immunodeficiency-virus-associated neurocognitive disorders." *J Neuroimmune Pharmacol* **4**(2): 161-162.
- Imaz, A., V. Falco, et al. (2009). "Impact of prior pneumococcal vaccination on clinical outcomes in HIV-infected adult patients hospitalized with invasive pneumococcal disease." *HIV Med* **10**(6): 356-363.
- Isakov, N. (1997). "Immunoreceptor tyrosine-based activation motif (ITAM), a unique module linking antigen and Fc receptors to their signaling cascades." *J Leukoc Biol* **61**(1): 6-16.
- Jacotot, E., L. Ravagnan, et al. (2000). "The HIV-1 viral protein R induces apoptosis via a direct effect on the mitochondrial permeability transition pore." *J Exp Med* **191**(1): 33-46.
- Jambo, K. C., D. H. Banda, et al. (2014). "Small alveolar macrophages are infected preferentially by HIV and exhibit impaired phagocytic function." *Mucosal Immunol*.
- Janas, A. M. and L. Wu (2009). "HIV-1 interactions with cells: from viral binding to cell-cell transmission." *Curr Protoc Cell Biol* **Chapter 26**: Unit 26 25.
- Janeway, C. A. (2001). *Immunobiology 5 : the immune system in health and disease*. New York, Garland ; Edinburgh : Churchill Livingstone.
- Janeway, C. A., Jr. and R. Medzhitov (2002). "Innate immune recognition." *Annu Rev Immunol* **20**: 197-216.
- Janoff, E. N., R. F. Breiman, et al. (1992). "Pneumococcal disease during HIV infection. Epidemiologic, clinical, and immunologic perspectives." *Ann Intern Med* **117**(4): 314-324.
- Janoff, E. N., C. Fasching, et al. (1997). "Responsiveness of human immunodeficiency virus type 1-infected Kenyan women with or without prior pneumococcal disease to pneumococcal vaccine." *J Infect Dis* **175**(4): 975-978.
- Janoff, E. N., J. O'Brien, et al. (1993). "Streptococcus pneumoniae colonization, bacteremia, and immune response among persons with human immunodeficiency virus infection." *J Infect Dis* **167**(1): 49-56.
- Jeffrey, A. A., D. Israel-Biet, et al. (1991). "HIV isolation from pulmonary cells derived from bronchoalveolar lavage." *Clin Exp Immunol* **84**(3): 488-492.
- Jenkins, S. J., D. Ruckerl, et al. (2011). "Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation." *Science* **332**(6035): 1284-1288.
- Johnston, M. I. and A. S. Fauci (2007). "An HIV vaccine--evolving concepts." *N Engl J Med* **356**(20): 2073-2081.
- Jonsson, S., D. M. Musher, et al. (1985). "Phagocytosis and killing of common bacterial pathogens of the lung by human alveolar macrophages." *J Infect Dis* **152**(1): 4-13.
- Jordano, Q., V. Falco, et al. (2004). "Invasive pneumococcal disease in patients infected with HIV: still a threat in the era of highly active antiretroviral therapy." *Clin Infect Dis* **38**(11): 1623-1628.
- Kadioglu, A. and P. W. Andrew (2004). "The innate immune response to pneumococcal lung infection: the untold story." *Trends Immunol* **25**(3): 143-149.
- Kadioglu, A., W. Coward, et al. (2004). "CD4-T-lymphocyte interactions with pneumolysin and pneumococci suggest a crucial protective role in the host response to pneumococcal infection." *Infect Immun* **72**(5): 2689-2697.
- Kadioglu, A., N. A. Gingles, et al. (2000). "Host cellular immune response to pneumococcal lung infection in mice." *Infect Immun* **68**(2): 492-501.
- Kahn, J. O. and B. D. Walker (1998). "Acute human immunodeficiency virus type 1 infection." *N Engl J Med* **339**(1): 33-39.
- Kalsdorf, B., T. J. Scriba, et al. (2009). "HIV-1 infection impairs the bronchoalveolar T-cell response to mycobacteria." *Am J Respir Crit Care Med* **180**(12): 1262-1270.

- Kaplan, R. C., L. A. Kingsley, et al. (2007). "Ten-year predicted coronary heart disease risk in HIV-infected men and women." *Clin Infect Dis* **45**(8): 1074-1081.
- Karn, J. and C. M. Stoltzfus (2012). "Transcriptional and posttranscriptional regulation of HIV-1 gene expression." *Cold Spring Harb Perspect Med* **2**(2): a006916.
- Kedzierska, K., P. Ellery, et al. (2002). "HIV-1 down-modulates gamma signaling chain of Fc gamma R in human macrophages: a possible mechanism for inhibition of phagocytosis." *J Immunol* **168**(6): 2895-2903.
- Kedzierska, K., N. J. Vardaxis, et al. (2001). "FcgammaR-mediated phagocytosis by human macrophages involves Hck, Syk, and Pyk2 and is augmented by GM-CSF." *J Leukoc Biol* **70**(2): 322-328.
- Keele, B. F., E. E. Giorgi, et al. (2008). "Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection." *Proc Natl Acad Sci U S A* **105**(21): 7552-7557.
- Kerkau, T., I. Bacik, et al. (1997). "The human immunodeficiency virus type 1 (HIV-1) Vpu protein interferes with an early step in the biosynthesis of major histocompatibility complex (MHC) class I molecules." *J Exp Med* **185**(7): 1295-1305.
- Kerr, J. F., A. H. Wyllie, et al. (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." *Br J Cancer* **26**(4): 239-257.
- Khor, C. C., S. J. Chapman, et al. (2007). "A Mal functional variant is associated with protection against invasive pneumococcal disease, bacteremia, malaria and tuberculosis." *Nat Genet* **39**(4): 523-528.
- Kilian, M., J. Mestecky, et al. (1980). "IgA1 proteases from Haemophilus influenzae, Streptococcus pneumoniae, Neisseria meningitidis, and Streptococcus sanguis: comparative immunochemical studies." *J Immunol* **124**(6): 2596-2600.
- Kinabo, G. D., A. van der Ven, et al. (2013). "Dynamics of nasopharyngeal bacterial colonisation in HIV-exposed young infants in Tanzania." *Trop Med Int Health* **18**(3): 286-295.
- Kirby, A. C., J. G. Raynes, et al. (2005). "The role played by tumor necrosis factor during localized and systemic infection with Streptococcus pneumoniae." *J Infect Dis* **191**(9): 1538-1547.
- Kirchhoff, F., T. C. Greenough, et al. (1995). "Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection." *N Engl J Med* **332**(4): 228-232.
- Kjellstrom, C., K. Ichimura, et al. (2000). "The origin of alveolar macrophages in the transplanted lung: a longitudinal microsatellite-based study of donor and recipient DNA." *Transplantation* **69**(9): 1984-1986.
- Klase, Z., R. Winograd, et al. (2009). "HIV-1 TAR miRNA protects against apoptosis by altering cellular gene expression." *Retrovirology* **6**: 18.
- Klasse, P. J. and J. P. Moore (2004). "Is there enough gp120 in the body fluids of HIV-1-infected individuals to have biologically significant effects?" *Virology* **323**(1): 1-8.
- Klein, M., B. Obermaier, et al. (2008). "Innate immunity to pneumococcal infection of the central nervous system depends on toll-like receptor (TLR) 2 and TLR4." *J Infect Dis* **198**(7): 1028-1036.
- Klugman, K. P., S. A. Madhi, et al. (2003). "A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection." *N Engl J Med* **349**(14): 1341-1348.
- Koedel, U., W. M. Scheld, et al. (2002). "Pathogenesis and pathophysiology of pneumococcal meningitis." *Lancet Infect Dis* **2**(12): 721-736.
- Koenig, S., H. E. Gendelman, et al. (1986). "Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy." *Science* **233**(4768): 1089-1093.
- Kogan, M. and J. Rappaport (2011). "HIV-1 accessory protein Vpr: relevance in the pathogenesis of HIV and potential for therapeutic intervention." *Retrovirology* **8**: 25.

- Koziel, H., Q. Eichbaum, et al. (1998). "Reduced binding and phagocytosis of *Pneumocystis carinii* by alveolar macrophages from persons infected with HIV-1 correlates with mannose receptor downregulation." *J Clin Invest* **102**(7): 1332-1344.
- Koziel, H., X. Li, et al. (2000). "Alveolar macrophages from human immunodeficiency virus-infected persons demonstrate impaired oxidative burst response to *Pneumocystis carinii* in vitro." *Am J Respir Cell Mol Biol* **23**(4): 452-459.
- Kroon, F. P., J. T. van Dissel, et al. (2000). "Enhanced antibody response to pneumococcal polysaccharide vaccine after prior immunization with conjugate pneumococcal vaccine in HIV-infected adults." *Vaccine* **19**(7-8): 886-894.
- Kuritzkes, D. R. (2000). "Neutropenia, neutrophil dysfunction, and bacterial infection in patients with human immunodeficiency virus disease: the role of granulocyte colony-stimulating factor." *Clin Infect Dis* **30**(2): 256-260.
- Kyaw, M. H., P. Christie, et al. (2003). "Invasive pneumococcal disease in Scotland, 1999-2001: use of record linkage to explore associations between patients and disease in relation to future vaccination policy." *Clin Infect Dis* **37**(10): 1283-1291.
- Kyaw, M. H., C. E. Rose, Jr., et al. (2005). "The influence of chronic illnesses on the incidence of invasive pneumococcal disease in adults." *J Infect Dis* **192**(3): 377-386.
- Kyei, G. B., C. Dinkins, et al. (2009). "Autophagy pathway intersects with HIV-1 biosynthesis and regulates viral yields in macrophages." *J Cell Biol* **186**(2): 255-268.
- Labbe, K. and M. Saleh (2008). "Cell death in the host response to infection." *Cell Death Differ* **15**(9): 1339-1349.
- Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." *Nature* **227**(5259): 680-685.
- Laforge, M., L. Campillo-Gimenez, et al. (2011). "HIV/SIV Infection Primes Monocytes and Dendritic Cells for Apoptosis." *PLoS Pathog* **7**(6): e1002087.
- Landay, A., B. A. da Silva, et al. (2007). "Evidence of ongoing immune reconstitution in subjects with sustained viral suppression following 6 years of lopinavir-ritonavir treatment." *Clin Infect Dis* **44**(5): 749-754.
- Lassiter, C., X. Fan, et al. (2009). "HIV-1 transgene expression in rats causes oxidant stress and alveolar epithelial barrier dysfunction." *AIDS Res Ther* **6**: 1.
- Lazzarin, A., C. Uberti Foppa, et al. (1986). "Impairment of polymorphonuclear leucocyte function in patients with acquired immunodeficiency syndrome and with lymphadenopathy syndrome." *Clin Exp Immunol* **65**(1): 105-111.
- Le Moing, V., C. Rabaud, et al. (2006). "Incidence and risk factors of bacterial pneumonia requiring hospitalization in HIV-infected patients started on a protease inhibitor-containing regimen." *HIV Med* **7**(4): 261-267.
- Learmont, J. C., A. F. Geczy, et al. (1999). "Immunologic and virologic status after 14 to 18 years of infection with an attenuated strain of HIV-1. A report from the Sydney Blood Bank Cohort." *N Engl J Med* **340**(22): 1715-1722.
- Lee, K. S., C. A. Scanga, et al. (2007). "TLR2 synergizes with both TLR4 and TLR9 for induction of the MyD88-dependent splenic cytokine and chemokine response to *Streptococcus pneumoniae*." *Cell Immunol* **245**(2): 103-110.
- Leeansyah, E., B. D. Wines, et al. (2007). "The mechanism underlying defective Fcγ receptor-mediated phagocytosis by HIV-1-infected human monocyte-derived macrophages." *J Immunol* **178**(2): 1096-1104.
- LeMessurier, K., H. Hacker, et al. (2010). "Inhibition of T cells provides protection against early invasive pneumococcal disease." *Infect Immun* **78**(12): 5287-5294.
- Lerner, C. W. and M. L. Tapper (1984). "Opportunistic infection complicating acquired immune deficiency syndrome. Clinical features of 25 cases." *Medicine (Baltimore)* **63**(3): 155-164.

- Lesprit, P., G. Pedrono, et al. (2007). "Immunological efficacy of a prime-boost pneumococcal vaccination in HIV-infected adults." *AIDS* **21**(18): 2425-2434.
- Levy, D. N., G. M. Aldrovandi, et al. (2004). "Dynamics of HIV-1 recombination in its natural target cells." *Proc Natl Acad Sci U S A* **101**(12): 4204-4209.
- Lewin, S. R., J. Kiriwara, et al. (1998). "HIV-1 DNA and mRNA concentrations are similar in peripheral blood monocytes and alveolar macrophages in HIV-1-infected individuals." *AIDS* **12**(7): 719-727.
- Lifson, A. R. and H. A. Lando (2012). "Smoking and HIV: prevalence, health risks, and cessation strategies." *Curr HIV/AIDS Rep* **9**(3): 223-230.
- Liu, H., H. Perlman, et al. (2001). "Constitutively activated Akt-1 is vital for the survival of human monocyte-differentiated macrophages. Role of Mcl-1, independent of nuclear factor (NF)-kappaB, Bad, or caspase activation." *J Exp Med* **194**(2): 113-126.
- Liu, R., W. A. Paxton, et al. (1996). "Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection." *Cell* **86**(3): 367-377.
- Liu, X., V. S. Chauhan, et al. (2010). "NOD2 mediates inflammatory responses of primary murine glia to *Streptococcus pneumoniae*." *Glia* **58**(7): 839-847.
- Lore, K., A. Smed-Sorensen, et al. (2005). "Myeloid and plasmacytoid dendritic cells transfer HIV-1 preferentially to antigen-specific CD4+ T cells." *J Exp Med* **201**(12): 2023-2033.
- Lu, Y. J., J. Gross, et al. (2008). "Interleukin-17A mediates acquired immunity to pneumococcal colonization." *PLoS Pathog* **4**(9): e1000159.
- Lum, J. J. and A. D. Badley (2003). "Resistance to apoptosis: mechanism for the development of HIV reservoirs." *Curr HIV Res* **1**(3): 261-274.
- Lum, J. J., A. A. Pilon, et al. (2001). "Induction of cell death in human immunodeficiency virus-infected macrophages and resting memory CD4 T cells by TRAIL/Apo2l." *J Virol* **75**(22): 11128-11136.
- Madeddu, G., A. G. Fois, et al. (2013). "Chronic obstructive pulmonary disease: an emerging comorbidity in HIV-infected patients in the HAART era?" *Infection* **41**(2): 347-353.
- Madeddu, G., E. M. Porqueddu, et al. (2008). "Bacterial community acquired pneumonia in HIV-infected inpatients in the highly active antiretroviral therapy era." *Infection* **36**(3): 231-236.
- Madesh, M. and G. Hajnoczky (2001). "VDAC-dependent permeabilization of the outer mitochondrial membrane by superoxide induces rapid and massive cytochrome c release." *J Cell Biol* **155**(6): 1003-1015.
- Madhi, S. A., P. Adrian, et al. (2007). "Long-term immunogenicity and efficacy of a 9-valent conjugate pneumococcal vaccine in human immunodeficient virus infected and non-infected children in the absence of a booster dose of vaccine." *Vaccine* **25**(13): 2451-2457.
- Madhi, S. A., O. S. Levine, et al. (2008). "Vaccines to prevent pneumonia and improve child survival." *Bull World Health Organ* **86**(5): 365-372.
- Malley, R. (2010). "Antibody and cell-mediated immunity to *Streptococcus pneumoniae*: implications for vaccine development." *J Mol Med (Berl)* **88**(2): 135-142.
- Malley, R., P. Henneke, et al. (2003). "Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection." *Proc Natl Acad Sci U S A* **100**(4): 1966-1971.
- Malley, R., K. Trzcinski, et al. (2005). "CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization." *Proc Natl Acad Sci U S A* **102**(13): 4848-4853.
- Mantovani, A., A. Sica, et al. (2004). "The chemokine system in diverse forms of macrophage activation and polarization." *Trends Immunol* **25**(12): 677-686.
- Marriott, H. M. (2010). Personal communication.

- Marriott, H. M., F. Ali, et al. (2004). "Nitric oxide levels regulate macrophage commitment to apoptosis or necrosis during pneumococcal infection." *FASEB J* **18**(10): 1126-1128.
- Marriott, H. M., C. D. Bingle, et al. (2005). "Dynamic changes in Mcl-1 expression regulate macrophage viability or commitment to apoptosis during bacterial clearance." *J Clin Invest* **115**(2): 359-368.
- Marriott, H. M., M. Daigneault, et al. (2012). "A decoy receptor 3 analogue reduces localised defects in phagocyte function in pneumococcal pneumonia." *Thorax* **67**(11): 985-992.
- Marriott, H. M., P. G. Hellewell, et al. (2006). "Decreased alveolar macrophage apoptosis is associated with increased pulmonary inflammation in a murine model of pneumococcal pneumonia." *J Immunol* **177**(9): 6480-6488.
- Marriott, H. M., P. G. Hellewell, et al. (2007). "Contrasting roles for reactive oxygen species and nitric oxide in the innate response to pulmonary infection with *Streptococcus pneumoniae*." *Vaccine* **25**(13): 2485-2490.
- Marriott, H. M., L. E. Jackson, et al. (2008). "Reactive oxygen species regulate neutrophil recruitment and survival in pneumococcal pneumonia." *Am J Respir Crit Care Med* **177**(8): 887-895.
- Mathers, B. M., L. Degenhardt, et al. (2008). "Global epidemiology of injecting drug use and HIV among people who inject drugs: a systematic review." *Lancet* **372**(9651): 1733-1745.
- Maus, U. A., S. Janzen, et al. (2006). "Resident alveolar macrophages are replaced by recruited monocytes in response to endotoxin-induced lung inflammation." *Am J Respir Cell Mol Biol* **35**(2): 227-235.
- McElrath, M. J., J. E. Pruett, et al. (1989). "Mononuclear phagocytes of blood and bone marrow: comparative roles as viral reservoirs in human immunodeficiency virus type 1 infections." *Proc Natl Acad Sci U S A* **86**(2): 675-679.
- McElrath, M. J., R. M. Steinman, et al. (1991). "Latent HIV-1 infection in enriched populations of blood monocytes and T cells from seropositive patients." *J Clin Invest* **87**(1): 27-30.
- Meddows-Taylor, S., D. J. Martin, et al. (1999). "Impaired interleukin-8-induced degranulation of polymorphonuclear neutrophils from human immunodeficiency virus type 1-infected individuals." *Clin Diagn Lab Immunol* **6**(3): 345-351.
- Meddows-Taylor, S., S. Pendle, et al. (2001). "Altered expression of CD88 and associated impairment of complement 5a-induced neutrophil responses in human immunodeficiency virus type 1-infected patients with and without pulmonary tuberculosis." *J Infect Dis* **183**(4): 662-665.
- Mellors, J. W., A. Munoz, et al. (1997). "Plasma viral load and CD4+ lymphocytes as prognostic markers of HIV-1 infection." *Ann Intern Med* **126**(12): 946-954.
- Meltzer, M. S. and H. E. Gendelman (1992). "Mononuclear phagocytes as targets, tissue reservoirs, and immunoregulatory cells in human immunodeficiency virus disease." *Curr Top Microbiol Immunol* **181**: 239-263.
- Merino, J., M. A. Martinez-Gonzalez, et al. (1998). "Progressive decrease of CD8high+ CD28+ CD57- cells with ageing." *Clin Exp Immunol* **112**(1): 48-51.
- Mientjes, G. H., I. J. Spijkerman, et al. (1996). "Incidence and risk factors for pneumonia in HIV infected and non-infected drug users." *J Infect* **32**(3): 181-186.
- Miuro, G., H. Kayhty, et al. (2005). "Conjugate pneumococcal vaccine in HIV-infected Ugandans and the effect of past receipt of polysaccharide vaccine." *J Infect Dis* **192**(10): 1801-1805.
- Miles, A. A., S. S. Misra, et al. (1938). "The estimation of the bactericidal power of the blood." *J Hyg (Lond)* **38**(6): 732-749.
- Milner, J. D., J. M. Brenchley, et al. (2008). "Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome." *Nature* **452**(7188): 773-776.

- Moberley, S., J. Holden, et al. (2013). "Vaccines for preventing pneumococcal infection in adults." *Cochrane Database Syst Rev* **1**: CD000422.
- Moberley, S. A., J. Holden, et al. (2008). "Vaccines for preventing pneumococcal infection in adults." *Cochrane Database Syst Rev*(1): CD000422.
- Moir, S., A. Malaspina, et al. (2001). "HIV-1 induces phenotypic and functional perturbations of B cells in chronically infected individuals." *Proc Natl Acad Sci U S A* **98**(18): 10362-10367.
- Mook-Kanamori, B. B., M. Geldhoff, et al. (2011). "Pathogenesis and pathophysiology of pneumococcal meningitis." *Clin Microbiol Rev* **24**(3): 557-591.
- Moorjani, H., B. P. Craddock, et al. (1996). "Impairment of phagosome-lysosome fusion in HIV-1-infected macrophages." *J Acquir Immune Defic Syndr Hum Retrovirol* **13**(1): 18-22.
- Morrow, G., L. Vachot, et al. (2007). "Current concepts of HIV transmission." *Curr HIV/AIDS Rep* **4**(1): 29-35.
- Mosser, D. M. and J. P. Edwards (2008). "Exploring the full spectrum of macrophage activation." *Nat Rev Immunol* **8**(12): 958-969.
- Muller, F., H. Rollag, et al. (1990). "Reduced oxidative burst responses in monocytes and monocyte-derived macrophages from HIV-infected subjects." *Clin Exp Immunol* **82**(1): 10-15.
- Munoz, J. F., S. Salmen, et al. (1999). "Effect of human immunodeficiency virus type 1 on intracellular activation and superoxide production by neutrophils." *J Infect Dis* **180**(1): 206-210.
- Mureithi, M. W., A. Finn, et al. (2009). "T cell memory response to pneumococcal protein antigens in an area of high pneumococcal carriage and disease." *J Infect Dis* **200**(5): 783-793.
- Murphy, J., R. Summer, et al. (2008). "The prolonged life-span of alveolar macrophages." *Am J Respir Cell Mol Biol* **38**(4): 380-385.
- Nakamura, H., S. De Rosa, et al. (1996). "Elevation of plasma thioredoxin levels in HIV-infected individuals." *Int Immunol* **8**(4): 603-611.
- Neil, S. J., T. Zang, et al. (2008). "Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu." *Nature* **451**(7177): 425-430.
- Nelson, A. L., A. M. Roche, et al. (2007). "Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance." *Infect Immun* **75**(1): 83-90.
- NIAID. (2012, April 2012). "HIV Replication Cycle." Retrieved June 2013, 2013, from <http://www.niaid.nih.gov/topics/hiv/understanding/biology/Pages/hivReplicationCycle.aspx>.
- Nicol, M. Q., J. M. Mathys, et al. (2008). "Human immunodeficiency virus infection alters tumor necrosis factor alpha production via Toll-like receptor-dependent pathways in alveolar macrophages and U1 cells." *J Virol* **82**(16): 7790-7798.
- Nicoletti, C., M. C. Brandileone, et al. (2007). "Prevalence, serotypes, and risk factors for pneumococcal carriage among HIV-infected adults." *Diagn Microbiol Infect Dis* **57**(3): 259-265.
- Nicoletti, I., G. Migliorati, et al. (1991). "A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry." *J Immunol Methods* **139**(2): 271-279.
- Nijhawan, D., M. Fang, et al. (2003). "Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation." *Genes Dev* **17**(12): 1475-1486.
- Nottet, H. S., L. de Graaf, et al. (1993). "Phagocytic function of monocyte-derived macrophages is not affected by human immunodeficiency virus type 1 infection." *J Infect Dis* **168**(1): 84-91.

- Noursadeghi, M., J. Tsang, et al. (2009). "Genome-wide innate immune responses in HIV-1-infected macrophages are preserved despite attenuation of the NF-kappa B activation pathway." *J Immunol* **182**(1): 319-328.
- Novak, R. M., T. J. Holzer, et al. (1990). "The effect of interleukin 4 (BSF-1) on infection of peripheral blood monocyte-derived macrophages with HIV-1." *AIDS Res Hum Retroviruses* **6**(8): 973-976.
- O'Brien, T. R., W. A. Blattner, et al. (1996). "Serum HIV-1 RNA levels and time to development of AIDS in the Multicenter Hemophilia Cohort Study." *JAMA* **276**(2): 105-110.
- Oh, S. K., W. W. Cruikshank, et al. (1992). "Identification of HIV-1 envelope glycoprotein in the serum of AIDS and ARC patients." *J Acquir Immune Defic Syndr* **5**(3): 251-256.
- Olivetta, E. and M. Federico (2006). "HIV-1 Nef protects human-monocyte-derived macrophages from HIV-1-induced apoptosis." *Exp Cell Res* **312**(6): 890-900.
- Olivetta, E., D. Pietraforte, et al. (2005). "HIV-1 Nef regulates the release of superoxide anions from human macrophages." *Biochem J* **390**(Pt 2): 591-602.
- Opferman, J. T. and S. J. Korsmeyer (2003). "Apoptosis in the development and maintenance of the immune system." *Nat Immunol* **4**(5): 410-415.
- Orlikowsky, T., Z. Q. Wang, et al. (1997). "Cytotoxic monocytes in the blood of HIV type 1-infected subjects destroy targeted T cells in a CD95-dependent fashion." *AIDS Res Hum Retroviruses* **13**(11): 953-960.
- Palecanda, A., J. Paulauskis, et al. (1999). "Role of the scavenger receptor MARCO in alveolar macrophage binding of unopsonized environmental particles." *J Exp Med* **189**(9): 1497-1506.
- Patel, N. R., K. Swan, et al. (2009). "Impaired M. tuberculosis-mediated apoptosis in alveolar macrophages from HIV+ persons: potential role of IL-10 and BCL-3." *J Leukoc Biol* **86**(1): 53-60.
- Patel, N. R., J. Zhu, et al. (2007). "HIV impairs TNF-alpha mediated macrophage apoptotic response to Mycobacterium tuberculosis." *J Immunol* **179**(10): 6973-6980.
- Paterson, G. K. and C. J. Orihuela (2010). "Pneumococci: immunology of the innate host response." *Respirology* **15**(7): 1057-1063.
- Patki, A. H., S. P. Zielske, et al. (2000). "Preferential S phase entry and apoptosis of CD4(+) T lymphocytes of HIV-1-infected patients after in vitro cultivation." *Clin Immunol* **97**(3): 241-247.
- Paul, J. (1997). "Royal Society of Tropical Medicine and Hygiene Meeting at Manson House, London, 12 December 1996. HIV and pneumococcal infection in Africa. Microbiological aspects." *Trans R Soc Trop Med Hyg* **91**(6): 632-637.
- Pemba, L., S. Charalambous, et al. (2008). "Impact of cotrimoxazole on non-susceptibility to antibiotics in Streptococcus pneumoniae carriage isolates among HIV-infected mineworkers in South Africa." *J Infect* **56**(3): 171-178.
- Penaranda, M., V. Falco, et al. (2007). "Effectiveness of polysaccharide pneumococcal vaccine in HIV-infected patients: a case-control study." *Clin Infect Dis* **45**(7): e82-87.
- Perciavalle, R. M., D. P. Stewart, et al. (2012). "Anti-apoptotic MCL-1 localizes to the mitochondrial matrix and couples mitochondrial fusion to respiration." *Nat Cell Biol* **14**(6): 575-583.
- Perelson, A. S., P. Essunger, et al. (1997). "Decay characteristics of HIV-1-infected compartments during combination therapy." *Nature* **387**(6629): 188-191.
- Perelson, A. S., A. U. Neumann, et al. (1996). "HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time." *Science* **271**(5255): 1582-1586.
- Perfetto, S. P., P. K. Chattopadhyay, et al. (2006). "Amine reactive dyes: an effective tool to discriminate live and dead cells in polychromatic flow cytometry." *J Immunol Methods* **313**(1-2): 199-208.

- Phillips, A. and P. Pezzotti (2004). "Short-term risk of AIDS according to current CD4 cell count and viral load in antiretroviral drug-naive individuals and those treated in the monotherapy era." *AIDS* **18**(1): 51-58.
- Pietraforte, D., E. Tritarelli, et al. (1994). "gp120 HIV envelope glycoprotein increases the production of nitric oxide in human monocyte-derived macrophages." *J Leukoc Biol* **55**(2): 175-182.
- Pinti, M., P. Biswas, et al. (2003). "Different sensitivity to apoptosis in cells of monocytic or lymphocytic origin chronically infected with human immunodeficiency virus type-1." *Exp Biol Med (Maywood)* **228**(11): 1346-1354.
- Pitrak, D. L. (1999). "Filgrastim treatment of HIV-infected patients improves neutrophil function." *AIDS* **13 Suppl 2**: S25-30.
- Plata, F., B. Autran, et al. (1987). "AIDS virus-specific cytotoxic T lymphocytes in lung disorders." *Nature* **328**(6128): 348-351.
- Polsky, B., J. W. Gold, et al. (1986). "Bacterial pneumonia in patients with the acquired immunodeficiency syndrome." *Ann Intern Med* **104**(1): 38-41.
- Preston, B. D., B. J. Poiesz, et al. (1988). "Fidelity of HIV-1 reverse transcriptase." *Science* **242**(4882): 1168-1171.
- Public_Health_England. (2013). "Cumulative weekly number of reports of Invasive Pneumococcal Disease due to any of the seven serotypes in Prevenar 7™ : Persons aged >=5 Years in England and Wales by Epidemiological Year: July-June (2005- To Date) ", from <http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/Pneumococcal/EpidemiologicalDataPneumococcal/CurrentEpidemiologyPneumococcal/InPrevenar7/pneumo03Cummulativeweekly5INPrevenar7vacc/>.
- Public_Health_England. (2013). "Cumulative weekly number of reports of Invasive Pneumococcal Disease due to any of the six serotypes in Prevenar13™ but not in PCV7 : Children aged < 2 Years in England and Wales by Epidemiological Year: July-June (2007- To Date) ", from <http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/Pneumococcal/EpidemiologicalDataPneumococcal/CurrentEpidemiologyPneumococcal/InPrevenar13NotInPrevenarPCV7/pneumo07Cummulativeweeklyunder2IN13NOTIN7vacc/>.
- Public_Health_England. (2013). "General Information on Pneumococcal Disease." from http://www.hpa.org.uk/web/HPAweb&HPAwebStandard/HPAweb_C/1203008864027.
- Pugliese, A., C. Cantamessa, et al. (1999). "Effects of the exogenous Nef protein on HIV-1 target cells." *Cell Biochem Funct* **17**(3): 183-192.
- Radin, J. N., C. J. Orihuela, et al. (2005). "beta-Arrestin 1 participates in platelet-activating factor receptor-mediated endocytosis of Streptococcus pneumoniae." *Infect Immun* **73**(12): 7827-7835.
- Raoul, H., R. Le Naour, et al. (1998). "HIV type 1 infection of human macrophages induces an upregulation of manganese superoxide dismutase gene that may protect cells from death." *AIDS Res Hum Retroviruses* **14**(5): 427-434.
- Reardon, C. C., S. J. Kim, et al. (1996). "Phagocytosis and growth inhibition of Cryptococcus neoformans by human alveolar macrophages: effects of HIV-1 infection." *AIDS* **10**(6): 613-618.
- Regev-Yochay, G., M. Raz, et al. (2004). "Nasopharyngeal carriage of Streptococcus pneumoniae by adults and children in community and family settings." *Clin Infect Dis* **38**(5): 632-639.
- Rello, J., M. Lujan, et al. (2010). "Why mortality is increased in health-care-associated pneumonia: lessons from pneumococcal bacteremic pneumonia." *Chest* **137**(5): 1138-1144.

- Ricci, C., V. Pastukh, et al. (2008). "Mitochondrial DNA damage triggers mitochondrial-superoxide generation and apoptosis." *Am J Physiol Cell Physiol* **294**(2): C413-422.
- Richman, D. D., T. Wrin, et al. (2003). "Rapid evolution of the neutralizing antibody response to HIV type 1 infection." *Proc Natl Acad Sci U S A* **100**(7): 4144-4149.
- Rijkers, G. T., L. A. Sanders, et al. (1993). "Anti-capsular polysaccharide antibody deficiency states." *Immunodeficiency* **5**(1): 1-21.
- Ring, A., J. N. Weiser, et al. (1998). "Pneumococcal trafficking across the blood-brain barrier. Molecular analysis of a novel bidirectional pathway." *J Clin Invest* **102**(2): 347-360.
- Robbins, G. K., J. G. Spritzler, et al. (2009). "Incomplete reconstitution of T cell subsets on combination antiretroviral therapy in the AIDS Clinical Trials Group protocol 384." *Clin Infect Dis* **48**(3): 350-361.
- Robinson, K. A., W. Baughman, et al. (2001). "Epidemiology of invasive Streptococcus pneumoniae infections in the United States, 1995-1998: Opportunities for prevention in the conjugate vaccine era." *JAMA* **285**(13): 1729-1735.
- Robinson, K. M., M. S. Janes, et al. (2008). "The selective detection of mitochondrial superoxide by live cell imaging." *Nat Protoc* **3**(6): 941-947.
- Rodriguez-Barradas, M. C., J. Goulet, et al. (2008). "Impact of pneumococcal vaccination on the incidence of pneumonia by HIV infection status among patients enrolled in the Veterans Aging Cohort 5-Site Study." *Clin Infect Dis* **46**(7): 1093-1100.
- Rodriguez-Barradas, M. C., R. A. Tharapel, et al. (1997). "Colonization by Streptococcus pneumoniae among human immunodeficiency virus-infected adults: prevalence of antibiotic resistance, impact of immunization, and characterization by polymerase chain reaction with BOX primers of isolates from persistent S. pneumoniae carriers." *J Infect Dis* **175**(3): 590-597.
- Roederer, M., J. G. Dubs, et al. (1995). "CD8 naive T cell counts decrease progressively in HIV-infected adults." *J Clin Invest* **95**(5): 2061-2066.
- Ronsholt, F. F., H. Ullum, et al. (2012). "T-cell subset distribution in HIV-1-infected patients after 12 years of treatment-induced viremic suppression." *J Acquir Immune Defic Syndr* **61**(3): 270-278.
- Rosenberg, E. S., A. M. Caliendo, et al. (1999). "Acute HIV infection among patients tested for mononucleosis." *N Engl J Med* **340**(12): 969.
- Rudd, C. E., A. Taylor, et al. (2009). "CD28 and CTLA-4 coreceptor expression and signal transduction." *Immunol Rev* **229**(1): 12-26.
- Rychert, J., D. Strick, et al. (2010). "Detection of HIV gp120 in plasma during early HIV infection is associated with increased proinflammatory and immunoregulatory cytokines." *AIDS Res Hum Retroviruses* **26**(10): 1139-1145.
- Said, E. A., F. P. Dupuy, et al. (2010). "Programmed death-1-induced interleukin-10 production by monocytes impairs CD4+ T cell activation during HIV infection." *Nat Med* **16**(4): 452-459.
- Salmen, S., G. Teran, et al. (2004). "Increased Fas-mediated apoptosis in polymorphonuclear cells from HIV-infected patients." *Clin Exp Immunol* **137**(1): 166-172.
- Sanchez, C. J., C. A. Hinojosa, et al. (2011). "Changes in capsular serotype alter the surface exposure of pneumococcal adhesins and impact virulence." *PLoS One* **6**(10): e26587.
- Savarino, A., F. Bottarel, et al. (2000). "Role of CD38 in HIV-1 infection: an epiphenomenon of T-cell activation or an active player in virus/host interactions?" *AIDS* **14**(9): 1079-1089.
- Schneider, E., S. Whitmore, et al. (2008). "Revised surveillance case definitions for HIV infection among adults, adolescents, and children aged <18 months and for HIV infection and AIDS among children aged 18 months to <13 years--United States, 2008." *MMWR Recomm Rep* **57**(RR-10): 1-12.
- Selwyn, P. A., A. R. Feingold, et al. (1988). "Increased risk of bacterial pneumonia in HIV-infected intravenous drug users without AIDS." *AIDS* **2**(4): 267-272.

- Sharma, G., N. A. Hanania, et al. (2009). "The aging immune system and its relationship to the development of chronic obstructive pulmonary disease." *Proc Am Thorac Soc* **6**(7): 573-580.
- Sharova, N., C. Swingler, et al. (2005). "Macrophages archive HIV-1 virions for dissemination in trans." *EMBO J* **24**(13): 2481-2489.
- Shaykhiev, R., A. Krause, et al. (2009). "Smoking-dependent reprogramming of alveolar macrophage polarization: implication for pathogenesis of chronic obstructive pulmonary disease." *J Immunol* **183**(4): 2867-2883.
- Sheehy, A. M., N. C. Gaddis, et al. (2002). "Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein." *Nature* **418**(6898): 646-650.
- Shen, X. and G. D. Tomaras (2011). "Alterations of the B-cell response by HIV-1 replication." *Curr HIV/AIDS Rep* **8**(1): 23-30.
- Shirai, A., M. Cosentino, et al. (1992). "Human immunodeficiency virus infection induces both polyclonal and virus-specific B cell activation." *J Clin Invest* **89**(2): 561-566.
- Sierra-Madero, J. G., Z. Toossi, et al. (1994). "Relationship between load of virus in alveolar macrophages from human immunodeficiency virus type 1-infected persons, production of cytokines, and clinical status." *J Infect Dis* **169**(1): 18-27.
- Silvestri, G., D. L. Sodora, et al. (2003). "Nonpathogenic SIV infection of sooty mangabeys is characterized by limited bystander immunopathology despite chronic high-level viremia." *Immunity* **18**(3): 441-452.
- Sloan, R. D. and M. A. Wainberg (2011). "The role of unintegrated DNA in HIV infection." *Retrovirology* **8**: 52.
- Smith, P. D., C. H. Fox, et al. (1994). "Quantitative analysis of mononuclear cells expressing human immunodeficiency virus type 1 RNA in esophageal mucosa." *J Exp Med* **180**(4): 1541-1546.
- Sogaard, O. S., N. Lohse, et al. (2008). "Hospitalization for pneumonia among individuals with and without HIV infection, 1995-2007: a Danish population-based, nationwide cohort study." *Clin Infect Dis* **47**(10): 1345-1353.
- Spear, G. T., C. Y. Ou, et al. (1990). "Analysis of lymphocytes, monocytes, and neutrophils from human immunodeficiency virus (HIV)-infected persons for HIV DNA." *J Infect Dis* **162**(6): 1239-1244.
- Srivastava, A., P. Henneke, et al. (2005). "The apoptotic response to pneumolysin is Toll-like receptor 4 dependent and protects against pneumococcal disease." *Infect Immun* **73**(10): 6479-6487.
- Staal, F. J., S. W. Ela, et al. (1992). "Glutathione deficiency and human immunodeficiency virus infection." *Lancet* **339**(8798): 909-912.
- Steinbock, M. C., B. S. Auerbach, et al. (1991). "Antibody responses to Haemophilus influenzae type B vaccines in men with human immunodeficiency virus infection." *N Engl J Med* **325**(26): 1837-1842.
- Sterne, J. A., M. A. Hernan, et al. (2005). "Long-term effectiveness of potent antiretroviral therapy in preventing AIDS and death: a prospective cohort study." *Lancet* **366**(9483): 378-384.
- Stoler, M. H., T. A. Eskin, et al. (1986). "Human T-cell lymphotropic virus type III infection of the central nervous system. A preliminary in situ analysis." *JAMA* **256**(17): 2360-2364.
- Stone, B., D. Dockrell, et al. (2010). "HIV and bone disease." *Arch Biochem Biophys* **503**(1): 66-77.
- Stremlau, M., C. M. Owens, et al. (2004). "The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys." *Nature* **427**(6977): 848-853.
- Strober, W. (2001). "Trypan blue exclusion test of cell viability." *Curr Protoc Immunol* **Appendix 3**: Appendix 3B.

- Sundstrom, C. and K. Nilsson (1976). "Establishment and characterization of a human histiocytic lymphoma cell line (U-937)." *Int J Cancer* **17**(5): 565-577.
- Suresh, D. R., V. Annam, et al. (2009). "Total antioxidant capacity--a novel early bio-chemical marker of oxidative stress in HIV infected individuals." *J Biomed Sci* **16**: 61.
- Swingler, S., B. Brichacek, et al. (2003). "HIV-1 Nef intersects the macrophage CD40L signalling pathway to promote resting-cell infection." *Nature* **424**(6945): 213-219.
- Swingler, S., A. M. Mann, et al. (2007). "Apoptotic killing of HIV-1-infected macrophages is subverted by the viral envelope glycoprotein." *PLoS Pathog* **3**(9): 1281-1290.
- Tachado, S. D., J. Zhang, et al. (2005). "HIV impairs TNF-alpha release in response to Toll-like receptor 4 stimulation in human macrophages in vitro." *Am J Respir Cell Mol Biol* **33**(6): 610-621.
- Takahashi, H., K. Oishi, et al. (2003). "Decreased serum opsonic activity against Streptococcus pneumoniae in human immunodeficiency virus-infected Ugandan adults." *Clin Infect Dis* **37**(11): 1534-1540.
- Takashima, K., K. Tateda, et al. (1997). "Role of tumor necrosis factor alpha in pathogenesis of pneumococcal pneumonia in mice." *Infect Immun* **65**(1): 257-260.
- Teixeira, L., H. Valdez, et al. (2001). "Poor CD4 T cell restoration after suppression of HIV-1 replication may reflect lower thymic function." *AIDS* **15**(14): 1749-1756.
- Teshale, E. H., D. Hanson, et al. (2008). "Effectiveness of 23-valent polysaccharide pneumococcal vaccine on pneumonia in HIV-infected adults in the United States, 1998-2003." *Vaccine* **26**(46): 5830-5834.
- Tesoriero, J. M., S. M. Gieryic, et al. (2010). "Smoking among HIV positive New Yorkers: prevalence, frequency, and opportunities for cessation." *AIDS Behav* **14**(4): 824-835.
- Thompson, C. B. (1995). "Apoptosis in the pathogenesis and treatment of disease." *Science* **267**(5203): 1456-1462.
- Thornberry, N. A. and Y. Lazebnik (1998). "Caspases: enemies within." *Science* **281**(5381): 1312-1316.
- Tilling, R., S. Kinloch, et al. (2002). "Parallel decline of CD8+/CD38++ T cells and viraemia in response to quadruple highly active antiretroviral therapy in primary HIV infection." *AIDS* **16**(4): 589-596.
- Titanji, K., A. De Milito, et al. (2006). "Loss of memory B cells impairs maintenance of long-term serologic memory during HIV-1 infection." *Blood* **108**(5): 1580-1587.
- Tocheva, A. S., J. M. Jefferies, et al. (2011). "Declining serotype coverage of new pneumococcal conjugate vaccines relating to the carriage of Streptococcus pneumoniae in young children." *Vaccine* **29**(26): 4400-4404.
- Tomlinson, G. S., H. Booth, et al. (2012). "Adherent human alveolar macrophages exhibit a transient pro-inflammatory profile that confounds responses to innate immune stimulation." *PLoS One* **7**(6): e40348.
- Towbin, H., T. Staehelin, et al. (1979). "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications." *Proc Natl Acad Sci U S A* **76**(9): 4350-4354.
- Tsang, J., B. M. Chain, et al. (2009). "HIV-1 infection of macrophages is dependent on evasion of innate immune cellular activation." *AIDS*.
- Twigg, H. L., 3rd, G. K. Iwamoto, et al. (1992). "Role of cytokines in alveolar macrophage accessory cell function in HIV-infected individuals." *J Immunol* **149**(4): 1462-1469.
- Twigg, H. L., 3rd, M. F. Lipscomb, et al. (1989). "Enhanced accessory cell function by alveolar macrophages from patients infected with the human immunodeficiency virus: potential role for depletion of CD4+ cells in the lung." *Am J Respir Cell Mol Biol* **1**(5): 391-400.
- Twigg, H. L., 3rd, B. A. Spain, et al. (1999). "Production of interferon-gamma by lung lymphocytes in HIV-infected individuals." *Am J Physiol* **276**(2 Pt 1): L256-262.

- Twigg, H. L., C. T. Schnizlein-Bick, et al. (2010). "Measurement of antiretroviral drugs in the lungs of HIV-infected patients." *HIV Ther* **4**(2): 247-251.
- Twigg, H. L., D. M. Soliman, et al. (1999). "Lymphocytic alveolitis, bronchoalveolar lavage viral load, and outcome in human immunodeficiency virus infection." *Am J Respir Crit Care Med* **159**(5 Pt 1): 1439-1444.
- Twigg, H. L., M. Weiden, et al. (2008). "Effect of highly active antiretroviral therapy on viral burden in the lungs of HIV-infected subjects." *J Infect Dis* **197**(1): 109-116.
- UNAIDS. (2012). "GLOBAL FACT SHEET." Retrieved 15 October 2013, 2013, from http://www.unaids.org/en/media/unaids/contentassets/documents/epidemiology/2012/gr2012/20121120_FactSheet_Global_en.pdf.
- Van den Bergh, R., E. Florence, et al. (2010). "Transcriptome analysis of monocyte-HIV interactions." *Retrovirology* **7**: 53.
- van oud Alblas, A. B. and R. van Furth (1979). "Origin, Kinetics, and characteristics of pulmonary macrophages in the normal steady state." *J Exp Med* **149**(6): 1504-1518.
- Vaux, D. L., G. Haeccker, et al. (1994). "An evolutionary perspective on apoptosis." *Cell* **76**(5): 777-779.
- Vazquez, N., T. Greenwell-Wild, et al. (2005). "Human immunodeficiency virus type 1-induced macrophage gene expression includes the p21 gene, a target for viral regulation." *J Virol* **79**(7): 4479-4491.
- Verhoven, B., R. A. Schlegel, et al. (1995). "Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes." *J Exp Med* **182**(5): 1597-1601.
- Vieira, H. L., D. Haouzi, et al. (2000). "Permeabilization of the mitochondrial inner membrane during apoptosis: impact of the adenine nucleotide translocator." *Cell Death Differ* **7**(12): 1146-1154.
- Vigerust, D. J., B. S. Egan, et al. (2005). "HIV-1 Nef mediates post-translational down-regulation and redistribution of the mannose receptor." *J Leukoc Biol* **77**(4): 522-534.
- Vilhardt, F., O. Plastre, et al. (2002). "The HIV-1 Nef protein and phagocyte NADPH oxidase activation." *J Biol Chem* **277**(44): 42136-42143.
- Vlahakis, S. R., S. A. Bennett, et al. (2007). "HIV protease inhibitors modulate apoptosis signaling in vitro and in vivo." *Apoptosis* **12**(5): 969-977.
- Wagner, R. P., S. M. Levitz, et al. (1992). "HIV-1 envelope protein (gp120) inhibits the activity of human bronchoalveolar macrophages against *Cryptococcus neoformans*." *Am Rev Respir Dis* **146**(6): 1434-1438.
- Wang, K. K., A. Posner, et al. (1996). "Total protein extraction from cultured cells for use in electrophoresis and western blotting." *Biotechniques* **20**(4): 662-668.
- Watera, C., J. Nakiyingi, et al. (2004). "23-Valent pneumococcal polysaccharide vaccine in HIV-infected Ugandan adults: 6-year follow-up of a clinical trial cohort." *AIDS* **18**(8): 1210-1213.
- Weaver, J. G., A. Tarze, et al. (2005). "Inhibition of adenine nucleotide translocator pore function and protection against apoptosis in vivo by an HIV protease inhibitor." *J Clin Invest* **115**(7): 1828-1838.
- Weber, S. E., H. Tian, et al. (2011). "CD8+ cells enhance resistance to pulmonary serotype 3 *Streptococcus pneumoniae* infection in mice." *J Immunol* **186**(1): 432-442.
- Wedderburn, L. R., A. Patel, et al. (2001). "The developing human immune system: T-cell receptor repertoire of children and young adults shows a wide discrepancy in the frequency of persistent oligoclonal T-cell expansions." *Immunology* **102**(3): 301-309.
- Weinberger, D. M., K. Trzcinski, et al. (2009). "Pneumococcal capsular polysaccharide structure predicts serotype prevalence." *PLoS Pathog* **5**(6): e1000476.

- Weiser, J. N., R. Austrian, et al. (1994). "Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization." *Infect Immun* **62**(6): 2582-2589.
- Weiser, J. N., D. Bae, et al. (2003). "Antibody-enhanced pneumococcal adherence requires IgA1 protease." *Proc Natl Acad Sci U S A* **100**(7): 4215-4220.
- West, A. P., I. E. Brodsky, et al. (2011). "TLR signalling augments macrophage bactericidal activity through mitochondrial ROS." *Nature* **472**(7344): 476-480.
- Westendorp, M. O., R. Frank, et al. (1995). "Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120." *Nature* **375**(6531): 497-500.
- Whetter, L. E., I. C. Ojukwu, et al. (1999). "Pathogenesis of simian immunodeficiency virus infection." *J Gen Virol* **80 (Pt 7)**: 1557-1568.
- WHO. (2005). "Pneumococcal disease." Retrieved 29 June 2013, from <http://www.who.int/ith/diseases/pneumococcal/en/>
- WHO (2007). WHO case definitions of HIV for surveillance and revised clinical staging and immunological classification of HIV-related disease in adults and children., World Health Organization.
- WHO. (2013, July 2013). "Factsheet 310: The top 10 causes of death." Retrieved October, 2013, from www.who.int/mediacentre/factsheets/fs310/en/index.html.
- WHO. (2013). "HIV Fact File." Retrieved 15 October 2013, 2013, from <http://www.who.int/features/factfiles/hiv/facts/en/index1.html>.
- Wileman, T. E., M. R. Lennartz, et al. (1986). "Identification of the macrophage mannose receptor as a 175-kDa membrane protein." *Proc Natl Acad Sci U S A* **83**(8): 2501-2505.
- Williams, I., D. Churchill, et al. (2012). "British HIV Association guidelines for the treatment of HIV-1-positive adults with antiretroviral therapy 2012." *HIV Med* **13 Suppl 2**: 1-85.
- Williams, K. C. and T. H. Burdo (2009). "HIV and SIV infection: the role of cellular restriction and immune responses in viral replication and pathogenesis." *APMIS* **117**(5-6): 400-412.
- Witt, D. J., D. E. Craven, et al. (1987). "Bacterial infections in adult patients with the acquired immune deficiency syndrome (AIDS) and AIDS-related complex." *Am J Med* **82**(5): 900-906.
- Witzenrath, M., F. Pache, et al. (2011). "The NLRP3 inflammasome is differentially activated by pneumolysin variants and contributes to host defense in pneumococcal pneumonia." *J Immunol* **187**(1): 434-440.
- Wolf, D., V. Witte, et al. (2001). "HIV-1 Nef associated PAK and PI3-kinases stimulate Akt-independent Bad-phosphorylation to induce anti-apoptotic signals." *Nat Med* **7**(11): 1217-1224.
- Wu, L., C. L. Gabriel, et al. (2009). "Invariant natural killer T cells: innate-like T cells with potent immunomodulatory activities." *Tissue Antigens* **73**(6): 535-545.
- Yoshimura, A., E. Lien, et al. (1999). "Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2." *J Immunol* **163**(1): 1-5.
- Yukl, S. A., S. Gianella, et al. (2010). "Differences in HIV burden and immune activation within the gut of HIV-positive patients receiving suppressive antiretroviral therapy." *J Infect Dis* **202**(10): 1553-1561.
- Yuste, J., M. Botto, et al. (2005). "Additive inhibition of complement deposition by pneumolysin and PspA facilitates *Streptococcus pneumoniae* septicemia." *J Immunol* **175**(3): 1813-1819.
- Zhang, J., X. Xu, et al. (2004). "Activation-induced cell death in T cells and autoimmunity." *Cell Mol Immunol* **1**(3): 186-192.
- Zhang, J. R., K. E. Mostov, et al. (2000). "The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells." *Cell* **102**(6): 827-837.

- Zhang, M., X. Li, et al. (2002). "Bcl-2 upregulation by HIV-1 Tat during infection of primary human macrophages in culture." J Biomed Sci **9**(2): 133-139.
- Zhang, Q., L. Bagrade, et al. (2007). "Low CD4 T cell immunity to pneumolysin is associated with nasopharyngeal carriage of pneumococci in children." J Infect Dis **195**(8): 1194-1202.
- Zhang, Z., T. B. Clarke, et al. (2009). "Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice." J Clin Invest **119**(7): 1899-1909.
- Zhou, Y., H. Zhang, et al. (2005). "Kinetics of human immunodeficiency virus type 1 decay following entry into resting CD4+ T cells." J Virol **79**(4): 2199-2210.
- Zhu, D. M., J. Shi, et al. (2011). "HIV infection enhances TRAIL-induced cell death in macrophage by down-regulating decoy receptor expression and generation of reactive oxygen species." PLoS One **6**(4): e18291.

Appendix 1. Buffers

Phosphate Buffered Saline (PBS)

1 x tablet (Oxoid) per 100 mL glass distilled water (dH₂O), autoclaved.

Tris buffered saline (TBS, 10 x concentration)

	volume / weight
1M Tris –HCl pH8.0	100 mL
Sodium chloride (NaCl, Fisher)	97.3 g
dH ₂ O	900 mL

TBS-Tween

10x TBS 1000mL + Tween (Sigma-Aldrich) 5 mL

Paraformaldehyde

Made up as 4% - 0.4g PFA (BDH) in 100 mL dH₂O heated to 60°C in a water bath, then stored at -20°C in 10 mL aliquots. Thawed and diluted in PBS to desired concentration on day of use.

Polyacrylamide gels

	Stacking gel	Resolving gel
dH ₂ O	3 mL	6.6 mL
40% acrylamide (Bio Rad)	620 µL	4.5 mL
0.5M Tris pH 6.8 (Bio Rad)	1260 µL	-
1.5M Tris pH 8.8 (Bio Rad)	-	3.8 mL
20% sodium dodecyl sulphate (SDS, Fisher)	25 µL	75 µL
Ammonium persulphate (APS, Sigma)	50 µL	150 µL
Tetramethylethylenediamine (TEMED, Sigma-Aldrich)	5 µL	6 µL

Running Buffer (10x)

	volume / weight
Tris-Base (Fisher)	30.3 g
Glycine (Fisher)	190 g
20% SDS	50 mL
dH ₂ O	Up to 1000 mL

Towbin transfer buffer

	volume / weight
Tris-Base (Fisher)	2.9 g
Glycine (Fisher)	1.45 g
20% SDS	925 µL
Methanol	100 mL
dH ₂ O	Up to 500 mL

SDS lysis buffer (Tris-EDTA-SDS)

	Volume
1M Tris-HCl pH 7.4 (Fluka)	2 mL
1M NaCl	15 mL
0.5M EDTA (Sigma)	1 mL
0.5M EGTA (Sigma)	1 mL
20% SDS	5 mL
H ₂ O	72ml

25% weight for weight sucrose solution

	Weight
Sucrose (Sigma)	12.5 g
PBS	47.5 g

FACS buffer

	Volume
30% Bovine Serum Albumin (BSA, First Link)	150 μ L
PBS	50 mL

Galactosidase substrate solution (100 x solution)

	weight / volume	Final concentration
Magnesium chloride (MgCl, Sigma)	0.0952115 g	1 mM
Potassium-ferricyanide ($K_3[Fe(CN)_6]$ Fluka)	0.98772 g	3 mM
Potassium-ferrocyanide ($K_4[Fe(CN)_6]$ Fluka)	1.26717 g	3 mM
PBS	10 mL	

Appendix 2. Publications & Awards

Peer reviewed articles

P Collini, M Noursadeghi, I Sabroe, R F Miller, D H Dockrell

Monocyte and macrophage dysfunction as a cause of HIV-1 induced dysfunction of innate immunity

Current Molecular Medicine 2010 Nov 1;10(8):727-40

PubMed PMID: 20937022

Presented abstracts

Invasive pneumococcal disease among HIV-1 seropositive individuals in the era of highly active antiretroviral therapy: does HIV-1 impair the macrophage host response to pneumococci?

PJ Collini, DH Dockrell.

Poster 55 Spring Meeting for Clinician Scientists in Training, Academy of Medical Sciences, London, February 27th 2013

HIV-1 alters macrophage apoptosis in response to *Streptococcus pneumoniae*.

P Collini, R Read, D Dockrell

Poster 57 18th Annual Conference Of The British HIV Association (BHIVA)

18-20 April 2012, Birmingham, UK

HIV-1 modulates macrophage death responses to *S. pneumoniae* infection.

P Collini, D Dockrell

Poster 455 49th Annual Meeting of Infectious Diseases Society of America (IDSA), 20-23

October 2011, Boston, USA

HIV-1 is associated with reduced macrophage apoptosis in response to *S. pneumoniae*.

P Collini, R Read, D Dockrell

Oral presentation. British Infection Association (BIA) 14th Annual Scientific Meeting, 20 May 2011, London, UK

Awards

1st Prize Oral presentation, Medical School Research Meeting 2013, University of Sheffield:

Invasive pneumococcal disease among HIV-1 seropositive individuals in the era of highly active antiretroviral therapy: does HIV-1 impair the macrophage host response to pneumococci?

BHIVA/MSD CROI 2013 conference Travel Scholarship

IDSA/HIVMA International HIV Travel Grants for the IDSA 2011

MRC Clinical Training Fellowship G0901963 2010-2013