The impact of HIV-1 disease and its treatment on Mycobacterium tuberculosis-specific immunity

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

by

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ABSTRACT

Human immunodeficiency virus-1 (HIV-1) and tuberculosis (TB) are significant global health issues today and the immunological correlates of protection to both diseases remain poorly defined. HIV-1 is the greatest risk factor for the development of TB and HIV-associated TB contributes significantly to the global TB burden, particularly in sub-Saharan Africa. The host T cell response to Mycobacterium tuberculosis (Mtb) is critical for control and is weakened in HIV-1 disease. However, the extent and precise mechanisms remain incompletely elucidated. Antiretroviral therapy (ART) for HIV-1 reduces TB risk in treated individuals significantly, although not to levels seen in HIV-uninfected individuals.

This thesis studies HIV- and Mtb-specific T cell responses in 2 cohorts of individuals from a community with high HIV-1 and TB prevalence in Bloemfontein, South Africa. An analysis of HIV-specific CD8 T cell responses in Chapter 4, confirms the superior role of HIV-1 Gag-specific CD8 responses in controlling HIV-1 viraemia and demonstrates that the loss of such responses contributes to HIV-1 disease progression and likely susceptibility to opportunistic infection.

I investigated the impact of HIV-1 infection on the Mtb-specific T cell response through a cross-sectional comparison of T cell responses in HIV-infected and HIV-uninfected individuals (Chapters 5 and 6). HIV-infected individuals had a significant depletion of both Th_1 and Th_{17} CD4 responses to Mtb-specific antigens as well inhibition of Mtb-specific CD8 T cell responses, in comparison to those uninfected with HIV-1. PPD- and Rv2031c-specific responses were particularly reduced in HIV-infected individuals.

Over a 12 month period of therapy, ART partially restored the Mtb-specific CD4 T cell response (Chapters 5 and 7). This effect was greater for Th_1 than Th_{17} responses and had no detectable effect on the Mtb-specific CD8 response. However, despite some evident restoration, there remains a significant quantitative deficit in individuals on ART that is likely to contribute to persistent elevated TB risk.

Overall, these data contribute to a better understanding of the mechanisms of susceptibility to TB during HIV-1 disease and ART, as well as of the correlates of protective immune responses to both pathogens.

ACKNOWLEDGEMENTS

First and foremost, this research would never have been possible had it not been for the willingness of all the study participants attending the health care clinics in Bloemfontein to enrol in and engage with our research studies. I am particularly grateful to them. A particular thanks should also be extended to all the clinical staff that helped to foster a flexible and collaborative environment that was essential to the successful implementation of this project.

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CONTRIBUTIONS TO THIS THESIS

All of the studies detailed in this thesis relied heavily upon the support from our research team in Oxford and in Bloemfontein. Dr John Frater and Professor Rodney Phillips supervised the project from the very beginning and were critically involved in the planning, funding and execution of all the research involved. Professor Helen McShane and Dr Iman Satti provided a valuable sounding board for discussion of relevant aspects of Mtb-specific immunology whilst Dr Nickie Goedhals and Dr Cloete van Vuuren managed all research activities in Bloemfontein. A list of collaborators is provided and the specific contributions to aspects of the thesis are detailed below.

Collaborators:

- **University of the Free State, Bloemfontein, South Africa.**

Dr Nickie Goedhals, Dr Cloete van Vuuren, Sr Boniswa Malefane, Professor Felicity Burt and Mitta Kesentseng

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Prof Helen McShane, Dr Iman Satti and Rachel Tanner.

Specific contributions:

All chapters

CD4 T cell counts and HIV-1 viral load measurements: CD4 T cell counts and HIV-1 viral load testing were carried out by the South African National Health Laboratory Service (NHLS) at Pelonomi and Universitas Hospitals as part of routine patient care. Where viral load data were not required as per South African ART treatment guidelines, Dr Nickie Goedhals performed HIV-1 quantitation assays specifically for our study purposes using assay kits generously supplied by Abbott Laboratories, South Africa Ltd.

Statistical analyses: Dr Jacob Hurst contributed his statistical and analytical advice to analyses in all chapters of this thesis. His assistance in establishing a database for analysis of the complex Boolean data generated using multiparameter flow cytometry was particularly helpful.

Chapters 4 and 5

IFNγ ELISPOT assays: HIV- and Mtb-specific IFNγ ELISPOT assays formed the basis of studies described in Chapters 4 and 5. HIV-1 Clade C peptides were kindly supplied by Professor Philip Goulder and the ESAT-6 and CFP-10 recombinant proteins supplied at cost price through a MTA with Lionex GmbH, Germany. Mathew Jones and Jodi Meyerowitz contributed significantly to the running of the assays at the baseline and 12 month time points respectively.

MIG/IP10 qPCR assays: Professor Paul Klenerman and Dr Narayan Ramamurthy provided valuable advice and assistance in developing the MIG/IP10 qPCR assay. Robert Hohan, a 3rd year biomedical sciences FHS student, completed the bulk of the assays for samples collected at baseline and 6 months and also contributed valuably to the development of the assay.

Chapters 6 and 7

WB ICS assays: These chapters include cross-sectional and longitudinal data obtained through a WB ICS assay that was adapted from a protocol edited by Dr Iman Satti. She advised us on techniques to adapt the assay to conditions in Bloemfontein as well as

on how to complete the high-throughput analysis of the many flow cytometry samples in Oxford. Antibody panels were adjusted from those used by Dr Satti at the Jenner Institute, Oxford University. Dr Chris Wilberg and Dr James Ussher were extremely generous with their time and expertise in assisting us with optimisation of the flow cytometry protocol on the MACSQuant flow cytometer at the Peter Medawar Building. Jodi Meyerowitz spent countless hours running the flow cytometry samples and thereby played an extremely important role in generating the data in these chapters. Professor Philip Goulder generously supplied the Gag OPAL peptide pool.

For Mom and Dad

ABBREVIATIONS

°C	Degrees Celsius
3TC	Lamivudine
ACD	Acid citrate dextrose
AIDS	Acquired immune deficiency syndrome
ART	Antiretroviral therapy
BL	Baseline
BMI	Body mass index
BCG	Bacillus Calmette-Guerin
CA	Capsid
CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
cDNA	Complementary DNA
CFP-10	Cultured filtrate protein 10kDa
CI	Confidence interval
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CTL	Cytotoxic T lymphocyte
CXCR4	C-X-C chemokine receptor type 4
DC	Dendritic cell
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
EC	ESAT-6 and CFP-10 recombinant protein
EFV	Efavirenz
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
ESAT-6	Early secreted antigenic target 6kDa
Env	Envelope protein
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FSC	Forward scatter
g	Gram
Gag	Group-specific antigen
GALT	Gut-associated lymphoid tissue
HAART	Highly active antiretroviral therapy
Gp120	120kDa Envelope Glycoprotein Surface Subunit
Gp41	41kDa Envelope Glycoprotein Transmembrane Subunit
HIV-1	Human Immunodeficiency Virus type 1
HLA	Human Leukocyte Antigen
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
ICS	Intracellular cytokine staining
IFNγ	Interferon gamma
IL-2	Interleukin 2
IL-17	Interleukin 17
INH	Isoniazid

INT	Integrase
IP-10	Interferon gamma inducible protein-10
IPT	Isoniazid preventative therapy
IQR	Interquartile range
kDa	Kilo Dalton
L	Litre
LTBI	Latent tuberculosis infection
MA	Matrix protein
MAC	Mycobacterium avium complex
µg	Microgram
Mg	Milligram
µL	Microlitre
mL	Millilitre
µM	Micromolar
MIG	Monokine induced by interferon gamma
Mtb	Mycobacterium tuberculosis
mRNA	Messenger RNA
NaCl	Sodium chloride
NC	Nucleocapsid
Nef	Negative regulatory factor
NHDF	Normal Human Dermal Fibroblasts
NHLS	National Health Laboratory Service
NRTI	Nucleoside reverse transcriptase inhibitor
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
OI	Opportunistic infection
OLP	Overlapping peptide
OR	Odds ratio
Pol	Polymerase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PIC	Pre-integration complex
PPD	Purified protein derivative
PR	Protease
P-TEFb	Positive transcription elongation factor b
pVL	Plasma viral load
qPCR	Quantitative polymerase chain reaction
Rev	Regulator of Expression of Virion Proteins
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institution medium
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
SFC	Spot forming cell
SPARTAC	Short Pulse Antiretroviral Therapy at HIV Seroconversion
SSC	Side-scatter

Tat	Transactivator of transcription
TB	Tuberculosis
TDF	Tenofovir
Th_1	CD4 T helper Type 1 response
Th_{17}	CD4 T helper Type 17 response
Tm	Melting temperature
TNF-α	Tumour necrosis factor alpha
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
WB ICS	Whole blood ICS

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The impact of HIV-1 disease and its treatment on Mycobacterium tuberculosis-specific immunity

1 INTRODUCTION

Mycobacterium tuberculosis (Mtb) is an ancient human pathogen that emerged, and accompanied modern humans as they migrated out of Africa 70000 years ago (Comas et al. 2013). Mtb is the main causative agent of tuberculosis disease (TB) and there have been two particular periods in recent history during which TB has had especially devastating consequences for the human race. The first was between the 17th and 19th centuries, when one in five adults in Europe and North America died of TB, whilst the second has been more recent during the past 30 years within communities ravaged by the human immunodeficiency type 1 virus (HIV-1)(Wilson 2005; Harries et al. 2010). HIV-1 however, unlike TB, traces its relatively recent origin to zoonotic transmission from African apes in southern Cameroon less than a century ago (Keele et al. 2006).

Today TB and HIV-1 infection remain the two leading infectious causes of mortality worldwide despite the availability of effective chemotherapy for both diseases (WHO 2014b). Medical interventions against TB and HIV-1 are challenged by slow changes in the social conditions driving their spread, increasing levels of drug resistance, a poor understanding of the immunological correlates of protection and the current lack of effective vaccines. Separate to these shared challenges, HIV-1 disease and TB form a particularly devastating combination for the host during co-infection. HIV-infected individuals are more than 20 times more likely to develop active TB than individuals without HIV and, as a result, the highest TB incidence is found in areas with high HIV-1 prevalence (WHO 2013b).

The development of antiretroviral therapy (ART) heralded a huge breakthrough in reducing HIV-related morbidity and mortality, particularly within the context of TB (Lawn, Badri, et al. 2005). Although ART significantly reduces TB risk in HIV-infected individuals, that risk remains higher than in HIV-uninfected individuals despite long term ART (Gupta et al. 2012).

This thesis explores the host response to HIV-1 and Mtb, during HIV-1 disease and its treatment, within a community that is particularly affected by the HIV/TB co-epidemic in Bloemfontein, South Africa.

1.1 HIV-1

1.1.1 The HIV/AIDS epidemic

The Acquired Immune Deficiency Syndrome (AIDS) is one of the most devastating infectious diseases in human history. HIV-1, is its causative agent and despite its discovery in the early-1980's it continues to be a major health problem, having claimed approximately 39 million lives and infected 75 million people globally (Barré-Sinoussi et al. 1983; Gallo et al. 1984; WHO 2014a). In 2013 there were an estimated 2.1 million new HIV-1 infections and 1.5 million deaths attributed to HIV-related causes (WHO 2014a). Although a problem worldwide, sub-Saharan Africa is the epicentre of the HIV pandemic, accounting for almost 70% of those infected and only 12% of the world's population. Within South Africa, the worst affected country in the region, an estimated 6.3 million people are currently living with HIV including 20% of all adults aged 15-49 years (UNAIDS 2013b). The Free State Province of South Africa,

where our study is based, has the third highest HIV prevalence in South Africa (Fig. 1.1)(South African National Department of Health 2013a).

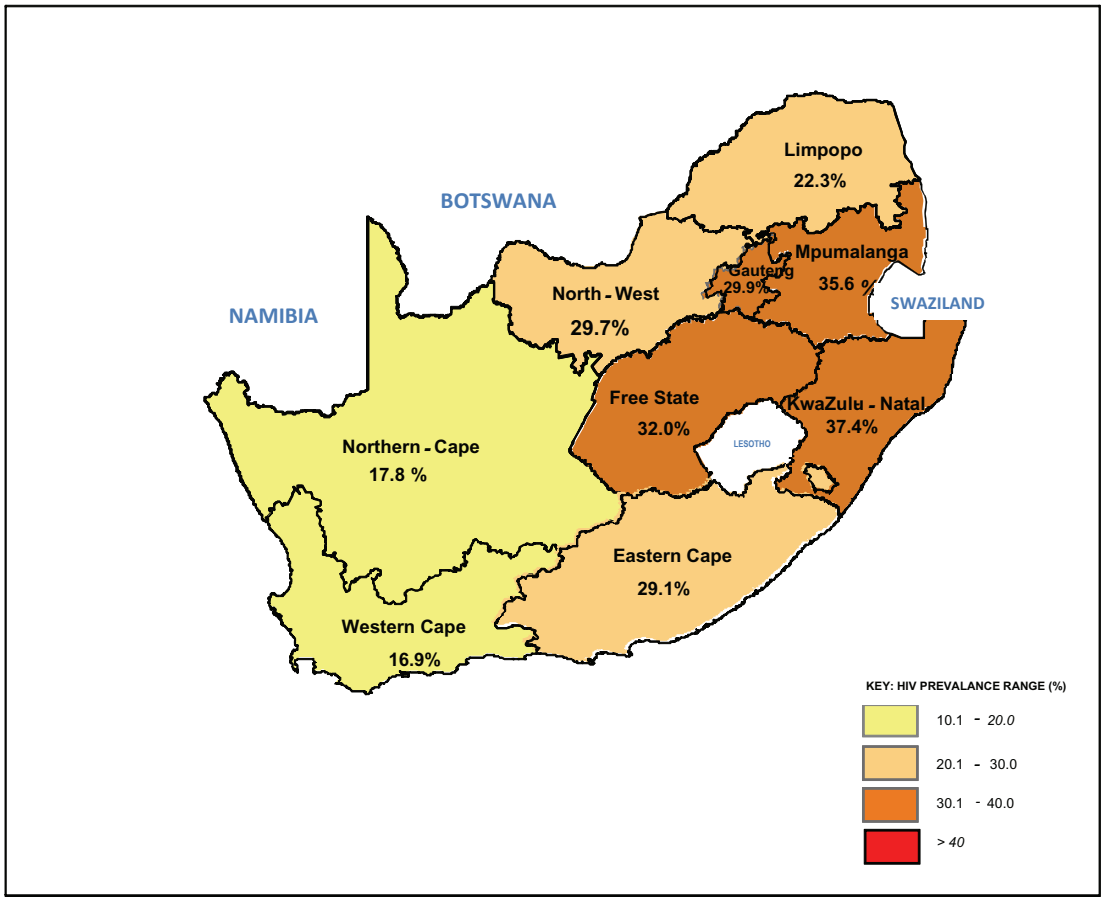


Figure 1.1 HIV-1 prevalence by province, South Africa, 2012.

Image source: The 2012 National Antenatal Sentinel HIV and Herpes Simplex Type-2 Prevalence Survey in South Africa (South African National Department of Health 2013a)

The disproportionate prevalence of HIV-1 infection in South Africa has its origin in the social, economic and environmental conditions created by the political system of apartheid during the mid-twentieth century (Coovadia et al. 2009). Over-crowded living conditions, under-developed healthcare facilities for black people and migrant labour practices created an environment for efficient transmission of HIV-1 infection (Abdool Karim et al. 2009). In addition, a sluggish government response during the

1980's and 1990's was compounded by the "denialism" of President Thabo Mbeki's government of the early 2000's and consequently worsened an already escalating problem - leading in part to the epidemic being faced today.

1.1.2 HIV-1 transmission, pathogenesis and clinical course

HIV-1 is transmitted by infected bodily fluids, primarily through sexual intercourse. Less frequent routes of transmission include transfusion of infected blood products and mother-to-child transmission during pregnancy, peripartum and breastfeeding (Levy 2007). Upon crossing the mucosal barrier the virus primarily infects Langerhans Cells, dendritic cells, macrophages and CD4+ T cells and then migrates via the draining lymph nodes to the gut-associated lymphoid tissue (GALT). There it causes significant depletion of memory CD4 T cells (Guadalupe et al. 2003; Brenchley et al. 2004; Mehandru et al. 2004).

Following transmission the untreated viral illness manifests in 3 stages, classically described as acute infection, a variable period of chronic infection and eventually AIDS (Fig. 1.2) (Levy 2007). Acute HIV-1 infection is often accompanied by an acute HIV syndrome manifesting as a "flu-like illness" occurring two to four weeks following transmission of the virus (Gurunathan et al. 2009). Fiebig et al. revealed that the first weeks following infection can be further subdivided based on a stepwise gain in positivity of commonly used diagnostic assays (Fiebig et al. 2003). The period between initial infection, when infection is established in local tissues but systemic dissemination is yet to occur to detectable levels, is known as the "eclipse" phase and may last up to 10 days (Cohen et al. 2010). The acute phase of the illness is also

accompanied by the depletion of CD4 T cells in the peripheral blood, likely related to the profound depletion within the GALT.

Continuous viral replication and establishment of a viral set point is accompanied by progressive depletion of CD4 T cells, resultant susceptibility to opportunistic infections and the development of malignancies (Marriott & McMurchie 1996). Despite the obvious affect of depletion and dysregulation of CD4 T cells, HIV-1 also induces dysfunction in CD8 T cells, B cells, natural killer cells and non-lymphoid cells (Moir et al. 2011).

Clinical symptoms of immune-compromise as well as a declining CD4 T-lymphocyte count are core markers of disease progression with “AIDS” being defined by the presence of specific opportunistic infections and malignancies or by a CD4 T-lymphocyte count below 200 cells/uL (Castro, K.G., Ward, J.W., Slutsker, L., James, M.P.H, Buehler, W., Jaffe, H.W., Berkelman 1993).

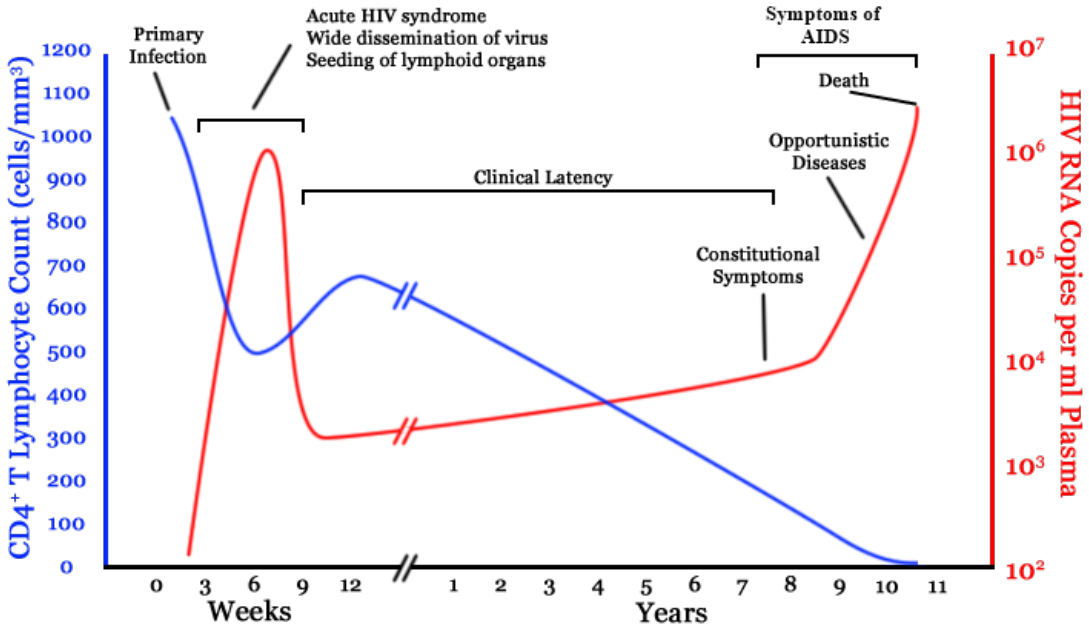


Figure 1.2 Figure depicting a typical course of HIV-1 infection.

(Image is adapted from Pantaleo et al. 1993 (Pantaleo et al. 1993), image source Wikimedia Commons)

1.1.3 HIV-1 structure and life cycle

HIV-1 is a Lentivirus within the Retrovirus genus and contains two copies of a single-stranded, positive-sense RNA genome about 10kb in size. Each virion is approximately 100 – 120 nm in diameter and the RNA genome encodes 9 genes (*gag, pol, env, tat, rev, nef, vif, vpr* and *vpu*)(Kuznetsov et al. 2003).

Gag, pol and *env* encode the structural proteins of the virus. The major Gag proteins include matrix (MA, p17), capsid (CA, p24) and nucleocapsid (NC, p7)(Levy 2007). *Pol* encodes for viral enzymes reverse transcriptase (RT), RNase H, integrase (INT) and HIV protease (PR) whilst *env* encodes for the envelope protein gp160. The glycoproteins gp120 and gp41 are essential in viral attachment and fusion with host cells and are cleaved from gp160 post-translationally (McCune et al. 1988).

Rev, vpr and *tat* are essential proteins involved in the regulation of HIV replication whilst *vif, nef* and *vpu* are accessory regulatory proteins.

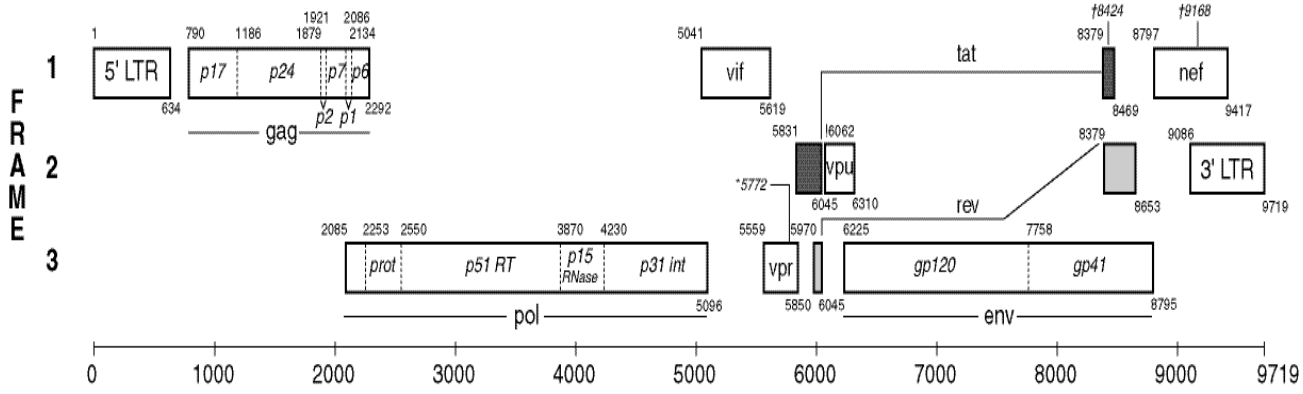


Figure 1.3 Landmarks of the HIV-1 genome. This is a schematic representation of the 10kb HIV-1 genome, detailing the 9 genes and 15 proteins encoded. Key abbreviations: LTR – long terminal repeat; gag – p17 (matrix), p24 (capsid), p7 (nucleocapsid) and p6; pol – prot (protease), p51RT (reverse transcriptase), p15RNase, p31int (integrase); vif (viral infectivity factor); vpr (viral protein R); tat (transactivator of transcription); rev (regulation of expression of virion proteins);vpu (viral protein U); env – gp120 (glycoprotein 120), gp41 (transmembrane glycoprotein 41) and nef (negative regulatory factor). (Image source: HIV sequence database (Los Alamos National Laboratory 2014)

1.1.3.1 Structure of HIV-1

The RNA genome is contained within a capsid composed of Gag p24. Gag p17 forms a matrix around this capsid, which is in turn enveloped by the viral Env protein. Env contains a lipid bilayer derived from the host cell along with the surface lipoprotein gp120 and the transmembrane protein gp41 (Gomez & Hope 2005).

The viral enzymes RT and INT and the nucleocapsid proteins are closely associated with the RNA strands as are the accessory regulatory proteins vif, nef and vpr (Fig. 1.4) (Gelderblom et al. 1989).

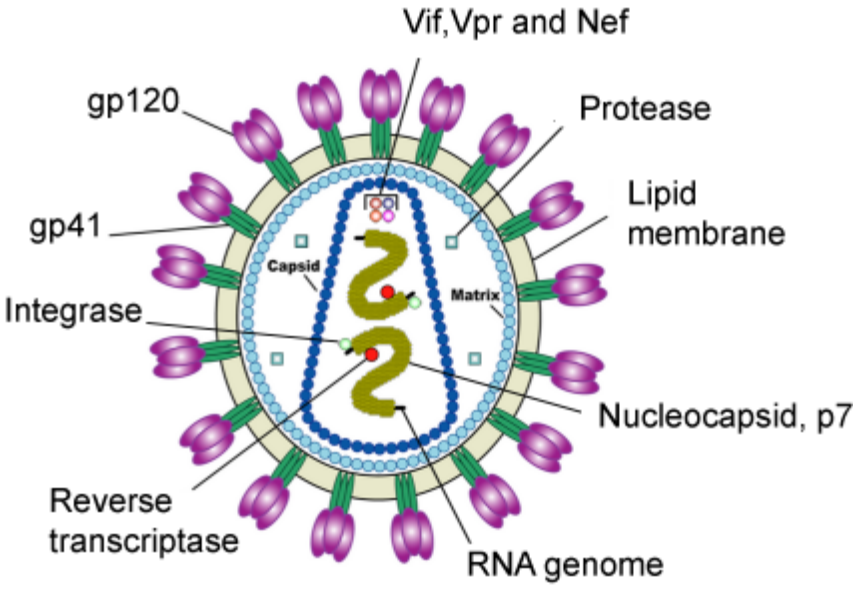


Figure 1.4 A schematic representation of the structure of HIV-1.

(Image adapted from US National Institute of Health, Image source: Wikimedia Commons)

1.1.3.2 Life cycle

HIV-1 primarily infects CD4 T cells and macrophages (Fig. 1.5). Fusion of the viral and host cellular membranes is facilitated by the attachment of the viral gp120 to the host CD4 receptor and membrane-spanning chemokine co-receptor CCR5 or CXCR4 (Klatzmann et al. 1984; Feng et al. 1996; Choe et al. 1996). This facilitates entry of the capsid into the cell where it is uncoated and viral RNA is reverse transcribed by viral reverse transcriptase to form a double stranded DNA copy of its genome (cDNA) (Fitzon et al. 2000). This is part of the pre-integration complex (PIC) that is transported into the cell nucleus where PIC-associated integrase facilitates integration into the host cell chromosome forming a provirus. Proviral transcription is mediated by host RNA polymerase II and positive transcription elongation factor b (P-TEFb) to form viral mRNAs, some of which are cleaved and assembled by viral

protease into mature HIV virions (Engelman & Cherepanov 2012). These virions bud off and are released from the host cell as infectious virus.

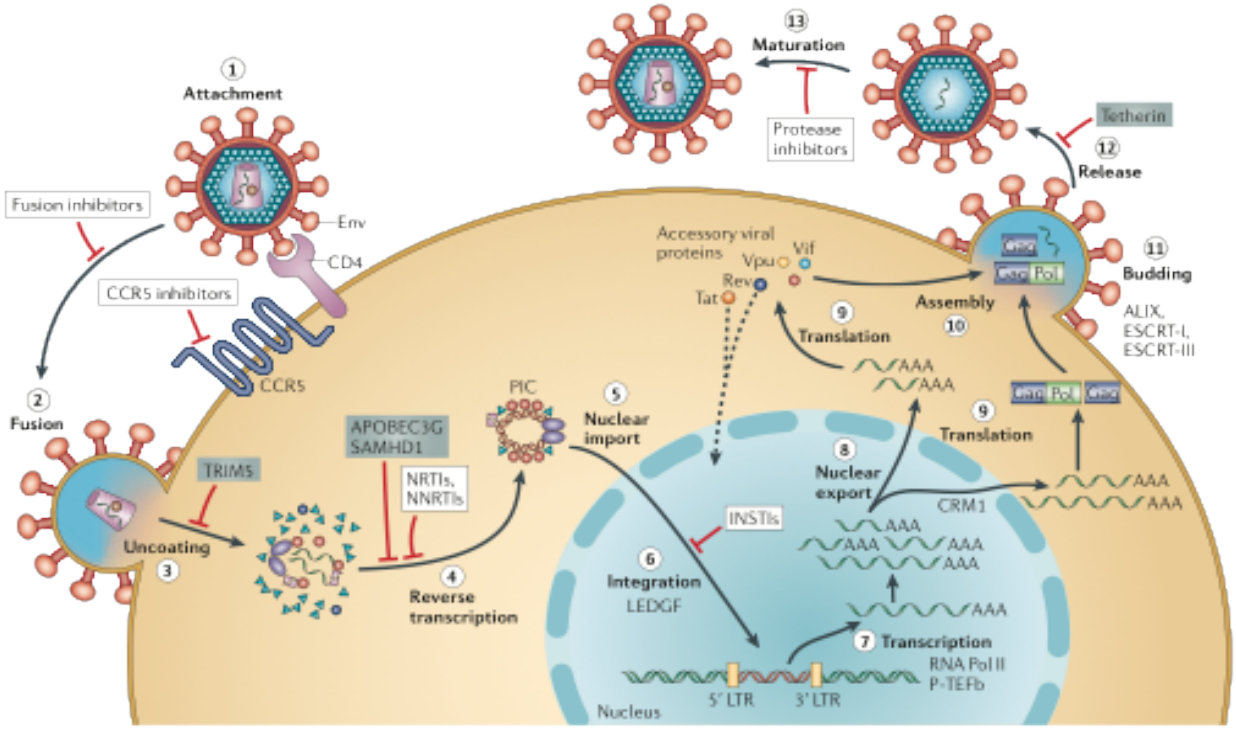


Figure 1.5 Schematic overview of the HIV-1 replication cycle and steps targeted by current antiretroviral therapies. NRTIs – nucleoside reverse transcriptase inhibitors, NNRTIs – non-nucleoside reverse transcriptase inhibitors, INSTIs – integration strand transfer inhibitors.

(Image adapted from Engelman and Cherepanov, 2012 (Engelman & Cherepanov 2012)

1.1.4 Antiretroviral therapy for HIV-1

The advent of antiretroviral therapy (ART) has significantly reduced HIV-associated morbidity and mortality (Egger et al. 2002; Palella et al. 1998; Mocroft et al. 2003). ART has also reduced rates of HIV-1 transmission: from mother-to-child, due to accidental needlestick injury, between sero-discordant couples and within communities with high HIV prevalence (Brocklehurst & Volmink 2002; Gerberding 2003; Cohen et al. 2011; Thigpen et al. 2012; Porco et al. 2004).

Despite the major benefits of ART, in 2012 only 61% of people estimated to be eligible for ART in middle to low-income countries were receiving treatment (UNAIDS 2013a). This number represents just 36% of all those living with HIV-1 in these countries and highlights the need for scaling up ART provision.

The anti-cancer drug Zidovudine (AZT) was the first drug found to be effective against HIV-1 when it was shown to inhibit the viral reverse transcriptase (RT) and to delay the onset of AIDS (Mitsuya et al. 1985; Dournon et al. 1988). AZT is a member of the class of nucleoside RT inhibitors (NRTIs). NRTIs are analogues of naturally occurring deoxynucleotides and inhibit RT by substrate competition and resultant chain termination during DNA synthesis. Despite initial optimism, the effect of AZT when first used as monotherapy was short-term, resulting in rapid development of viral drug resistance. This necessitated the development of other drugs and new classes that inhibited additional essential steps in viral replication such as the non-nucleoside RT inhibitors (NNRTIs), protease inhibitors (PIs), integrase strand transfer inhibitors (INSTIs), fusion inhibitors and chemokine CCR5-receptor blockers. The NNRTIs is another class of drug that interferes with RT but acts via a different mechanism that involves binding directly to the enzyme. The stage in viral replication inhibited by each of these classes of drugs is shown in Fig. 1.5. The aforementioned rapid loss of clinical efficacy observed when using antiretroviral drugs as mono- and dual-therapy was overcome by the combination of 3 drugs, normally from at least 2 different classes, which form the basis of ART today (Hammer et al. 1996; Cameron et al. 1998). The recommended 1st line combination of antiretroviral drugs today is comprised of a backbone of 2 NRTIs and 1 NNRTI (WHO 2013a). Table 1.1 describes common

antiretroviral drugs and their combinations, currently in use by at South African governmental antiretroviral initiation sites.

Table 1.1 Antiretroviral drugs commonly used in South Africa today.

ART drug class	ART drug name	Abbreviation	Commonly used ART drug combinations
NRTIs	Tenofovir	**TDF**	***1st Line***
	Stavudine	**d4T**	TDF + FTC/3TC + EFV or NVP
	Lamivudine	**3TC**	OR
	Zidovudine	**AZT**	d4T + 3TC + EFV or NVP
	Emtricitabine	**FTC**	OR
NNRTIs	Nevirapine	**NVP**	ABC + 3TC + EFV or NVP
	Efavirenz	**EFV**	***2nd Line***
	Etravirine	**ETR**	AZT + 3TC + LPV/r
PIs	Lopinivir/Ritonivir	**LPV/r**	OR
	Darunavir	**DRV**	TDF + 3TC/FTC + LPV/r
INSTIs	Raltegravir	**RGV**	***3rd Line***
			RGV + DRV + ETR
			- adjusted according to genotype interpretation and prior drug exposures

Table 1.1 details the commonly used ART drugs and their combinations currently used in South Africa. 2nd and 3rd line drug combinations are used in the context of severe adverse effects or treatment failure. NRTIs – nucleoside reverse transcriptase inhibitors, NNRTIs – non-nucleoside reverse transcriptase inhibitors, PIs – protease inhibitors, INSTIs – integration strand transfer inhibitors (South African National Department of Health 2013b).

1.1.5 Determinants of HIV-1 disease progression

The rate of decline to AIDS varies between individuals, and involves a poorly defined interplay of viral and immunological host factors.

1.1.5.1 HIV-specific CD8 T cells

The only clear associations with durable HIV-1 control in multiple genome-wide association studies (GWASs) are single-nucleotide polymorphisms (SNPs) within the

HLA class 1 region linked to HLA-B*57 and HLA-C expression (Fellay et al. 2007; Pereyra et al. 2010). Furthermore, the robust association of certain beneficial HLA class 1 alleles such as HLA B*57 and B*27 in Caucasian individuals and HLA B*58 and B*81 in Southern African individuals highlights the role of cell-mediated immunity in HIV-1 control (Carrington & O'Brien 2003; Kiepiela et al. 2004). A detailed account of the role of HIV-specific CD8 T cell responses in HIV-1 disease progression is presented in Chapter 4.

1.1.5.2 Immune activation

The level of generalized immune activation is a well-recognized feature of chronic HIV-1 infection and along with the HIV-1 set point viraemia, influences the rate of CD4 T cell decline and consequent progression to AIDS (Douek 2003; Deeks et al. 2004). Although highly debated, immune activation is believed to be the consequence of gut translocation of bacterial products such as lipopolysaccharide (LPS) through a disrupted mucosal barrier (Brenchley et al. 2006). The mediators of immune activation include pro-inflammatory cytokines, chemokines and interferon-α (IFNα) and their effect at a cellular level manifests as an up-regulation of activation markers on all major lymphocyte populations (Moir et al. 2011). The eventual consequence is increased cell turnover; increased activation-induced-apoptosis of CD4 and CD8 T cells as well as exhaustion and functional impairment of HIV-specific T and B cells (Moir et al. 2011).

1.1.5.3 The role of viral adaptation in progression to AIDS

HIV-1 is able to escape pressures exerted by both the immune system and antiretroviral therapy through the development of escape mutations. The interactions between HLA-mediated CD8 T cell selection pressure, viral escape and viral fitness and the relation of these factors to HIV-1 progression have been previously postulated, but remain poorly defined (Frater et al. 2007; Huang et al. 2011). Viral adaptation to the selection pressure imposed by an effective immune response involves the development of escape mutations which may have associated fitness costs and consequences for disease progression (Phillips et al. 1991; Goulder & Watkins 2004; Frater et al. 2007). The development of compensatory mutations in some instances and the reversion of escape mutations to wild-type virus in others is associated with decline to AIDS (Huang et al. 2011). Reversion of escape mutations may result from a weakening of cell-mediated immunity associated with quantitative and qualitative T cell loss and resultant disease progression.

1.1.5.4 Co-infection

HIV-1 co-infection with pathogens such as cytomegalovirus and Mycobacterium tuberculosis (Mtb) has also been shown to be associated with faster progression to AIDS and may reflect higher levels of immune activation (Báfica et al. 2003; Robain et al. 2001). HIV-1 replication is increased at sites of TB disease and may contribute to higher HIV-1 viral loads and accelerated CD4 T cell loss (Toossi et al. 2007).

1.2 Tuberculosis

1.2.1 The tuberculosis epidemic

TB accounts for a huge burden of disease around the world with an estimated 8.6 million TB incident cases and 1.3 million TB-associated deaths in 2012 (WHO 2014b). Low- and middle-income countries, particularly in sub-Saharan Africa, carry a disproportionate burden of the disease (Fig. 1.6).

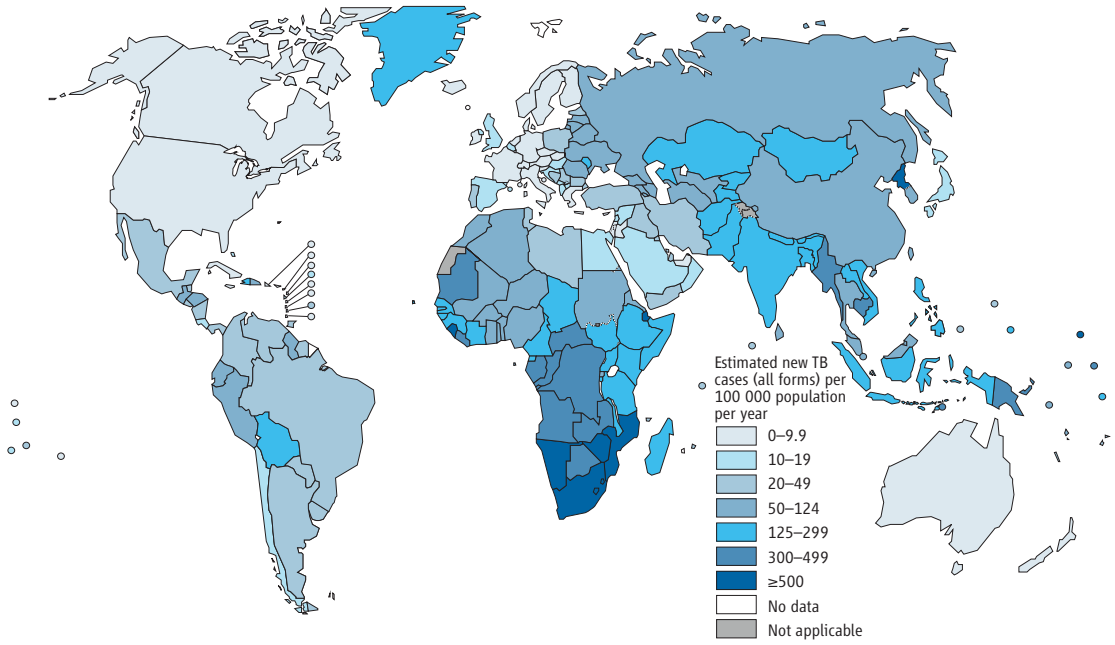


Figure 1.6 Estimated TB Incidence rates, 2012. Graphic showing the disproportionate burden of TB disease in low- and middle-income countries, particularly in sub-Saharan Africa.

(Image source: World Health Organisation, 2013 (WHO 2013b))

South Africa has one of the worst TB epidemics in the world with high incident TB and HIV-1 co-infection rates accompanied by increasing levels of multidrug-resistant and extensively-drug resistant TB.

Mtb was first introduced to South Africa during the 17th century by Dutch and British settlers, many of whom had been infected during the TB epidemic that was

devastating Europe at the time (Packard 1987). The ensuing discovery of mineral wealth and rapid explosion of mining during the late 19th century facilitated the spread of the disease to previously unexposed black South Africans through silica dust exposure, cramped communal living conditions and poor nutrition (Abdool Karim et al. 2009). Migrant workers duly transmitted the mycobacterium to rural communities when returning home and TB incidence rates rose steadily during the 20th century, peaking first in the 1960s and then again from the late 1990s with the convalescence of the HIV-1 epidemic. The consequence is that in 2012, South Africa reported over 500000 incident TB cases placing it 3rd globally, behind the far more populous nations of India and China respectively (WHO 2013b).

1.2.2 Mtb infection and the host immunological response

1.2.2.1 Infection and the innate immune response

Mycobacterium Tuberculosis (Mtb) transmits by aerosol spread and infects via the respiratory tract. When the bacillus is inhaled into the lungs it infects the invading innate immune cells, including alveolar macrophages, neutrophils, monocytes and dendritic cells (DCs) (Wolf et al. 2007; Kang et al. 2011). The mycobacterium enters macrophages utilising CR3 and mannose receptors whilst it gains entry to DCs through binding to the DC-SIGN receptor (Tailleux et al. 2002). Similar to other intracellular organisms, immunity to Mtb is primarily cell-mediated. Due to the resistant nature of the waxy cell wall of the Mtb bacillus, it is transported intact to the lung parenchyma. The trafficking of myeloid DCs to the draining lymph nodes is typically delayed, resulting in the appearance of adaptive immune responses as late as 6 weeks after Mtb exposure (Ernst 2012). This delay is believed to be key in permitting mycobacterial persistence (Reiley et al. 2008).

1.2.2.2 The adaptive immune response

DCs present mycobacterial antigens with HLA class-I or -II presentation molecules and CD80 and CD86 co-stimulatory molecules to T cells resulting in an initial CD4 and a later CD8 T cell response (Mellman & Steinman 2001). These antigen specific T cells form a granuloma around infected macrophages and activate the macrophages through the production of cytokines. Studies in mice using gene-disruption, in vivo antibody depletion or adoptive transfer have established that although both CD4 and CD8 T cells play a role in susceptibility to Mtb, it is primarily the CD4 T cell response that is key to controlling infection (Orme 1988; Feng & Britton 2000; Caruso et al. 1999; Scanga et al. 2000). This is highlighted in humans by the increased susceptibility of individuals living with HIV to Mtb disease (Kwan & Ernst 2011).

The CD4 Th_1 response

The role of Th_1 cytokines in protection against Mtb is well established. Humans and mice unable to produce IFNγ or with defects in IFNγ signalling are extremely susceptible to TB (Flynn et al. 1993; Jouanguy et al. 1999). IFNγ is believed to augment macrophage function and killing by enhancing phagosome maturation and activation of oxidative burst (MacMicking et al. 2003; Chan et al. 1992). Interestingly, in the mouse model, IFNγ produced by CD4 T cells is essential to host survival and has also been shown to augment CD8 T cell function in the context of Mtb infection (Green et al. 2013). The therapeutic use of tumour necrosis factor α (TNFα)-blocking agents and the resultant increased TB risk highlights the importance of TNFα in anti-tuberculous immunity (Harris & Keane 2010). TNFα regulates chemokine induction, up-regulates monocyte adhesion molecules and promotes macrophage apoptosis

(Roach et al. 2002; López Ramírez et al. 1994; Keane et al. 1997). IL-2 is also believed to be important through its role in secondary expansion of memory T cells (Johnson et al. 1997; Williams et al. 2006). The ability of T cells to co-express IFNγ, TNFα and IL-2 (referred to as "polyfunctionality") has been shown to be protective in murine models of Mtb and Leishmania major and is believed to be important in humans too (Darrah et al. 2007; Forbes et al. 2008; Aagaard et al. 2009).

The CD4 Th_{17} response

Whilst the importance of the Th_1 response in anti-TB immunity is undisputed, evidence points to CD4 mycobacterial functions that are independent of the production of Th_1 cytokines (Gallegos et al. 2011). IL-17 producing T cells are induced during mycobacterial infection and appear to play a complex protective role (Scriba et al. 2008; O'Garra et al. 2013). IL-17 has been implicated in a number of autoimmune disorders and studies in mice have shown a potential detrimental role through associating an increase in IL-17 producing T cells with excessive inflammation and lung pathology (Cruz et al. 2006). However, other studies have shown a protective effect that is possibly mediated by the interplay between IL-17 and the induction of Th_1 responses (Wozniak et al. 2010; Gopal et al. 2012). IL-17 is has also been shown to play a protective role through neutrophil recruitment during mycobacterial disease (Scriba et al. 2008).

The CD8 T cell response

Mycobacterial infection induces a CD8 T cell response in humans, however the response is generally of lower magnitude than the CD4 response and its importance in containment of Mtb infection is uncertain (Walzl et al. 2011). Data from murine and non-human primate studies suggest a significant role for Mtb-specific CD8 responses and this is supported by some human studies and (Flynn et al. 1992; Sousa et al. 2000; Chen et al. 2009). Where the importance of a Mtb-specific CD8 T cell response in human mycobacterial infection has been demonstrated, it has been associated with in vitro CD8 expression of granulysin and perforin which are active against Mtb (Stenger et al. 1998; Stegelmann & Bastian 2005; Bruns et al. 2009). Lewinsohn et al. proposed a particular role for CD8 T cells in immune surveillance and lysis of cells that are heavily infected with Mtb, a role which may be particularly important in advanced HIV-1 infection (Lewinsohn et al. 2003).

The non-classical T cell, B cell and NK cell response

Responses to mycobacterial antigens by T cell subsets in addition to classical HLA class I- or class II-restricted αβ T cells have been observed and are thought to play a protective role. These subsets include HLA-E-restricted CD8 T cells, mucosa-associated invariant T (MAIT) cells, gamma-delta (γδ) T cells and CD1-restricted mycobacterial lipid-specific T cells (Ernst 2012).

Natural killer (NK) cells are also bactericidal against mycobacteria and help to regulate the innate response as well as CD8 and γδ T cells against Mtb infection (Zhang et al. 2006; Gerosa et al. 2002). Although antibodies to Mtb do not allow the

transfer of immunity, they do appear to have an opsonising role and are capable of enhancing both innate and cell-mediated immune responses to mycobacteria (De Valliere et al. 2005). In addition, B cell follicle aggregates have been found in lungs of humans and mice infected with Mtb possibly indicating a more important role for B cells in TB disease than was previously understood (Kozakiewicz et al. 2013).

1.2.3 TB latency

The induction of an efficient adaptive immune response does not typically eliminate all mycobacteria. It instead results in arrestment of bacterial proliferation and when combined with the bacterial induction of a "dormancy regulon" establishes an equilibrium between bacteria and host known as latent TB infection (LTBI) (Sherman et al. 2001). The mycobacterial DosS-DosR regulon is induced by stimuli present during LTBI such as hypoxia, nitrous oxide and carbon monoxide and causes the up-regulation of genes that facilitate survival in a hypoxic and nutrient-starved environment (Ernst 2012). In addition, the mycobacterium can secrete a resuscitation-promoting factor (Rpf) that accelerates recovery from this dormant state and may contribute to progression to active TB disease (Chao & Rubin 2010).

1.2.4 TB reactivation

Of the 2 billion people worldwide currently with LTBI, 10% will develop active TB disease during their lifetimes (Sutherland 1976; Vynnycky & Fine 1997). Reactivation of latent TB disease, as opposed to reinfection with a new strain of Mtb, accounts for most of the cases of active TB worldwide and can occur decades after initial infection (Lillebaek et al. 2003). The best-established risk factors for TB reactivation are defects in CD4 T cell quantity and function associated with HIV-1 disease as well as

therapeutic TNFα-blockade (Kwan & Ernst 2011; Harris & Keane 2010). The precise mechanisms associated with CD4-associated Mtb control are incompletely explored and constitute a major component of this thesis. Additional risk factors associated with the reactivation of TB are shown in Table 1.2.

Table 1.2 Common risk factors associated with reactivation of TB

Associated medical conditions	HIV, diabetes mellitus, uraemia, gastrectomy, silicosis, haematological malignancies, vitamin D deficiency, alcoholism, malnutrition
Immunosuppressive medications	TNFα monoclonal antibodies, glucocorticoids, chemotherapy
Thin body habitus	
Advanced age	
Smoking	

(Table adapted from Ernst, 2012 and Lin & Flynn, 2010 (Ernst 2012; Lin & Flynn 2010))

1.2.5 TB Antigens

The first mycobacterial extracts were obtained by Robert Koch as early as 1880 and at the time were proposed to both cure and prevent TB (Yang et al. 2012). Tuberculin or purified protein derivative (PPD) has been used for the past 50 years in the Tuberculin Skin Test (TST) to support the diagnosis of TB and is a precipitation of proteins from heat-killed cultures of Mtb. Most of the protein components of PPD are shared between different mycobacterial species or with species of unrelated bacteria (Chaparas et al. 1970). The result is that individuals vaccinated with BCG respond to PPD in many cases better than individuals infected with Mtb itself, posing a significant impediment to its use for the specific diagnosis of Mtb infection (Fine et al. 1994; Bosman et al. 1998).

The antigens early secreted antigenic target-6kDa (ESAT-6) and cultured filtrate protein-10kDa (CFP-10) were later found to be largely restricted to the tuberculosis complex and absent in all strains of BCG (Andersen et al. 1995; Morten Harboe et al. 1996; Berthet et al. 1998). ESAT-6 and CFP-10 are therefore used extensively today for diagnosis of LTBI and for enumeration of Mtb-specific immune responses ex vivo.

A number of Mtb proteins are expressed to a significant degree in LTBI (Leyten et al. 2006). These "latency antigens" include α-crystallin (Rv2031c), Rv2659c and Rv2660c amongst others. The first experiments exploring mycobacterial latency induced a stationary phase of growth by simulating hypoxic environments. Rv2031c was shown to be up-regulated and expressed on the thickened bacterial cell wall which is thought to play a protective role within hostile growth environments (Cunningham & Spreadbury 1998). Immune responses to latency antigens may be important in maintaining control of LTBI and novel vaccine approaches are targeting such antigens in the hope of creating a more broadly active candidate vaccine (Aagaard et al. 2011; Dey et al. 2011). Rv2031c is one of the most highly expressed of these proteins during LTBI and the loss of Rv2031c-specific T cell responses may lead to active TB disease (Dey et al. 2011).

1.2.6 TB chemoprophylaxis

TB chemoprophylaxis is the use of anti-TB medications on their own or in combinations for the treatment of LTBI and the prevention of subsequent active tuberculosis. Placebo-controlled trials involving the administration of Isoniazid for 6-12 months have shown significant reductions in the development of active TB in both HIV-uninfected and HIV-infected individuals (Akolo et al. 2010). The effect of TB

chemoprophylaxis was greater amongst those with clinical evidence of LTBI as determined by Tuberculin skin test (TST). Isoniazid preventative therapy (IPT) is therefore recommended by the World Health Organization (WHO) as one of the main strategies for the prevention of active TB in HIV-infected individuals (WHO 2011b). Currently, South African national treatment guidelines recommend IPT for all HIV-infected individuals (with no signs of active tuberculosis disease) and a recent study confirmed both the efficacy and safety of the concurrent initiation of ART and IPT for the prevention of TB in HIV-infected individuals (South African National Department of Health 2013b; Rangaka et al. 2014).

The effect of IPT on the Mtb-specific T cell response is clinically important as it has implications for the utility of IGRAs and TSTs in monitoring the treatment response to IPT. IPT may also influence the evaluated extent to which ART restores Mtb-specific T cell responses in HIV-infected individuals, as investigated in this thesis. It is also important to bear in mind that Mtb-specific T cell responses have been shown to be dynamic, both in the presence and absence of IPT when longitudinally measured in the same individuals (Mitchell et al. 2012; J. L. Johnson et al. 2014).

When evaluating the effect of IPT on the reversion of positive TST and IGRA results, the data are inconclusive. Whilst one study noted TST reversion following IPT in 20.5% of participants, most studies have not shown significant proportions of individuals with IGRA reversion following IPT (D. F. Johnson et al. 2014). This may be due to the increased sensitivity of the IGRA's over the TST for detecting LTBI. Whilst not showing significant IGRA reversion on IPT, two studies have however shown a reduction in the level of IFNγ produced in response to Mtb-specific antigens whilst

one showed an increase (O'Shea et al. 2014; J. L. Johnson et al. 2014; Takenami et al. 2012). A detailed study by Wilkinson et al. revealed a transient early increase in the number of IFNγ-producing cells above baseline, as measured by ELISPOT in healthy IGRA+ individuals taking a combination of Isoniazid and Rifampin chemoprophylaxis. There was a consequent decrease in the number of IFNγ-producing cells below baseline after completing 3 months of treatment, although this decrease was not significant (Wilkinson et al. 2006). Furthermore, additional experiments showed that the increase in IFNγ-producing cells was associated with the use of Isoniazid and not Rifampin and postulated to be due to the mechanism of action of Isoniazid on the bacterial cell wall.

In summary, it is not completely clear what the effect of IPT on the character of the Mtb-specific T cell response is. These effects may be dependent on the duration of treatment, the level of immunosuppression of individuals undertaking IPT as well as the level of TB disease burden in the community.

1.3 HIV/TB co-infection

1.3.1 Epidemiology and burden of disease

Infection with HIV-1 predisposes to both infection with and reactivation of TB, thereby contributing to a HIV/TB epidemic that is a massive global health problem today. The impact of the HIV-1 epidemic in parts of eastern and southern Africa has seen the number of TB cases increase by up to 3-fold in the decade following the late 1980s (Raviglione & Harries 1997; WHO 2011a). In 2012 1.1 million (13%) TB incident cases and 320000 (25%) TB deaths were associated with HIV co-infection. This is particularly evident in Africa, which accounts for 75% of HIV-infected-TB cases

worldwide (Fig. 1.7 and 1.8)(WHO 2013b). Unsurprisingly, TB is the leading cause of death among HIV-infected individuals and in Africa accounts for more than 50% of HIV-related deaths (Bates et al. 2013; Getahun et al. 2010).

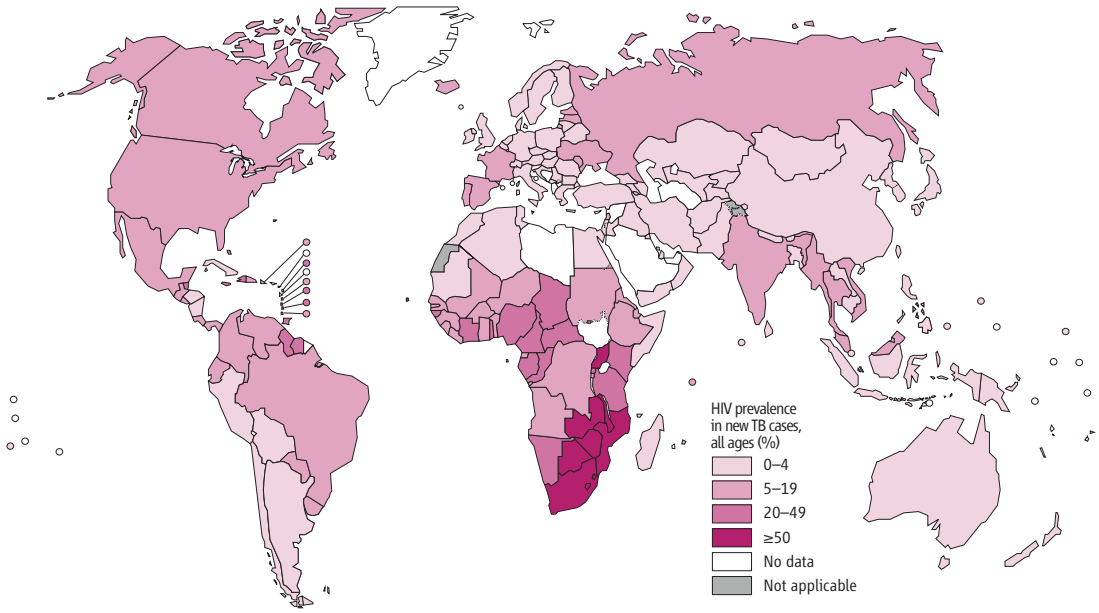


Figure 1.7 Estimated HIV-1 prevalence in new TB cases, 2012. This graphic highlights the convalescence of the HIV-1 and TB epidemics, particularly in sub-Saharan Africa. (Image source: World Health Organisation, 2013 (WHO 2013b)

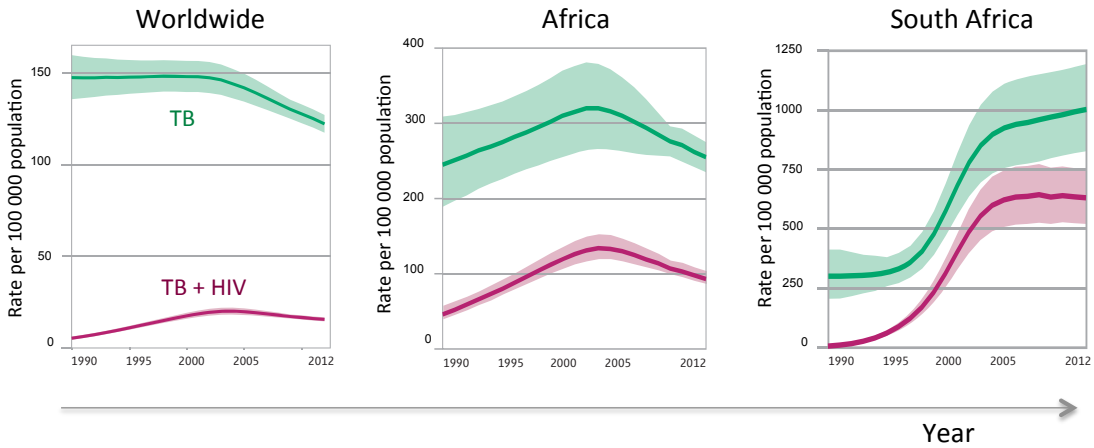


Figure 1.8 Estimated TB incidence rates by region, 1990 – 2012. The contrasting patterns of TB incidence rates indicate the particular severity of the TB and HIV/TB epidemic in South Africa. TB incidence rates are shown in green and HIV-infected TB incidence rates in red.(Image source: Adapted from World Health Organisation, 2013 (WHO 2013b)

1.3.2 Pattern of TB presentation within the context of HIV-1 disease progression

Most opportunistic infections typically cause disease only at extreme levels of immune suppression, after extended periods of HIV-1 disease progression. In contrast, TB can occur in HIV-infected persons at all stages of HIV-1 disease suggesting a unique increase in TB susceptibility early in HIV-1 infection (Figure 1.9) (Saharia & Koup 2013). TB risk was shown to double within a year of HIV-1 infection in mineworkers in South Africa and to continue to increase during chronic HIV infection (Sonnenberg et al. 2005; van Asten et al. 2003). Similar results were shown in a study in high-income countries (Lodi et al. 2013).

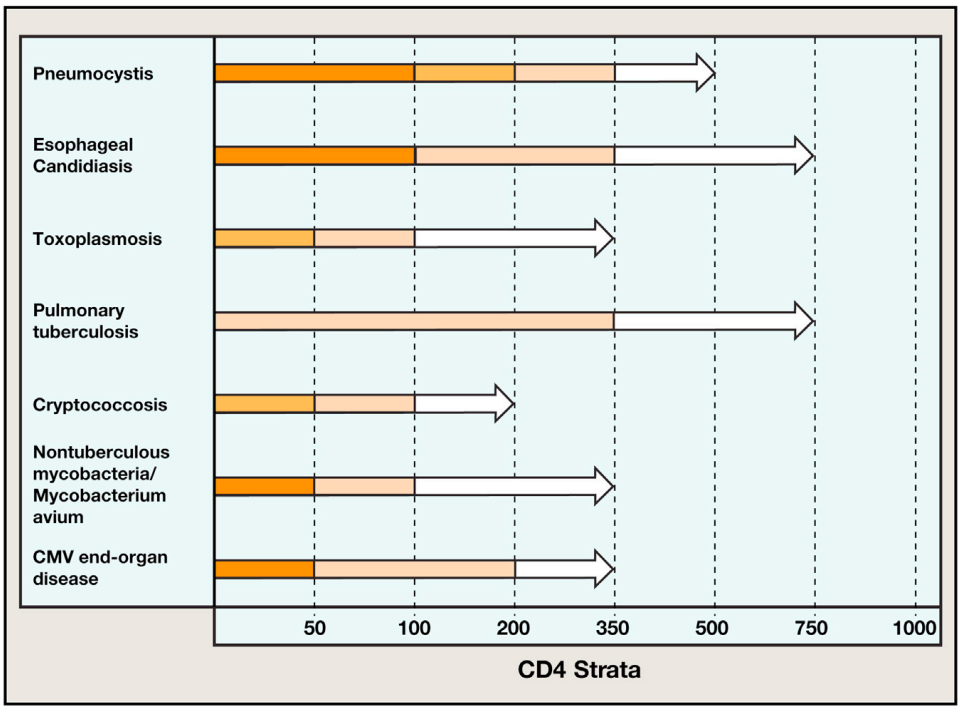


Figure 1.9 Incidence of select opportunistic diseases in HIV-infected individuals, stratified by CD4 T cell count. The colour of the bars indicates the approximate incidence rate (IR) per 100 person-years for each respective opportunistic pathogen - dark orange, IR >10; orange, IR = 5 – 10; light orange, IR = 1 – 5; white, IR <1. TB cases occur with CD4 T cell counts above 500 cells/uL whilst CMV end organ disease occurs predominantly in individuals with CD4 T cell counts below 50 cells/uL.

(Image source: Saharia and Koup, 2013 (Saharia & Koup 2013))

1.3.3 The effect of HIV-1 on the Mtb-specific immune response

The mechanism by which HIV-1 disturbs TB immunity is only partially understood and is based on the effect of HIV-1 viral replication on the cellular immune function in forming and maintaining the granuloma. HIV-1 replication leads to a decrease in total CD4 T cell numbers, a disturbance in macrophage function and changes in Mtb-specific T cell function (Diedrich & Flynn 2011).

1.3.3.1 Innate immunity

As macrophages are susceptible to infection with HIV-1, it is unsurprising that HIV-1 appears to manipulate Mtb-associated macrophage function by decreasing apoptosis of Mtb-infected macrophages as well as a decreasing the acidification of Mtb-infected phagosomes (Patel et al. 2009; Mwandumba et al. 2004). Both of these functions are regarded as critical responses during co-infection and may well result in decrease macrophage killing and persistence of Mtb-infection.

1.3.3.2 Adaptive immunity

Most obviously HIV-1 infection results in a quantitative decline in CD4 T cells that correlates with increased risk of TB and likelihood of extra-pulmonary dissemination of TB disease (O'Garra et al. 2013). However, the early increase in TB-susceptibility in HIV-infected individuals exists prior to profound CD4 T cell depletion and is likely to be indicative of additional early qualitative defects in CD4 T cell function (Figure 1.9). It appears that the cause of this may be related to the early preferential depletion of Mtb-specific CD4 T cells in acute HIV infection (Geldmacher et al. 2008). This effect is not seen to the same extent in other pathogen-specific T cell responses (such as for

cytomegalovirus (CMV)) and has been attributed to the specific cytokine expression and early differentiated phenotype of Mtb-specific CD4 T cells (Geldmacher et al. 2010). IL-2 production by Mtb-specific CD4 T cells predisposes these cells to HIV-1 infection and depletion whilst CMV-specific CD4 T cells secrete less IL-2 and higher levels of β chemokines such as MIP-1β rendering them less susceptible to HIV-1 infection (Geldmacher & Koup 2012).

Apart from a quantitative decline, HIV-1 infection also induces functional changes in the Mtb-specific CD4 T cell response. T cell proliferation in response to Mtb-associated antigens is impaired in HIV-infected subjects (Zhang et al. 1994; Mendonça et al. 2007). Mtb-specific CD4 T cells in HIV-infected individuals have also been shown to have decreased production of IFNγ, TNFα and IL-2. The decreased cytokine secretion in response to mycobacterial antigens such as purified protein derivative (PPD), ESAT-6 and CFP-10, has been shown in T cells from peripheral blood, bronchealveolar lavage specimens and pleural fluid (Diedrich & Flynn 2011). In addition, HIV-1 co-infection results in reduced frequencies of Mtb-specific polyfunctional T cells in peripheral blood as well as the lung (Day et al. 2008; Kalsdorf et al. 2009; Jambo et al. 2011).

Th_{17} CD4 cells are important for the maintenance of mucosal immunity and are believed to play a significant role in the immune response to Mtb (Scriba et al. 2008). Memory Th_{17} cells are found in high frequency in the GALT and are preferentially depleted during HIV-1 infection, possibly leading to increased susceptibility to TB (Prendergast et al. 2010; El Hed et al. 2010).

Little is known of the effect of HIV-1 infection on the Mtb-specific-CD8 T cell response, however evidence from the mouse model indicates that a loss of IFNγ from CD4 T cells may negatively impact CD8 T cell responses (Green et al. 2013). Additional impairments in T cell function may be associated with T cell exhaustion caused by HIV-1 infection as it has been shown to affect both HIV-specific and non-specific T cells before (Rosignoli et al. 2009; Ernst 2012).

1.3.4 HIV/TB and ART

ART is a fundamental component in the fight against TB in HIV-infected individuals and reduces the individual risk of TB disease by up to 65%, irrespective of CD4 count (Suthar et al. 2012). By disrupting HIV-1 viral replication, ART facilitates increases in CD4 T cells with a clear benefit in reducing mortality and morbidity in TB co-infected individuals. This effect appears to be greatest in patients initiating ART with CD4 counts below 200 cells/uL (Lawn, Bekker, et al. 2005; Havlir et al. 2011).

Despite the clear evidence of Mtb-specific immune restoration, TB incidence rates in HIV-infected individuals on ART are still higher than in HIV-uninfected individuals and lead us to believe that this restoration is only partial (Badri et al. 2002; Girardi et al. 2005). In a study in the Western Cape Province in South Africa, the incident rates of TB remained substantially higher than rates in the local HIV-uninfected population regardless of duration of ART or attainment of CD4 T cell counts exceeding 700 cells/uL (Gupta et al. 2012). The character of these incompletely restored responses is not fully known and is likely to be dependent on the status of immune suppression at the time at which ART was initiated.

1.3.4.1 The effect of ART on the Mtb-specific T cell response

ART improves the ability of Mtb-specific CD4 T cells to proliferate in response to PPD, as evidenced by an increase in the proportion of responders as well as the magnitude of response following initiation of ART (Autran et al. 1997; T. Li et al. 1998). Schluger et al. found that IFNγ production in response to Mtb, as opposed to proliferation, increased over 12 months of ART, although it still remained suppressed in comparison to HIV-uninfected individuals (Schluger et al. 2002).

Wilkinson et al. carried out a detailed study of 19 individuals undergoing ART for 48 weeks in Cape Town, South Africa. They showed that despite a decrease in proportion of IFNγ- and IL-2-expressing CD4 T cells after initiation of ART, there was an increase in the absolute number of IFNγ+ PPD-specific CD4 T cells over 48 weeks of ART (Wilkinson et al. 2009). Sutherland et al. in their study in The Gambia observed an increase in CD4 T cell production of IFNγ and TNFα in response to PPD by 6 months of ART, although not in response to EC. This increase was consistent for polyfunctional Th_1 responses to PPD and EC, thereby also suggesting a restoration of functionality of the Mtb-specific T cell response (Sutherland et al. 2010). Both of these studies showed that the reconstituting Mtb-specific CD4 T cells were initially comprised of memory CD4 T cells with later reconstitution of the naïve T cell population, a finding supported by other earlier studies (Autran et al. 1997; Evans et al. 1998). The effect of ART on Mtb-specific CD8 T cells is not well described with one study suggesting that CD8 T cell activation is reduced on ART whilst another described persistent CD8 maturational differences and increased activation despite 12 years of suppressive ART (Evans et al. 1998; Rönsholt et al. 2012).

Despite the clear reconstitution of components of the Mtb-specific T cell response, the magnitude of such responses has been consistently been shown to be significantly decreased when compared with healthy HIV-uninfected controls (Wilkinson et al. 2009; Sutherland et al. 2006; Sutherland et al. 2010). These findings suggest that defects remain in the Mtb-specific T cell response despite effective ART and may contribute to the elevated TB risk experienced by HIV-infected individuals on ART when compared with HIV-uninfected individuals.

1.4 Summary and Research questions

Both HIV-1 and TB remain significant global health issues today, despite the availability of effective chemotherapy for both diseases. In many parts of the developing world, where HIV-1 prevalence is high, the HIV/TB co-epidemic predominates with devastating impact. HIV-1 infection predisposes to active TB disease whilst TB disease also accelerates HIV-1 disease progression.

The mechanism by which HIV-1 infection impacts immunity to TB is only partly understood and appears to involve both quantitative and qualitative defects in the Mtb-specific T cell response. Evidence suggests that Mtb-specific CD4 T cell responses are preferentially depleted, however it is unclear which antigen-specific components of the Mtb-specific T cell responses are important and thereby contribute to the increased TB risk in HIV-infected individuals. The role of CD8 T cells within the Mtb-specific T cell response is also unclear, as is the effect of HIV-1 infection on this particular pathogen-specific cell population.

Whilst much is understood about the clinical progression to AIDS in HIV-1 disease, little is known about the precise pathogenesis. HIV-specific CD8 T cells play a central role in restricting HIV-1 viral replication, however it is unclear what characteristics of this response are effective or how progressive CD4 T cell depletion affects the CD8 T cell response. The link between an effective HIV-specific T cell response and Mtb-specific immunity is likely to be significant, although it has not been fully explored thus far.

ART restores CD4 T cell numbers contributing to a significant reduction in both TB- and non-TB related morbidity and mortality. The increased incidence of TB in HIV-

infected individuals on ART, compared to HIV-uninfected individuals, indicates that Mtb-specific immunity is not fully restored. This is supported by limited experimental data and the character of the "restored" Mtb-specific T cell response, therefore, has not been fully elucidated nor has it been clearly correlated with increased TB risk.

This thesis investigates key aspects of HIV-specific and Mtb-specific immunity during HIV-1 disease progression, and treatment with ART, in a South African population particularly affected by the HIV/TB co-epidemic. My aims include:

- To develop and investigate a cohort of HIV-infected African patients over 12 months of HIV-1 disease progression and ART.
- To characterise HIV-specific CD8 T cell responses that contribute to HIV-1 virological control.
- To evaluate the impact of HIV-mediated CD4 T cell depletion on the character of HIV-specific CD8 T cell responses.
- To explore the impact of HIV-1 disease on Mtb-specific T cell number and function.
- To better elucidate the impact of ART on restoration of the Mtb-specific T cell response.

2 MATERIALS AND METHODS

2.1 Preparation of samples

2.1.1 Venepuncture and initial treatment of blood samples

2.1.1.1 Cohort 1

A total of 50mls of blood was drawn from each participant at each clinic visit. 40mls was drawn into ACD-A tubes and stored at room temperature for separation of PBMCs within 6 hours of venepuncture. 10mls of blood was also drawn into sodium-heparin tubes and, within 2 hours of venepuncture, aliquoted and incubated on-site with antigen (as described in detail section 2.2.3) for use in the whole blood intracellular cytokine-staining assay (WB ICS).

2.1.1.2 Cohort 2

50mls of blood was taken into ACD-A tubes and processed within 6 hours of venepuncture. Following phlebotomy, blood samples were transported to the laboratory at the University of the Free State Department of Microbiology and Virology.

2.1.2 Separation of plasma and PBMC

Blood samples were initially centrifuged at 1800 rpm for 10 minutes, the plasma supernatant aliquoted into 3 separate cryotubes and immediately stored at -80°C.

The remaining blood components were diluted 2:1 with RPMI (RPMI 1640, Sigma-Aldrich UK) supplemented with penicillin/streptomycin (100 IU/mL) and L-glutamine (146mg/L), and layered over Lymphoprep (Alere, UK) in a ratio of 2:1. The samples were centrifuged at 2200rpm without brake for 20 minutes. The PBMC layer was then

removed by Pasteur pipette and washed twice with RPMI (first at 1800 rpm and then again at 1500 rpm) before being reconstituted in R10 medium (RPMI 1640 with penicillin/streptomycin (100IU/mL), L-glutamine (146mg/L) and 10% sterile filtered and heat-inactivated fetal bovine serum (FBS)). For Cohort 1, freshly isolated PBMCs were immediately used in ELISPOT and qPCR assays as described in 2.2.2 and 2.2.4 respectively. Unused PBMCs were cryopreserved thereafter. For Cohort 2, freshly isolated PBMCs were immediately cryopreserved.

2.1.3 PBMC cryopreservation

PBMCs were re-suspended in "freezing mix" (50% FBS, 40% RPMI and 10% Dimethyl Sulphoxide (DMSO)) at 5-10 million cells/mL and placed in a Mr Frosty freezing container (Nalgene, USA) overnight, before being transferred to liquid nitrogen storage the following day.

2.2 Immunology

2.2.1 Antigens

2.2.1.1 HIV-1 peptides

- HIV-1 Clade C whole proteome

414 individual 18-mer peptides, overlapping by 10 amino acids, and spanning the entire HIV proteome were synthesized based on the available 2006 HIV-1 Clade C consensus. These peptides were used in IFNγ ELISPOT assays as described below.

- HIV-1 OPAL - Gag peptide pool

120 individual 15-mer peptides, overlapping by 11 amino acids and spanning the HIV-1 Gag protein were synthesized based on the HIV-1 Clade C Durban Consensus. These peptides were used in the WB ICS assay described below.

2.2.1.2 Mycobacterial antigens

- Tuberculin purified protein derivative (PPD) (Statens Serum Institut, Denmark) – PPD is produced from cultures of virulent Mycobacterium Tuberculosis that are heat-sterilized, filtered and concentrated. PPD is primarily a mixture of proteins (>90%) with molecular mass greater than 10 kDa.

- Mycobacterium tuberculosis specific recombinant proteins (Lionex, Germany) – all proteins were expressed in and purified from E. coli, had purity of >95% (SDS-PAGE/Densitometry) and endotoxin content below 25 IU/mg (Limulus Amebocyte Lysate assay).

- ESAT-6 – Early secretory antigenic target-6 kDa is also known as Rv3875.
- CFP-10 – Culture filtrate protein-10 kDa is also known as Rv3874 and previously designated as “LHP’.
- Rv2031c 16kDa – Also known as Alpha crystallin, 16kDa antigen, hspX or HSP16.3.

2.2.1.3 Cytomegalovirus antigens

- Cytomegalovirus lysate (Virusys, USA) – Normal Human Dermal Fibroblasts (NHDF) were used as host cells to grow Cytomegalovirus strain AD169 to create an infected-cell extract enriched for viral proteins.

- Uninfected cell lysate (Virusys, USA) – Uninfected NHDFs that were confirmed HIV- and Hepatitis B-negative were used as an appropriate mock-enriched control extract.

2.2.2 ELISPOT assays

2.2.2.1 HIV-1 whole proteome

HIV-1 peptides were used in an initial "megamatrix" of peptide pools containing 10-12 peptides to screen for HIV-specific T cell responses by IFNγ ELISPOT assay. Responses to individual 18mer peptides in peptide pools were confirmed in a separate "confirmation" ELISPOT assay the following day in a method previously described (Addo et al. 2003).

96-well MultiScreen® polyvinylidene difluoride plates (Merck Millipore, UK) were coated with 100µL anti-human-IFNγ mAb 1-D1K (0.5 µg/mL; Mabtech, Sweden) overnight at 4°C. Plates were washed with blocking buffer (PBS with 1% FBS) and supplemented with 50µL R10 medium per well and 10µL of peptide (final peptide concentration was 2 µg/mL). 10µL of R10 media alone was added to 4 "negative control" wells and 10ul of phytohaemagglutinin was added to 2 "positive control" wells. PBMCs were added at a concentration of 50000 cells per well for the HIV-1 screening plate and 100000 cells per well for the HIV-1 "confirmation" plate in a volume of 100 µL of R10 medium per well. Plates were incubated for 16 hours at 37°C (with 5% CO_2) before being washed 7 times with 200µL PBS. 100µL of biotinylated anti-human-IFNγ mAb 7-B6-1 (0.5 µg/mL; Mabtech, Sweden) was then added to each well and incubated at room temperature for 90minutes. The plate was washed again with PBS and thereafter 100µL of streptavidin-alkaline phosphatase was added (0.5

μg/mL; Mabtech, Sweden) for 40 minutes before being washed again and developed using a chromogenic alkaline phosphatase substrate kit (Bio-Rad, UK).

Plates were counted using an AID version 3 ELISPOT Plate Reader (Autoimmune Diagnostika, Germany) and IFNγ-T cell responses were recorded as the number of spot forming cells (SFCs) per well per million PBMCs.

2.2.2.2 TB and cytomegalovirus

T cell responses to tuberculosis and cytomegalovirus (CMV) antigens were measured by IFNγ ELISPOT assay as described above on the same plate as the HIV-specific responses, where possible. Antigens that were used included PPD (20μg/mL), a combination of ESAT-6 and CFP-10 recombinant proteins in a single pool (10 μg/mL), Rv2031c recombinant protein (10μg/mL), CMV lysate (5μg/mL) and uninfected NHDF cell lysate (5μg/mL). All CMV and tuberculosis antigens were assayed in duplicate wells and reported as the mean.

2.2.2.3 Criteria for ELISPOT cut-off

For HIV-1 IFNγ ELISPOT responses, a positive result was considered to be any SFC/10^6 count above the sum of the mean number of SFCs/10^6 in each participant's 4 negative control wells and 3 times the standard deviation of the number of SFCs/10^6 in the negative wells of all assays across the entire cohort. An indeterminate result was defined as any assay with mean negative control counts above 50 SFCs/10^6.This definition is extremely rigorous and was recently used in the analysis of similar HIV-1 IFNγ ELISPOT data in the SPARTAC trial (Fidler et al. 2013).

I used different criteria when analysing TB and CMV IFNγ ELISPOT data due to the fact that different criteria are employed commercially for the diagnosis of LTBI and in most recent studies where ELISPOT has been used for enumeration of Mtb-specific responses (Smith et al. 2009). IFNγ ELISPOT responses to these antigens were therefore considered positive if the mean number of SFCs/10^6 per well was at least 20 SFCs/10^6 more than, and at least twice that of, the mean number of SFCs/million in the negative control wells (Chapman et al. 2002; Lalvani et al. 2001; Karam et al. 2008; Mitchell et al. 2012).

2.2.3 Whole blood ICS protocol

2.2.3.1 Whole blood antigen stimulation and sample processing

Within 2 hours of collection, 1mL of heparinized whole blood was incubated with antigen (Table 2.1) on site at the clinic at 37°C, in the presence of anti-CD28 and anti-CD49d antibodies (Becton Dickinson, UK). After 6 hours Brefeldin A (5 µg/mL; Sigma-Aldrich, UK) was added, the samples briefly vortexed and further incubated for 5 hours in a timed water bath.

Within 10 hours of the water bath having turned off, EDTA was added to each sample to a final concentration of 2mM and the sample vortexed. Red blood cells were then lysed and white cells fixed using BD FACS Lysing solution (Becton Dickinson, UK). White cells were washed twice with PBS and then cryopreserved in freezing mix (90% PBS, 10% DMSO) in a Mr Frosty freezing container (Nalgene, USA) at -80°C overnight. Samples were transferred to liquid nitrogen for later flow cytometric analysis in Oxford.

Table 2.1 Antigens used in the whole blood ICS assay

	Antigens used	Final concentration
Positive control	Staphylococcal enterotoxin B (SEB)	5 µg/mL
Negative control	Co-stimulatory antibodies alone	N/A
Mtb antigens	PPD	20 µg/mL
	ESAT-6/CFP-10	10 µg/mL
	Rv2031c	10 µg/mL
CMV antigens	CMV lysate	5 µg/mL
	Uninfected NHDF lysate	5 µg/mL
HIV antigens	HIV-1 OPAL Gag peptide pool	2 µg/mL

2.2.3.2 Intracellular cytokine staining

Cryopreserved white cells were thawed at 37°C, transferred to 4mL FACS tubes in 2mL PBS and then centrifuged for 5 minutes at 1500rpm. Cells were then resuspended in 200µL FACS buffer (0.1% bovine serum albumin (Sigma-Aldrich, UK), 0.01% sodium azide (Sigma-Aldrich, UK) in PBS), transferred to 96-well V-bottomed plates and centrifuged for 3 minutes at 1500rpm. 200µL BD Fix/Perm Buffer (Becton Dickinson, UK) was added to each well, mixed and incubated at 4°C for 20 minutes in the dark. After the 20 min incubation the plate was spun at 1800rpm for 3 minutes and washed twice with BD Perm Buffer (Becton Dickinson, UK) before being incubated with 50 µL of antibody mix (reconstituted in Perm Buffer) at room temperature for 30 minutes in the dark. After antibody incubation, samples were spun at 1800rpm for 3 minutes and washed twice before being resuspended in FACS buffer and transferred to a 96-well U-bottomed plate for acquisition on the flow cytometer.

Table 2.2 Antibodies used in the flow cytometric analysis

Marker	Concentration	Clone	Manufacturer
CD14-APC-Cy7	1:50	HCD14	BioLegend
CD19-APC-Cy7	1:50	HIB19	BioLegend
CD3-eFluor 450	1:200	UCHT1	eBioscience
CD4-APC	1:100	RPA-T4	BioLegend
CD8-VioGreen	1:100	BW135/80	Miltenyi Biotec
IFNγ-PE/Cy7	1:100	4S.B3	eBioscience
IL-17-Alexa Fluor 488	1:50	BL168	BioLegend
IL-2-PE	1:50	N7.48A	Miltenyi Biotec
TNFα-PerCP-Cy 5.5	1:50	MAb11	eBioscience

2.2.3.3 Multiparameter flow cytometry - acquisition

Stained cells were acquired on a MACSQuant flow cytometer (Miltenyi Biotec, Germany). The number of cells acquired varied depending on the stage of HIV-1 disease of each study participant and all events were acquired for each sample. Multiple samples from the same individual, taken at separate time points of the study were all run on the same plate using identical process and antibodies. Plate-to-plate variation was minimized by using the same antibody clone and lot, where possible, for all samples. In addition, a stimulated control sample from a single donor was run in duplicate on every plate in order to maintain consistency between assays. Single-stained mouse κ compensation beads were used on each plate for post-acquisition compensation controls.

2.2.3.4 Multiparameter flow cytometry – gating and data analysis

Flow cytometry data were analysed using FlowJo vX for Mac (Tree Star, USA). Doublets were excluded using forward scatter (FSC)-area vs. FSC-height (Fig 2.1 a-b). Lymphocytes were gated based on FSC and side-scatter (SSC) characteristics and cells

that were CD14+ or CD19+ were further excluded from the “Lymphocyte” gate (Fig 2.1 b-d). Cytokine-positive CD4 and CD8 T cells were identified after gating on CD3+ cells (Fig 2.1 d-g).

Unstimulated cells were used as a guideline to set cut-off gates for cytokine positive populations. A Boolean function was created in FlowJo using all the cytokine-negative cells for each sample. In order to be consistent between samples, the cytokine positive gate was set at a constant factor of 5x the 75th percentile of the defined Boolean negative function. The frequencies and absolute numbers of different combinations of IFNγ+, TNFα+, IL-2+ and IL-17+ CD4 and CD8 T cells after antigenic stimulation were calculated. Non-specific background was measured using the unstimulated sample and was subtracted from the values of each stimulated sample. Non-specific background for single cytokines was below 0.03% and extremely low when more than 1 cytokine was measured. A cut-off of 0.03% after background subtraction was used to define a positive single-cytokine response and is a more stringent criterion than used in other similar studies (Sutherland et al. 2010; Beveridge et al. 2007).

Boolean cytokine combination gates were made using FlowJo vX software and analysed in conjunction with PESTLE (version 1.7; National Institutes of Health, USA) and SPICE software (version 5.35; National Institutes of Health, USA).

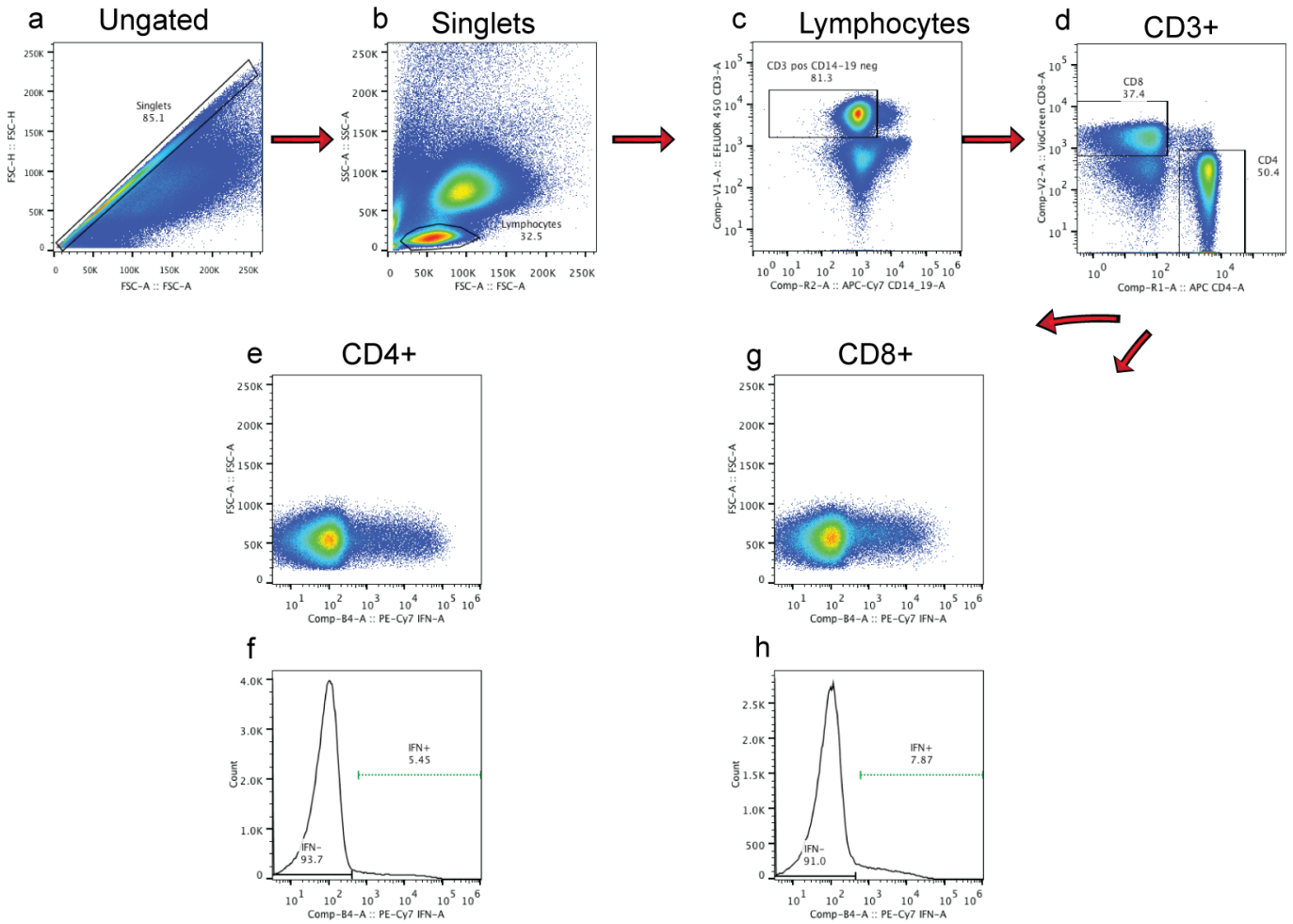


Figure 2.1 Flow cytometry gating strategy used to identify cytokine-positive CD4 and CD8 T cells. The gating strategy was followed in a stepwise manner from a – h. Cells were initially selected based on forward scatter height and forward scatter area to exclude doublets. The lymphocyte population was selected based on forward and side-scatter parameters and analysis then focused on CD3+ cells.

2.2.4 Quantitative PCR assay for monokine-induced by IFNγ (MIG) and IFNγ inducible protein-10 (IP10)

A novel and highly sensitive assay was used to measure Mtb-specific IFN-γ production by quantitative PCR (qPCR) for two reporters: monokine-induced by IFN-γ (MIG) and IFN-γ inducible protein-10 (IP10). The assay was developed by Kasprowicz et al. and optimized for our use (Kasprowicz et al. 2011). PBMC stimulation, lysis and cryopreservation were carried out in Bloemfontein with messenger RNA (mRNA) extraction, complementary DNA synthesis (cDNA) and qPCR being carried out in Oxford.

2.2.4.1 PBMC stimulation

1 million freshly isolated PBMCs per antigen stimulation were reconstituted in 500µL R10 medium and incubated at 37°C with 5% CO_2 (Table 2.3). After 16 hours PBMCs were lysed with RLT buffer supplemented with 1% β-mercaptoethanol (Qiagen, UK) and stored immediately at -80°C for later qPCR analysis.

Table 2.3 Antigens used in the MIG/IP10 qPCR assay

	Antigens used	Final concentration
Positive control	Recombinant IFNγ	5 µg/mL
Negative control	R10 medium alone	N/A
Mtb antigens	PPD	20 µg/mL
	ESAT-6/CFP-10	10 µg/mL
	Rv2031c	10 µg/mL

2.2.4.2 mRNA extraction and cDNA synthesis

The PBMC lysate was thawed and homogenized using a QIAshredder column (Qiagen, UK) by centrifuging at 13000rpm for 2 minutes. mRNA was extracted using a RNeasy®

Mini Kit as per the manufacturer's instructions and eluted in 35 µL RNase-free water. 10 µL mRNA was immediately used for cDNA synthesis via reverse-transcriptase PCR (RT-PCR) using the iScript cDNA synthesis kit (Bio-Rad, UK) as per the manufacturer's instructions. The remainder of the mRNA was stored at -80°C for repeat analysis if required. cDNA was stored at -20°C until qPCR analysis.

2.2.4.3 Quantitative PCR

qPCR was carried out for MIG, IP10 and "house-keeping" gene Hypoxanthine-guanine phosphoribosyltransferase (HPRT) on a Lightcycler®480 II system (Roche). The concentrations and sequences of primers used for each gene are shown in Table 2.4. The qPCR reaction used the Lightcycler®480 SYBR Green I Master Mix (Roche, UK) in a total preparation volume of 20 µL:

7 µL	H_2O (DNAse/RNase free; Roche)
10 µL	SYBR Green I Master Mix (Roche, UK)
1 µL	5' primer (Table 2.4)
1 µL	3' primer (Table 2.4)
1 µL	cDNA template

qPCR conditions were optimized for each gene of interest:

MIG	95°C for 10 min, then 45 cycles [95°C for 16 sec, 59°C for 10 sec and 72°C for 10 sec]
IP10	95°C for 10 min, then 45 cycles [95°C for 16 sec, 61°C for 20 sec and 72°C for 20 sec]
HPRT	95°C for 10 min then 45 cycles [95°C for 16 sec, 59°C for 20 sec and 72°C for 10 sec]

Table 2.4 Primers used for the MIG/IP10 qPCR assay

Gene	Primer name	Sequence	Tm (°C)
MIG	MIG1F	GTGGTGTTCTTTTCCTCTTG	59°C
	MIG1R	GTAGGTGGATAGTCCCTTGG	60°C
IP10	IP101F	TGATTTGCTGCCTTATCTTTCTGA	66°C
	IP101R	CAGCCTCTGTGTGGTCCATCCTTG	73°C
HPRT	HPRT2F	TGACCTTGATTTATTTTGCATACC	63°C
	HPRT2R	CGAGCAAGACGTTCAGTCCT	65°C

Standard curves for each gene of interest were created. cDNA for MIG, IP10 and HPRT was amplified using the conditions above and purified using a QIAquick® PCR Purification Kit as per the manufacturers instructions. The PCR products were quantified using Quanti-iT Pico green® DNA quantification kit (Invitrogen Molecular Probes, UK) as per the manufacturers instructions on a TBS-380 minifluorometer (Turner Biosystems, USA). The number of cDNA copies was calculated using the molecular weight of each gene amplicon and the following formula: RNA copy number = moles DNA x 6.02 x 10^{23}

The samples were then serially diluted, measured by qPCR and standard curves generated for application to patient sample quantification. A standard solution of $1x10^4$ copies was run in duplicate on each plate and the standard curve recalibrated accordingly.

All PCR reactions were carried out in duplicate and reported as the mean. Expression of MIG and IP10 were normalized to HPRT expression and a “fold increase” calculated through use of the following formula: Fold increase = ((MIG or IP10 stimulated / MIG or IP10 unstimulated) / (HPRT stimulated / HPRT unstimulated))

2.3 Virology

2.3.1 HIV-1 viral load measurement

The HIV viral load testing was performed by the National Health Laboratory Service (NHLS) in Bloemfontein using the Abbott RealTime HIV-1 assay (Abbott Molecular, USA) on the m2000 system. 0.6 mL of plasma was used, according to the manufacturer's instructions. This reverse transcriptase polymerase chain reaction (RT-PCR) assay targets the integrase region of the polymerase gene. Results are expressed as HIV-1 RNA copies/mL and the assay has a lower limit of detection of 25 copies/mL and a limit of quantitation of 40 copies/mL. The assay can detect HIV-1 group M (subtypes A-H), group N and group O isolates.

2.3.2 HIV-1 viral RNA extraction and synthesis of complementary DNA (cDNA)

In brief, virions from 500uL of plasma were pelleted by centrifugation at 23500g for 60minutes at 4°C. 360µL of supernatant was removed and HIV-1 viral RNA was isolated from the enriched 140µL using the QIAmp Viral RNA Mini Kit (Qiagen, UK) as per the manufacturer's instructions. The protocol used included the optional use of RNase-free DNAse to eliminate DNA contaminants. The extracted RNA was immediately used for synthesis of cDNA by reverse-transcriptase PCR (RT-PCR). RNA was reverse-transcribed using the Superscript III® RT enzyme (Invitrogen, UK) as per the manufacturer's protocol. The remaining RNA was stored at -80°C for repeated analysis if required.

2.3.3 PCR of HIV-1 Gag

Amplification of HIV-1 Gag was carried out with the use of a two-stage nested PCR reaction with two pairs of primers (using the outer primer set (OP) for the 1st round and the inner primer set (IP) for the 2nd round of nested PCR, Table 2.5). A second

primer set was used on samples not amplified using the first set. All primers were oligonucleotide primers purchased from MWG-Biotech, Germany. Random decamers were used to prime the synthesis of Gag.

The PCR reaction used the Platinum Taq DNA polymerase enzyme (Invitrogen, UK) in a preparation of 50 µL:

5 µL	10x PCR buffer
1.5 µL	$MgCl_2$ (50 mM; Invitrogen)
1 µL	dNTP (25 mM; Invitrogen)
38.3 µL	H_2O (DNAse/RNase free; Sigma-Aldrich)
1 µL	5' primer (20 pmol/µL)
1 µL	3' primer (20 pmol/µL)
0.2 µL	Platinum Taq (Invitrogen)
2 µL	cDNA template (for 1st round of nested PCR), or 1st round PCR product (for 2nd round of nested PCR)

The PCR reaction was performed on a Peltier Thermal Cycler (MJ Research, UK) using the following conditions:

Step 1	Denaturation	94°C	4 min
Step 2	Denaturation	94°C	30 sec
Step 3	Annealing	X°C	30 sec
Step 4	Elongation	72°C	45 sec
Step 5		Repeat steps 2-4 34 times	
Step 6	Elongation	72°C	7 min
Step 7	Storage	4°C	

The annealing temperature marked X°C was calculated by taking the average melting temperature (Tm) of the primer pairs used in each PCR reaction (Table 2.5).

Table 2.5 Primers used for PCR of HIV-1 Gag

Nested PCR primer name and pairs	Primer sequence	HXB2 nucleotide position	Tm (°C)	HIV Clade
GagC5OP	CTCTAGCAGTGGCGCCCGAA	627 → 646	64	C
GagC3OP	TCCTTTCCACATTTCCAACAGCC	2045 ← 2023	61	C
GagC5IP	ACTCGGCTTGCTGAAGTGC	696 → 714	59	C
GagC3IP	CAATTTCTGGCTATGTGCCC	2003 ← 1984	57	C
GagC15OP	CTAGCAGTGGCGCCCGAACA	629 → 648	64	C
GagC13OP	ACAGTCTTCATTTGGTGTCCTTC	2067 ← 2044	63	C
F1OLD5IP	TCTCTCGACGCAGGACTC	682 → 699	58	C
F1OLD3IP	TTTCCACATTTCCAACAGCC	2042 ← 2023	55	C

OP = outer primer (1st round of nested PCR), IP = inner primer (2nd round of nested PCR)

2.3.4 Sequencing of HIV-1 Gag

Sequencing was carried out using a set of 6 overlapping primers, 3 forward and 3 reverse. These primers consisted of the two inner primers used for the 2nd round of PCR (Table 2.5) and 4 additional inner primers, designed to provide good sequence overlap for subsequent sequence analysis (Table 2.6). Sequencing was performed using the Big Dye Terminator v3 reaction mix (Applied Biosystems, UK). The reaction mix used was as follows:

1.5 µL	BigDye buffer
1.0 µL	BigDye Terminator v3 reaction mix
1.5 µL	Primer (0.625mM)
4.0 µL	ddH_2O
2 µL	PCR template

The sequencing reaction was performed on Peltier Thermal Cycler (MJ Research, UK) using the following conditions: [96°C for 10 sec, 50°C for 5 sec, 60°C for 4 minutes] repeated 30 times.

The sequencing products were then cleaned by sodium acetate precipitation: 10µL of ddH_2O, 2µL of NaOAC (3M, pH 5.2; Sigma-Aldrich, UK) and 50µL of 100% ethanol were

added to the sequencing reaction, vortexed and then incubated at room temperature for 30 minutes. Following centrifugation for 90 minutes at 4000rpm at 4°C, the supernatant was removed and 150µL cold 70% ethanol added. This was centrifuged under the same conditions for 10 minutes and the 70% ethanol wash step repeated twice. The pellet was then dried at 94°C for 1 minute before the sequencing reaction was run on an ABI DNA Analyzer (Applied Biosystems, UK) by the Zoology Department, University of Oxford.

The sequences were assembled using Sequencher (version 4.8, Gene Codes Corporation, USA), manually aligned using Se-Al (version 2.0 a11, A. Rambaut, Department of Zoology, University of Oxford) and analysed using MacClade software (version 4.06; Sinauer Associates, USA)

Table 2.6 Additional HIV-1 Gag sequencing primers

Sequencing primer names	Primer sequence	HIV Clade
Gagseq2R	CTGCACTATAGGATAATTTTGAC	C
Gagseq3F	GACACCAAGGAAGCCTTAG	C
Gagseq4R	CTCCCACTGGAACAGGTG	C
Gagseq5F	GGAACAAATAGCATGGATGAC	C

2.4 Statistics

Standard statistical approaches for parametric and non-parametric data were used as appropriate. Specific tests are detailed in the results, and include Fisher-exact T tests, Wilcoxon signed-rank test, Spearman correlations, linear regressions, logistic regressions and Mann-Whitney tests. These were carried out using Microsoft Excel for Mac 2011 and GraphPad Prism 5 for Mac OS X (version 5.0a; GraphPad, USA). Logistic regressions were carried using IBM SPSS Statistics (version 22; IBM, USA).

3 DEVELOPMENT OF A CLINICAL COHORT FOR THE LONGITUDINAL EVALUATION OF HIV- AND MTB-SPECIFIC IMMUNITY

3.1 Background

The HIV/TB epidemic has affected sub-Saharan Africa with particularly devastating consequences (Harries et al. 2010). There is a dire need to understand the pathogenesis of HIV-associated TB as well as the reconstitution of TB-immunity and residual TB risk associated with ART within the context of a high HIV/TB incidence area. This has significant public health consequences as well as implications for better understanding immune correlates of protection against TB within communities particularly affected by the devastating co-epidemic.

3.1.1 Study setting

Our study was based in the city of Bloemfontein, located within the Mangaung Metropolitan Municipality in the Free State province of South Africa. The Free State is bordered by Gauteng, the Eastern Cape, Northern Cape, KwaZulu-Natal and North West provinces, as well as the neighbouring country of Lesotho. A population of 2.6 million makes it the second most sparsely populated province in South Africa.

Mangaung Metropolitan Municipality has a population of 750000, with a very high youth unemployment rate of 37,2% and only 60.7% of households currently living with access to full amenities (including electricity, piped water inside dwelling and flush toilet facilities connected to municipal sewage facilities) (Statistics South Africa 2014).

The Free State province is significantly affected by the HIV/TB pandemic with South Africa's third highest HIV prevalence rate in 2012 and one of the highest TB-associated

death rates (South African National Department of Health 2010b; Statistics South Africa 2010). Within the Mangaung municipality, 12.1% of all deaths in 2010 were caused by TB, ranking the municipality 9th highest of all South African municipalities for TB-associated mortality (Statistics South Africa 2014).

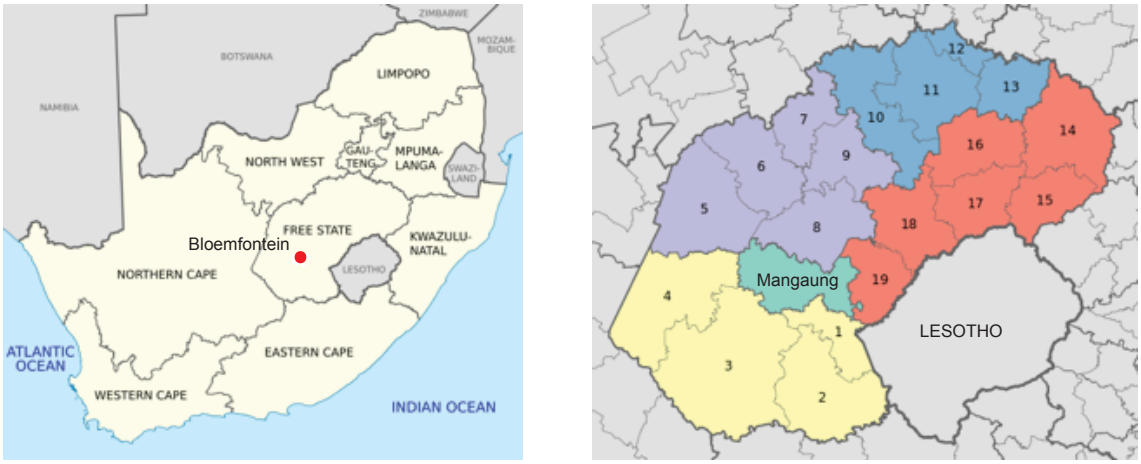
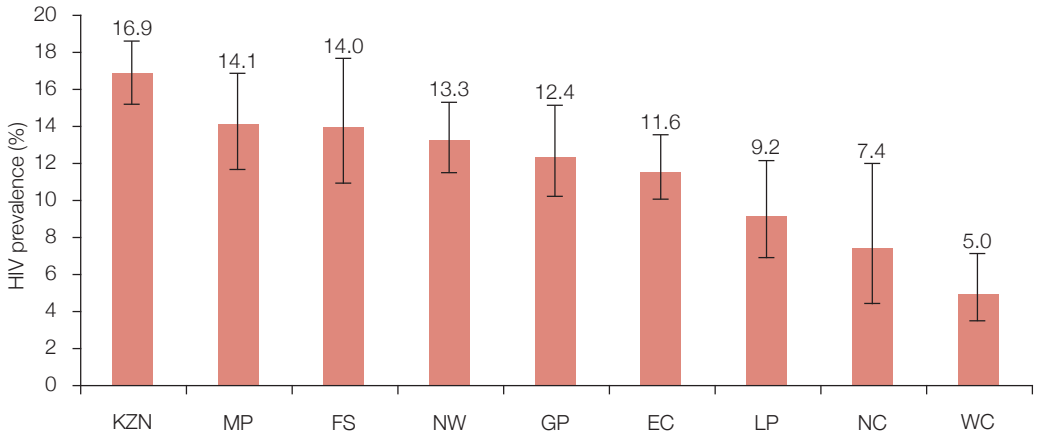


Figure 3.1 Map of South Africa (left panel) and the Free State province (right panel)

Left panel: Depicts South Africa and its provinces with the location of the Free State province and city of Bloemfontein clearly labelled. Right panel: Depicts the municipal districts of the Free State province. (Images are adapted from Wikimedia Commons (Wikimedia 2014))



Key: KZN – KwaZulu-Natal, MP – Mpumalanga, FS – Free State, NW – North West, GP – Gauteng, EC – Eastern Cape, LP – Limpopo, NC – Northern Cape, WC – Western Cape

Figure 3.2 Overall HIV prevalence by province, South Africa 2012. (Image source: South African National HIV Prevalence, Incidence and Behaviour Survey, 2012. (Shisana et al. 2014)

3.1.2 Study participant recruitment

3.1.2.1 Healthcare clinics

The majority of participants were recruited from Heidedal Primary Healthcare Clinic in Bloemfontein with 3 and 5 participants recruited from MUCCP and National Hospital Clinics respectively. All of these clinics serve as ART initiation sites as well as general primary healthcare clinics within the Mangaung Metropolitan Municipality health district. Heidedal Primary Healthcare Clinic is located on the site of the Pelonomi Regional Hospital, which serves as a secondary level referral hospital for the surrounding areas. Laboratory work was undertaken at the University of the Free State Virology Department located at the Universitas Hospital Complex site. The clinics are all within a 15 minute commute of the laboratory, thus providing fantastic opportunity for easy transport and rapid processing of blood specimens after venepuncture.

3.1.2.2 Study participant recruitment criteria

This study aimed to investigate antigen-specific T cell immunity within a community with high HIV and TB prevalence. In order to evaluate the effect of HIV-1 infection on the antigen-specific immunity, it was necessary to recruit HIV-uninfected individuals as a comparative group.

Inclusion criteria

The aim of recruitment was to include healthy HIV-infected and HIV-uninfected individuals with no other concurrent illnesses. HIV-infected individuals were included so as to reflect varied levels of HIV-1 disease progression. Individuals enrolled on the ART program at the clinics mentioned were recruited prior to commencing their ART initiation. HIV-uninfected individuals, as well as HIV-infected individuals with less

advanced HIV-1 disease progression (as reflected by a CD4 T cell count above 350 cells/uL) that did not meet criteria for ART initiation, were identified through the HIV counselling and testing services at the clinics involved. All participants were tested for HIV-1 using a point-of-care "HIV-1 rapid test" or laboratory-based HIV-1 ELISA. HIV-uninfected individuals were re-tested for HIV seropositivity at both the 6 and 12 month follow-up visits.

All participants recruited to the study were informed in their own language, provided written consent for inclusion and were longitudinally followed up at 6 months and 12 months after baseline sampling, where possible. Samples were labelled with study number only to ensure anonymity.

Exclusion criteria

Exclusion criteria at recruitment included active TB, current AIDS-defining illness, pregnancy, previous initiation on ART, current use of immunosuppressive medication and age younger than 18 years. Exclusion criteria during the follow-up period (for the purposes of the analyses in this thesis) were development of active TB and non-compliance on ART.

Active TB was excluded based on a TB symptom-orientated clinical history and physical examination (Getahun et al. 2011; WHO 2011b). Any individuals requiring further investigation for exclusion of active TB were referred to the clinic doctor. All other HIV-infected individuals were initiated on Isoniazid prophylaxis as per South African national guidelines (South African National Department of Health 2010a; South African National Department of Health 2013b).

Figure 3.3 Schematic showing 6 monthly longitudinal cohort sampling.

3.1.3 Cohort stratification by CD4 count

The studies in this thesis focus on virological and immunological characteristics of study participants at different stages of HIV-1 disease. As a result the cohort was stratified by CD4 cell count, which is detailed further in the following chapters.

3.2 Baseline clinical data

261 participants were enrolled in the study between May and July 2012. 243 (93.1%) participants were South African, 14 (5.4%) from Lesotho, 1 (0.4%) Zimbabwean, 1 (0.4%) Bangladeshi and 2 (0.8%) of unknown nationality. Many Basotho live, work or seek healthcare in the Free State due to the near proximity and perceived superior opportunities and health facilities in South Africa. 231 (88.5%) of the participants were Black African, 29 (11.1%) were Coloured and 1 was Asian (0.4%) whilst the predominant first languages spoken by participants were Sesotho, isiXhosa, Setswana and Afrikaans respectively. The study characteristics broadly represent the racial and ethnic composition of the Mangaung Metropolitan Municipality as well as the wider Free State population (Statistics South Africa 2014).

214 study participants were HIV-infected whilst 47 participants were HIV-uninfected at study enrolment. CD4 counts reflected the full range of HIV-1 disease progression

(Table 3.1). Unsurprisingly, most HIV-infected individuals had lower CD4 T cell counts, were more likely to be on INH prophylaxis and were more likely to have had previous active TB than the HIV-uninfected individuals (Fig. 3.4, Table 3.3; P<0.05 for all comparisons). Interestingly the median age of HIV-infected study participants was 10 years older (Table 3.4, P<0.0001) and the proportion of women higher (Table 3.4, P = 0.07), than that of the HIV-uninfected control group. This occurred due to the fact that there was an over-representation of young male patients attending HIV-1 voluntary counselling and testing at the recruitment clinics, due to the governmental circumcision program. I did not believe that the differences between the study and control groups had any bearing on the validity of any immunological comparisons made in this thesis.

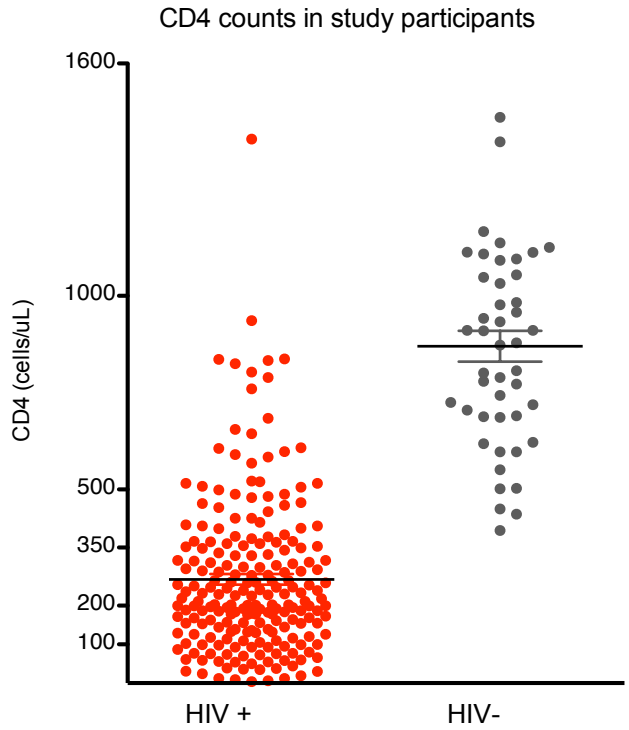


Figure 3.4 CD4 counts in HIV-infected and HIV-uninfected study participants (cells/uL)

Table 3.1. Baseline characteristics of all study participants

Characteristic	Total cohort	HIV-	HIV +				
	n= 261	n= 47	**All HIV+** n= 214	**CD4 >350** n= 55	**CD4 201 -350** n= 61	**CD4 101 – 200** n= 58	**CD4 <100** n= 40
Sex							
Female - n (%)	154 (59)	22 (47)	132 (62)	41 (75)	39 (64)	27 (47)	26 (65)
Male - n (%)	107 (41)	25 (53)	82 (38)	14 (25)	22 (36)	31 (53)	14 (35)
Age (years)[a]	34 (27 – 42)	25 (20 – 34)	35 (29 – 44)	35 (30 – 44)	34 (28 – 45)	35 (29 – 43)	35 (29 – 44)
CD4 count (cells/uL)[a]	263 (156 – 506)	876 (688 – 1064)	219 (132 – 354)	482 (399 – 606)	260 (231 – 302)	163 (133 – 187)	59 (31 – 79)
Viral load (RNA copies/ml)[a]	N/A	N/A	75136 (16045 – 275256)	14152 (5025 – 14152)	60990 (17234 – 205113)	132740 (34152 – 362089)	240373 (101579 – 624643)
BMI (kg/m^2)[a]	24.3 (22.3 – 27.7)	24.7 (22.8 – 28.8)	24.3 (22.1 – 27.3)	26.4 (22.5 – 31.9)	24.0 (22.1 – 27.5)	24.0 (22.3 – 26.7)	22.8 (20.4 – 20.7)
Prev TB - n (%)	17 (7)	0 (0)	17 (8)	6 (11)	6 (10)	3 (5)	2 (5)
BCG scar - n (%)	140 (54)	21 (45)	119 (56)	34 (62)	31 (51)	38 (68)	16 (40)
INH prophylaxis - n (%)	78 (30)	0 (0)	78 (36)	15 (27)	25 (41)	24 (43)	14 (35)
Time on INH prophylaxis[a b]	14 (7 – 28)	0 (0)	14 (7 – 28)	56 (22 – 169)	14 (7 – 28)	13 (7 – 14)	10 (6 – 16)

[a] Data expressed as median (interquartile range). [b] Duration of INH prophylaxis in individuals on IPT at the time of baseline sampling

Table 3.2 Comparison of clinical characteristics of HIV+ve and HIV-ve study participants

Characteristic	HIV- n= 47	HIV+ n= 214	P value
Sex [a]			
Female - n (%)	22 (47)	132 (62)	0.072
Male - n (%)	25 (53)	82 (38)	
Age (years) [b]	25 (20 – 34)	35 (29 – 44)	**<0.0001**
CD4 count (cells/uL) [b]	876 (688 – 1064)	219 (132 – 354)	**<0.0001**
BMI (kg/m^2) [b]	24.7 (22.8 – 28.8)	24.3 (22.1 – 27.3)	0.336
Prev TB - n (%) [a]	0 (0)	17 (8)	**0.049**
BCG scar - n (%) [a]	21 (45)	119 (56)	0.324
INH - n (%) [a]	0 (0)	78 (36)	**<0.0001**

[a] Data represented as number and percentage, P values derived using Fisher's exact tests.

[b] Data represented as median (interquartile range), P values derived using Mann-Whitney tests.

3.3 Longitudinal follow-up

Every attempt was made to follow up all 261 individuals enrolled in the study at the 6 and 12 month follow-up visits and where possible, the study visit was arranged to coincide with the regular 6 monthly ART clinic visit for HIV-infected individuals. In total 189 (72%) and 172 (66%) participants attended the 6 month and 12 month follow-up visits respectively. 204 (78%) individuals attended at least 1 follow-up visit whilst 160 (61%) individuals attended all 3 visits. The details of individuals followed-up at the 6 and 12 month time points are shown in Table 3.3.

Table 3.3 Longitudinal follow-up of study participants

Time point	Total	HIV-	HIV+				
			All HIV+	CD4>350	CD4 201 - 350	CD4 101-200	CD4<100
Baseline n	261	47	214	55	61	58	40
6 months n (%)	189 (72)	30 (64)	159 (74)	46 (84)	47 (77)	45 (80)	21 (53)
12 months n (%)	172 (66)	23 (49)	149 (70)	46 (84)	48 (79)	39 (70)	16 (40)
6 and 12 months	162 (62)	19 (40)	143 (67)	44 (80)	46 (75)	39 (70)	14 (35)

3.3.1 Reasons for loss to follow-up

Over 65% of all participants that failed to attend a follow up visit did so because they were unable to be contacted, had relocated, refused to take further part in the study or were unavailable for the study appointment. A high level of poverty with a paucity of formal housing and local work opportunities causes the local community served by the recruitment clinics to be extremely mobile and thus affected our ability to trace study participants after enrolment.

The loss to follow-up was greatest amongst HIV-uninfected individuals as well as HIV-infected individuals with CD4 T cell counts below 100cells/uL. Within the former group this occurred mostly due to refusal to further participate in the study and within the latter due to the development of active TB and increased mortality during the study. I perceived the HIV-uninfected individuals (unlike the HIV-infected individuals on or being follow-up in preparation for ART) to have little purpose in travelling to the clinic site in addition to that for study participation, thus affecting follow-up rates.

3.3.1.1 Deaths

18 study participants died during the duration of the follow-up period. 16 deaths occurred within 6 months of study enrolment and 2 deaths occurred between 6 and 12 months of follow up. All of the individuals that passed away were HIV-infected - 13 had CD4 T cell counts below 100 cells/uL at study enrolment, 4 had CD4 T cell counts 100-200 cells/uL and 1 individual had a CD4 T cell count of above 200 cells/uL (253 cells/uL). Determining the cause of death was very challenging. The next of kin of all deceased participants were contacted and, where appropriate, visited by the study nurse. 2 deceased participants had active TB as a contributing cause of death, 4 had pneumonia, 1 carcinoma, 1 chronic diarrhoea and 1 individual was killed in a motor vehicle accident. We were unable to get conclusive information on causes of death for 9 deceased study participants. In total, of the 167 ART-eligible individuals enrolled at baseline, 17 (10%) individuals died within 1 year of initiating ART due to presumed HIV-associated causes of death. Rates of early mortality on ART have been found to be up 3 fold higher in low- and middle-income countries, such as South Africa, when compared to high-income countries (Braitstein et al. 2006). These higher rates are associated with more advanced HIV-stage at presentation, higher incidences of communicable diseases as well as poor diagnostics and availability of health care resources (Abo et al. 2013). Mortality rates within a year of ART initiation in sub-Saharan Africa range between 8% and 26% depending on the study population (Lawn et al. 2008). The most commonly reported causes of early mortality post-ART include tuberculosis, wasting, advanced HIV and chronic diarrhoea, whilst factors associated with early mortality include: low baseline CD4 count, advanced WHO clinical stage, low body mass index and pre-ART quantitative HIV-1 RNA (Gupta et al. 2011).

3.3.1.2 Participants excluded from study

In total, 9 participants attending the 6 month follow-up visit were excluded from further participation in the study – 7 developed active TB and 2 defaulted ART soon after ART initiation. 1 additional participant was excluded at the 12 month visit due to ART default.

Of the 7 individuals that developed active TB during the study period, all developed TB within 2 months of initiating ART. These cases are likely to represent TB-immune reconstitution inflammatory syndrome (TB-IRIS) cases and highlights the need for thorough screening of immune-compromised individuals initiating ART for active TB (Meintjes et al. 2008).

Table 3.4 Reasons for loss to follow-up during study period

Reason	6 months Total - 72	12 months Total - 89
Death	16	18
Excluded from study	9	10
Refusal	20	24
Relocated	7	7
Unable to contact or unavailable for clinic visit	20	30

3.3.2 HIV-1 seroconversion of 2 individuals initially recruited in HIV-uninfected control group

Of the HIV-uninfected individuals, 2 participants became HIV-infected during the first 6 months of the study. Both individuals were followed up by the ART initiation team at the clinic and 1 individual initiated ART by the conclusion of the study.

3.3.3 ART initiation

159 HIV-infected individuals were seen at the 6 month follow-up visit and 118 individuals had been initiated on ART for a median duration of 196 days. 108 of the 113 individuals with a baseline CD4<350 (that were eligible for ART) had actually initiated ART along with 8 individuals with baseline CD4>350. At 12 months, 115 of the individuals followed up were on ART with a median duration of ART of 388 days. All 103 of the participants that had baseline CD4<350 and 12 individuals with baseline CD4>350 were part of the ART group.

All individuals initiating ART were started on 1st line therapy according to South African HIV treatment guidelines including Tenofovir (TDF), Lamivudine (3TC) and Efavirenz (EFV)/Nevirapine (NVP).

3.3.3.1 Incidence of self-reported adverse effects on ART

All study participants on ART were asked to report any adverse effects associated with ART over the preceding 6 month period. At least one adverse effect including a skin rash, nausea, dizziness, gastric upset and paraesthesia was reported by 8 individuals after 6 months of ART and 1 individual after 12 months.

3.3.3.2 ART adherence

ART adherence was assessed by a self-reported questionnaire and HIV-1 plasma viral load (pVL) measurement in all individuals on ART. Whilst self-reported adherence is prone to over-estimation of drug adherence, the data are interesting none-the-less. At the 6 month time point 93% (110/118) of individuals on ART reported that they "never" missed a dose, 4% (5/118) reported that they "occasionally" miss a dose and 2% (2/118) reported that they "regularly" miss a dose. Figures were similar at 12

months when 93% (107/115) reported that they “never” miss a dose, 4% (4/115) reported that they “occasionally” miss a dose and 4% (5/115) reported that they “regularly” miss a dose.

Current South African and WHO guidelines suggest HIV-1 viral load monitoring at 6 and 12 months after ART initiation and then yearly thereafter to measure ART treatment response and screen for virological failure (South African National Department of Health 2013b; WHO 2013a). A plasma viral load (pVL) above a threshold of 1000 HIV-1 RNA copies/mL on two separate occasions at least 3 months apart (after adequate adherence support) is used to define virological failure (WHO 2013a). Whilst this level may be practical within a poorly resourced setting such as Bloemfontein, guidelines in the United Kingdom, and other parts of the developed world, define virological failure in the same terms but with a pVL level above 400 copies/mL (Williams et al. 2014). Ideally a pVL below detectable limits of most conventional assays (<50 copies/mL) is recommended as the current standard of care.

Within our cohort, 133 individuals initiated ART during the course of the study and were followed up at 6 months, 12 months or both time points (Fig. 3.5). 86% (101/118) of individuals on ART at 6 months and 90% (104/115) of individuals on ART at 12 months had pVL below 1000 copies/mL (Table 3.5). When a more stringent pVL level of 400 copies/mL is employed, 83% and 90% of individuals at the 6 and 12 month time points respectively achieve virological suppression (Table 3.5). The proportions of individuals reaching virological suppression, at the levels defined in this chapter, are comparable to those found in ART-treated populations in the developed world (Lifson et al. 2011; Smith et al. 2004).

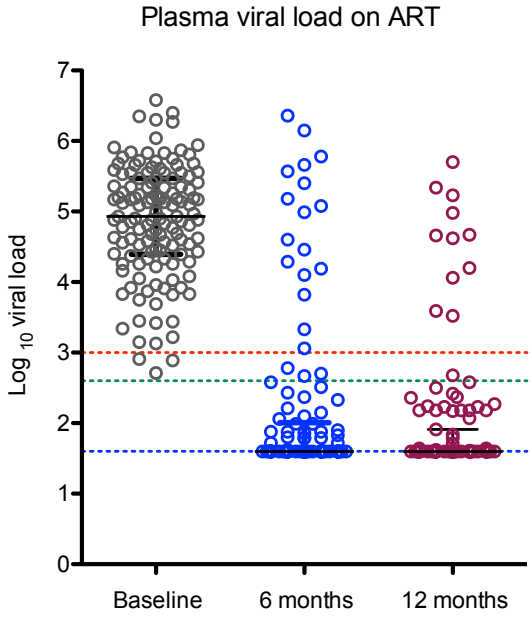


Figure 3.5 Viral loads of all individuals on ART. pVL was measured before initiation of ART and after 6 and 12 months of ART. All individuals that initiated ART and were followed up are represented in this figure (Baseline n = 133, 6 months n = 118, 12 months n = 115). The red, green and blue dotted lines indicate a pVL of 1000, 400 and 40 copies/mL respectively.

Table 3.5 Virological suppression in individuals on ART

Time point	Total number on ART at study visit	Median duration of ART Days (IQR)	Plasma Viral Load (HIV-1 RNA copies/mL)		
			< 1000 n (%)	< 400 n (%)	LDL n (%)
6 months	118	196 (184 – 202)	101 (86)	98 (83)	74 (63)
12 months	115	388 (367 – 399)	104 (90)	103 (90)	88 (77)

3.3.3.3 Changes in CD4 count and BMI on and off ART

Individuals that initiated ART had sustained increases in total CD4 T cell counts at both 6 and 12 months study visits as compared to baseline levels (Fig. 3.6a, Baseline – 6 months, $P < 0.0001$; Baseline – 12 months, $P < 0.0001$; 6 months – 12 months, $P = 0.024$). The median change in CD4 T cell count in individuals on ART after 6 months was 121 cells/uL (IQR: 49 – 216) and at 12 months it was 160 cells/uL (IQR: 43 – 258).

A concurrent increase in BMI was also observed at the 12 month time point (Fig. 3.6d, Baseline – 12 months, P = 0.035). By contrast individuals that didn't meet criteria for initiation of ART during this study, predictably exhibited decreases in CD4 count over 12 months of study observation (Fig 3.6b, Baseline – 12 months, P = 0.008) whilst no significant changes in BMI or CD4 T cell count were evident in the HIV-uninfected control group over the study period (Fig 3.6 c and f, P > 0.05 for all comparisons).

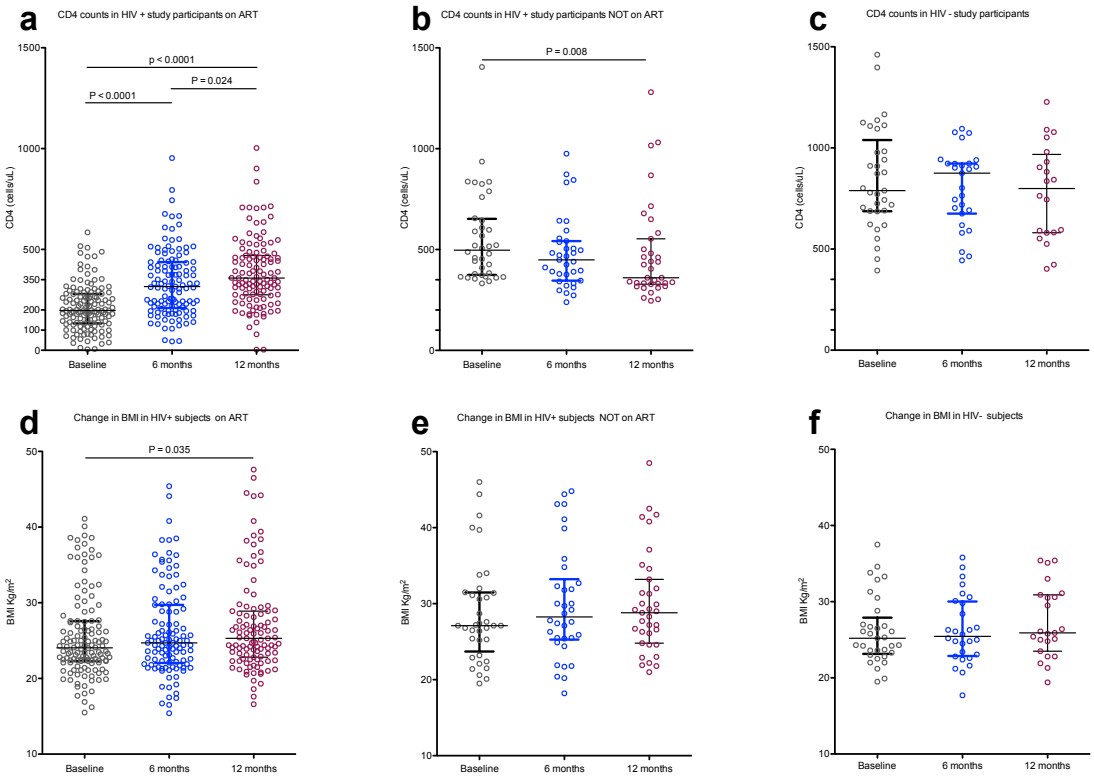


Figure 3.6 Changes in CD4 count and BMI in all individuals followed up at 6 and 12 month time points. P values indicated are for Mann Whitney tests.

3.3.4 Use of Isoniazid prophylaxis

Isoniazid preventative therapy (IPT) is recommended for all HIV-infected individuals, in whom active TB has been reasonably excluded (South African National Department of Health 2013b). Within our cohort 36% (78/214) of HIV-infected study participants were on IPT at the baseline visit for a mean duration of 39 days (95% CI 23.7 – 54.4).

67% (107/159) of HIV-infected individuals followed up at 6m and 77% (114/149) of HIV-infected individuals followed up at 12m had received or were receiving IPT at the time of sampling (6 months, mean: 153.0 (95% CI 2141.8 – 164.2) days; 12 months, mean: 181.2 (95% CI 176.5 – 185.9) days).

4 THE EFFECT OF CD4 T CELL DEPLETION ON CD8 T CELL-MEDIATED HIV-1 VIROLOGICAL CONTROL

4.1 Background

Most untreated individuals infected with HIV-1 inevitably progress to AIDS. Progression is associated with increased susceptibility to opportunistic infections, including tuberculosis, and has implications for increased transmission. By contrast, 1% of HIV-infected individuals achieve virological control below the level of detection of conventional assays and can thus be termed “elite controllers” (ECs) (Deeks & Walker 2007). Understanding the mechanisms of HIV-1 disease pathogenesis and consequently the correlates of a protective immune response will aid in the design of HIV-1 vaccines and other interventions. Of growing additional interest is the development of immune strategies to aid in the eradication of latently infected CD4 T cells, useful in potential HIV-1 cure strategies (Siliciano & Siliciano 2013).

Studies of ECs reveal an over-representation of certain alleles such as HLA-B*57 within HLA class 1 loci, suggesting that an effective HIV-specific CD8 T cell response is associated with non-progression. Indeed, the role of CD8 T cells in control of HIV-1 infection is well described (Walker et al. 1986; Schmitz et al. 1999; Kawashima et al. 2009; Goulder & Walker 2012). Within the HIV-specific CD8 T cell response, CD8 T cells directed at HIV-1 Gag epitopes appear to play a superior role in limiting progression as demonstrated by both Kiepiela et al. and Jia et al. in different populations (Kiepiela et al. 2007; Jia et al. 2012). This effect appears to be due to both early cell processing of Gag protein antigens and functional constraints formed by mutations within Gag CD8 epitopes that result in fitness costs for the virus (Tenzer et

al. 2009; Miura et al. 2009). In addition, HIV-specific CD8 T cells in ECs appear to be superior in terms of proliferative and cytolytic ability as well as polyfunctionality (Moir et al. 2011).

Interestingly, high-frequency HIV-specific CD8 responses are present in AIDS too, reinforcing the fact that CD8 T cells differ in their ability to inhibit viral replication. This is partly explained by the fact that although CD8 T cells in AIDS do appear to be functional, they are less differentiated, have reduced cytotoxicity, varied avidity and less polyfunctionality (Addo et al. 2007; Walker et al. 1987).

A hallmark of chronic HIV-1 infection is the quantitative and qualitative decline in CD4 T cell responses which potentially impacts on HIV-specific CD8 T cell responses (Matloubian et al. 1994; Kalams & Walker 1998; Altfeld & Rosenberg 2000). However, little is known of the effect of CD4 T cell loss on the ability of the HIV-specific CD8 T cell responses to inhibit HIV-1 viral replication. Some studies have suggested that with advancing HIV-1 disease, CD8 T cell responses are directed at Env rather than Gag, thereby reflecting the changing pattern of immune dominance with disease progression (Kiepiela et al. 2007; Jia et al. 2012; Altfeld et al. 2006).

In this chapter I investigate the patterns of HIV-specific CD8 T cell responses that are associated with control of HIV-1 viraemia in untreated individuals with chronic HIV-1 infection. I proceed to explore the impact of disease progression, as defined by a declining CD4 T cell count, on such HIV-specific CD8 T cell responses.

4.2 Hypothesis

Progressive CD4 T cell depletion in chronic HIV-1 disease results in a weakening of CD8 T cell-mediated immune selection pressure and loss of viral control. As risk for opportunistic infection is associated with pathogen-specific CD4 T cell loss, I hypothesize that loss of viral control leads to heightened risk for development of opportunistic infections such as tuberculosis.

4.3 Experimental aims

1. To study the specificity and breadth of HIV-specific CD8 T cell responses associated with HIV-1 viral control by mapping CD8 T cell responses to all expressed HIV-1 proteins through the use of ex vivo IFNγ CD8 T cell ELISPOT assays.
2. To study the effect of CD4 T cell decline on HIV-specific CD8 T cell responses by:
    a. Analysing differences in patterns of HIV-specific ex vivo IFNγ CD8 T cell ELISPOT responses in individuals stratified by CD4 T cell count
    b. Evaluating the CD8 T cell-imposed selection pressure in viral HIV-1 Gag sequences in two groups of HIV-infected individuals with contrasting CD4 T cell counts

4.4 Results

The results in this chapter are presented with an initial analysis of HIV-specific IFNγ ELISPOT responses across the whole HIV-1 proteome. Associations between specificity of ex vivo IFNγ CD8 T cell ELISPOT responses and HIV-1 viral load are investigated. The influence of declining CD4 T cell count on the HIV-specific CD8 T cell

response is then evaluated by stratifying individuals based on CD4 count. Finally an additional cohort of HIV-infected individuals is investigated in order to determine whether CD4 T cell decline is associated with loss of CD8-mediated selection pressure in the HIV-1 Gag gene.

4.4.1 IFN-γ ELISPOT assay reveals CD8 T cell responses across the entire HIV-1 Clade C proteome

IFN-γ ELISPOT assays were used to assess CD8 T cell responses across the whole HIV-1 Clade C proteome in 163 HIV-infected participants. 9 participants were excluded from the analysis as they had indeterminate results due to high background in the negative wells and 3 participants were found to have active TB shortly after recruitment and were thus excluded. The characteristics of the individuals included in this analysis are shown in Table 4.1 below. Individuals were stratified by CD4 T cell count, reflecting different stages of HIV-1 disease progression.

Table 4.1 Characteristics of HIV-infected individuals included in the IFNγ ELISPOT analysis

Characteristic	**Total HIV positive**	**HIV positive**			
		CD4 >350	**CD4 201 -350**	**CD4 101 – 200**	**CD4 <100**
	n= 151	n= 39	n= 41	n= 45	n= 26
Gender					
Female - n (%)	96 (64)	28(72)	27(66)	22(49)	19(73)
Male - n (%)	55 (36)	11(28)	14(34)	23(51)	7(27)
Mean age (years)	37.0	36.7	36.3	37.0	38.3
Mean CD4 count (cells/uL)	261.3	505.3	268.6	159.8	59.3
Viral load (RNA copies/ml)	249027.7	59254.3	162205.6	224965.9	704945.8

4.4.1.1 All HIV-1 proteins and the majority of HIV-1 peptides are targeted by HIV-1 –specific CD8 T cell responses

Of the 414 overlapping peptides (OLPs) used in the IFNγ ELISPOT assays, 311 (75%) were targeted by at least one individual, suggesting that most of the expressed HIV-1 proteome is able to elicit HLA class 1 restricted CD8 T cell responses. However, the number of individuals targeting peptides within each HIV-1 protein varied and clustered within immunodominant regions (Figure 4.1). Based on any peptide being recognised within each protein Pol, Nef, Gag and Env were targeted most frequently in 90.85%, 88.24%, 79.74% and 78.43% of the entire cohort respectively. Vpu and Tat were least frequently recognized at 4.58% and 24.18% respectively followed by Rev at 30.72% and Vif at 51.63% (Fig. 4.2a). When the frequency of recognition of the protein was adjusted for the size of the protein (by dividing it by the amino acid length) Nef, Vpr, Vif and Rev were most frequently targeted (Fig. 4.2b).

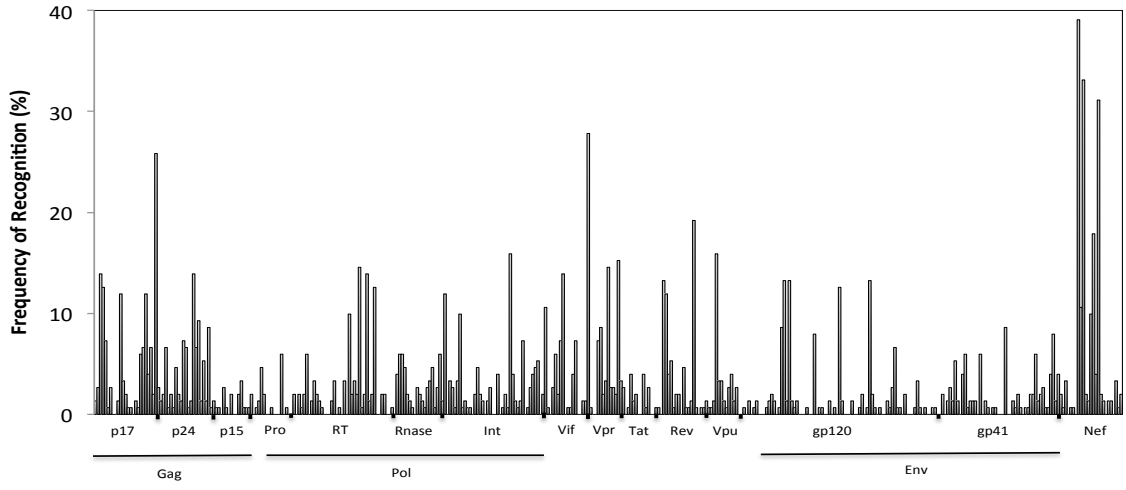


Figure 4.1 CD8 ELISPOT responses of 151 individuals across the whole HIV Clade C proteome. The figure shows the frequency with which each overlapping peptide (OLP) was recognized by the study population.

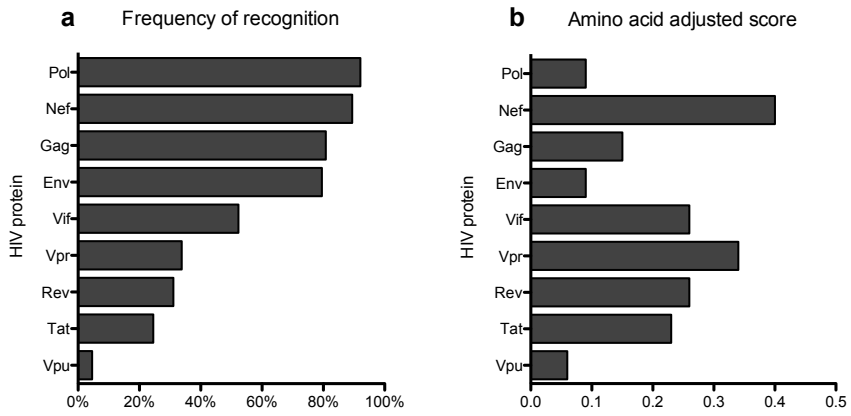


Figure 4.2 Frequency of HIV-1 protein recognition as measured by IFNγ ELISPOT assay a) The frequency of recognition of each HIV-1 protein. b) The amino acid adjusted score for HIV-1 protein recognition

The percentage of individual peptides targeted within each protein and each protein-subunit varied considerably with 100% (32 of 32) of peptides within Gag p24 being recognised at least once but only 50% (10 of 20) of peptides within Protease (Table 4.2). The individual peptides were targeted at different frequencies with the most commonly targeted peptides all being located in the highly conserved Nef protein (Table 4.3).

Table 4.2 The number of peptides recognised at least once per HIV protein/protein subunit

Protein	Protein subunit	No of overlapping peptides recognized per protein/subunit (%)
Gag		**58/66 (88)**
	p17	13/16 (81)
	p24	32/32 (100)
	p15	13/18 (72)
Pol		**98/133 (74)**
	Protease	10/20 (50)
	Reverse transcriptase	58/76 (76)
	Integrase	30/37 (81)
Vif		**18/24 (75)**
Vpr		**9/11 (82)**
Tat		**11/12 (92)**
Rev		**11/14 (79)**
Vpu		**5/9 (56)**
Env		**73/113 (65)**
	gp120	34/46 (74)
	gp41	39/67 (58)
Nef		**23/27 (85)**

Table 4.3 Summary of the most frequently targeted peptides in this study cohort

Rank	Protein	HXB2 Amino acid position	Peptide sequence	Study subjects with response (%)
1	Nef	65-82	EVGFPVRPQVPLRPMTYK	39
2	Nef	81-97	YKAAFDLSFFLKEKGGL	33
3	Nef	126-143	NYTPGPGVRYPLTFGWCF	31
4	Pol-Int	973-990	KVVPRRKVKIIKDYGKQM	28
5	Gag-p24	178-194	GATPQDLNTMLNTVGGH	26
6	Tat	32-49	YHCLVCFQTKGLGISYGR	19
7	Nef	112-127	LWVYHTQGFFPDWQNY	18
8	Pol-Int	737-753	MASEFNLPPIVAKEIVA	16
9	Rev	9-23	DEALLQAVRIIKILY	16
10	Vif	72-89	LQTGERDWHLGHGVSIEW	15
11	Pol-RT	288-304	PSINNETPGIRYQYNVL	15
12	Vif	41-57	RHHYESRHPKVSSEVHI	15
13	Gag-p17	17-34	EKIRLRPGGKKHYMLKHL	14
14	Gag-p24	288-305	GPKEPFRDYVDRFFKTLR	14
15	Pol-RT	306-323	QGWKGSPAIFQSSMTKIL	14

In summary these data indicate that while most of the HIV-1 proteome is able to generate HLA class-I restricted CD8 T cell responses, within our cohort the Nef protein (when adjusted for protein size) was most frequently recognised and contained the 3 most commonly recognised OLPs (Fig 4.2 and Table 4.3). Considering protein subunits, all Gag p24 peptides were recognised by at least one individual revealing it to be the most densely targeted protein (Table 4.2).

4.4.2 Associations between HIV-specific IFNγ CD8 ELISPOT responses and HIV-1 viral load

To explore the correlation between HIV-specific CD8 T cell responses and immune control of HIV-1, I used Spearman's rank order correlation analyses. I initially compared the absolute breadth and magnitude of IFNγ responses to HIV-1 viral load as described previously (Kiepiela et al. 2007; Addo et al. 2003). The breadth of response was defined as the total number of OLPs targeted by each individual and the magnitude as the total number of SFCs/10^6 PBMCs responding to each OLP. More recent approaches have proposed that the relative dominance of CD8 T cell targeting and not absolute magnitude is associated with viral control (Zuñiga et al. 2006; Jia et al. 2012). I therefore evaluated both absolute and relative contributions to the total HIV-specific CD8 T cell response in the following analyses.

4.4.2.1 HIV-1 cumulative breadth and magnitude associations with viral load

The proposed correlation between the overall magnitude of total HIV-specific CD8 T cell responses and HIV-1 viral load has shown different associations in different studies. Addo et al. and Jia et al. both used an OLP ELISPOT approach similar to that used here. Whilst Addo et al. found no significant correlations between the cumulative magnitude or breadth of the HIV-specific CD8 T cell response and viral load, Jia et al. found a positive correlation between the total cumulative magnitude and viral load (Addo et al. 2003; Jia et al. 2012). My data revealed no such significant correlations thus confirming a result from a larger study carried out in Durban, South Africa involving over 500 untreated individuals (Total HIV-1, magnitude: r = 0.089, P = 0.278, breadth: r = -0.0015, P = 0.985). (Kiepiela et al. 2007).

4.4.2.2 HIV-1 protein-specific associations with viral load: absolute breadth and magnitude

The correlations between the breadth and magnitude of protein-specific ELISPOT responses and HIV-1 viral load were then assessed. The only significant association between the magnitude of HIV-1-protein specific CD8 T cell responses and viral load was a positive correlation between Env-specific responses and viral load (Env – magnitude: r = 0.0475, P = 0.0072). The correlations between the breadth of protein-specific ELISPOT responses and viral load are shown in Figure 4.3 and revealed an inverse correlation between Gag-breadth and viral load (Gag-breadth: r = - 0.2447, P = 0.0025) and a positive correlation between Env-specific breadth and viral load (Env-breadth: r =0.1920, P = 0.0182).

In order to determine the influence of CD8 T cell responses directed at individual sub-proteins within Gag and Env on viral load, Gag-specific responses were separated based on CD8 T cell recognition of p24 and p17 proteins and Env-specific responses based on recognition of gp120 and gp41 proteins. When evaluating Gag-specific responses, only the breadth of p24-specific responses had a significant inverse correlation with viral load revealing that the Gag-specific effects shown above were mainly due to targeting of the Gag p24 sub-protein (Gag p24 – breadth: r = - 0.2742, P = 0.0007). Similarly only Env-gp41-specific breadth was positively correlated with viral load (Env gp41 – breadth: r = 0.2116, P = 0.0091).

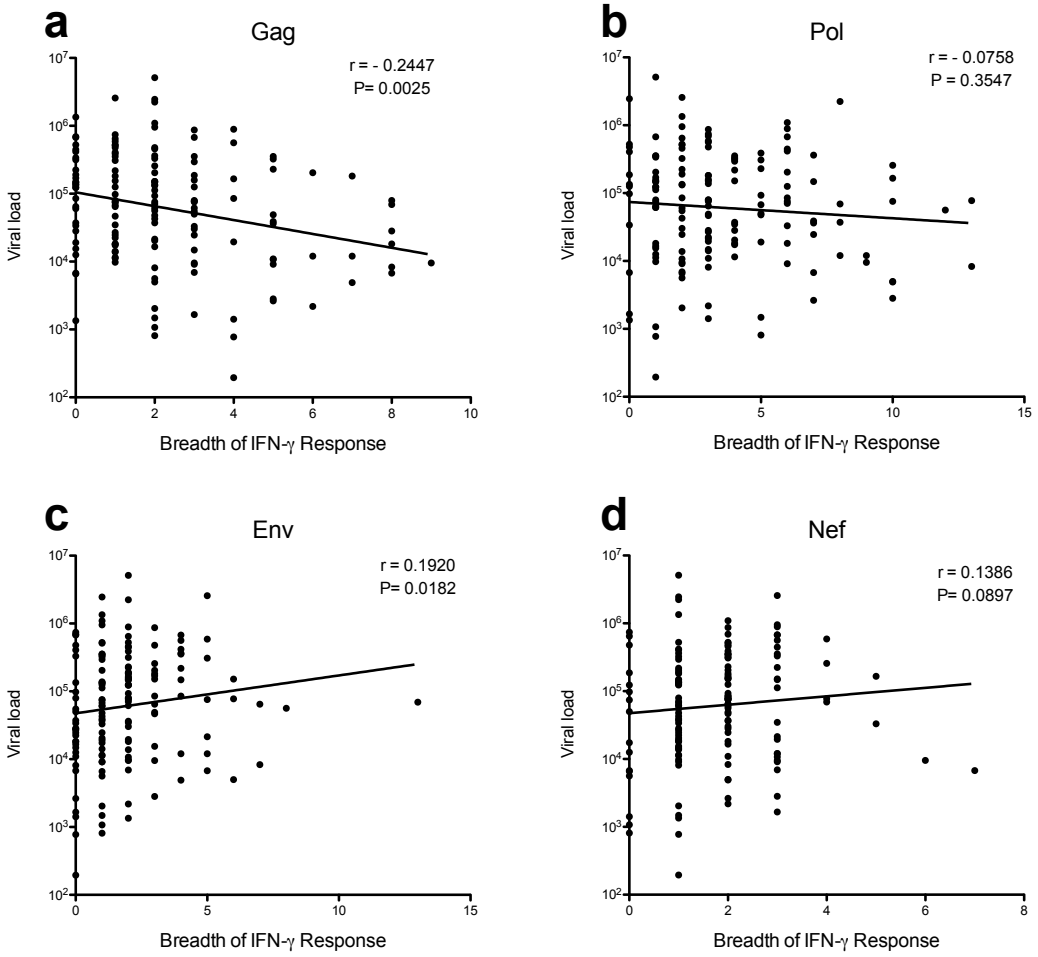


Figure 4.3 The correlation between breadth of HIV-1 protein-specific IFN-γ CD8 response and log_{10} viral load is shown in figures a – d. P values and r values are based on Spearman's rank test. Solid lines represent the linear trend.

Given the discordant associations between Gag- and Env-specific CD8 T cell responses with viral load, it was important to investigate the influence of the absolute number of epitopes targeted within these proteins on viral control (Fig 4.4). Having up to 3 Gag responses was not better than having none at all. However, having more than 3 responses was significantly better (P = 0.0089, Fig 4.4a). A similar effect on viral load was shown for Gag p24-specific CD8 T cell responses (P = 0.0006, Fig 4.4c).

By contrast, the targeting of 2 Env-epitopes was associated with a higher median viral load (P = 0.01, Fig 4.4b) than targeting none. Surprisingly this effect was not apparent when targeting 3 or more Env epitopes. This may be due to a combination of the fact that individuals with multiple Gag responses are also likely to have Env-specific responses and that Env-specific responses are driven by high levels of viraemia (Kiepiela et al. 2007). The difference in median viral load in relation to the number of Env epitope responses was more marked when gp41-specific Env epitope responses were considered. An increase in viral load with increasing number of gp41-specific responses was clear with the effect losing significance when more than 3 epitopes are targeted (P = 0.517, Fig. 4.4d).

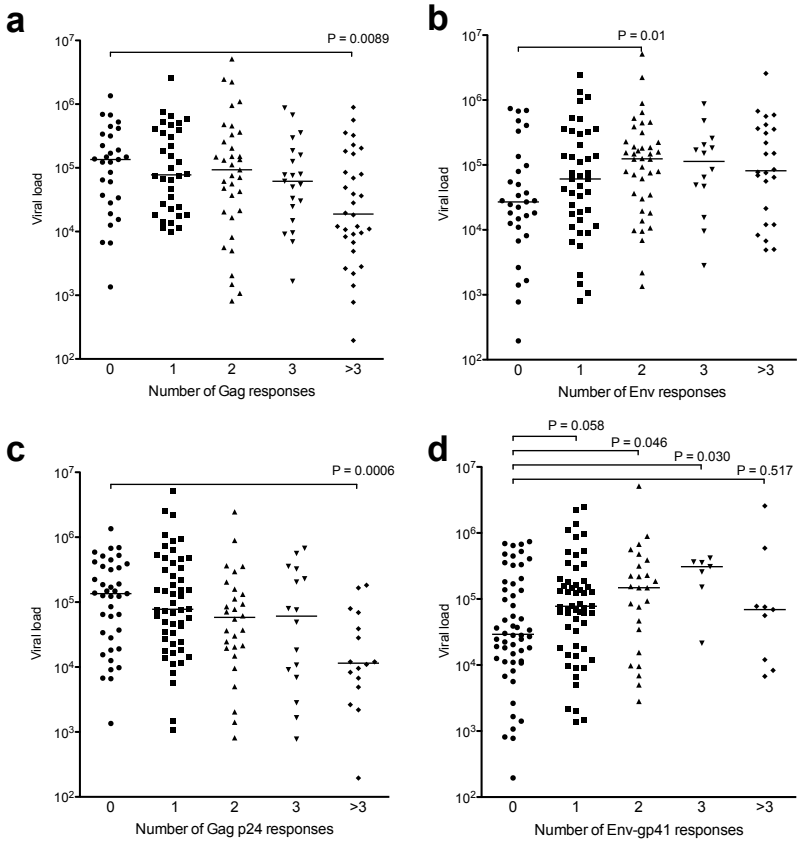


Figure 4.4 A comparison of median viral load between individuals with different no of Gag- and Env specific CD8 T cell responses. Horizontal lines show medians. P values indicated for Mann-Whitney tests

4.4.2.3 HIV-1 protein-specific associations with viral load: relative breadth and magnitude

Similar to previous studies, the relative contribution of protein-specific IFNγ ELISPOT responses to each individual's total HIV-responses (in terms of total magnitude and breadth) was calculated and measured against viral load (Jia et al. 2012; Zuñiga et al. 2006). Gag-relative breadth was inversely correlated with viral load (Gag rel breadth: r = - 0.2503 P = 0.0020) whilst Env-relative breadth and Nef-relative breadth had positive correlations (Figure 4.5 a – d). No significant associations between protein-specific relative magnitude and viral load were detectable in this data set.

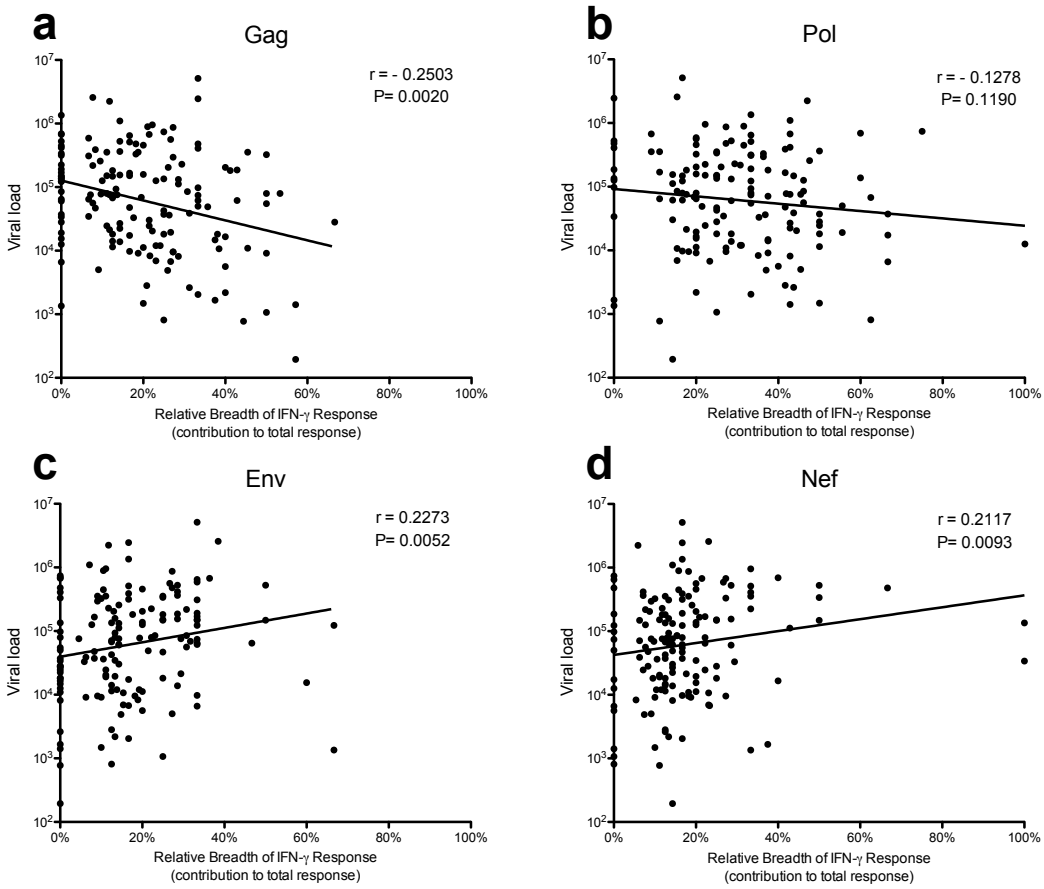


Figure 4.5 The correlation between relative breadth of HIV-1 protein-specific (proportion of total breadth of each protein) IFN-γ CD8+ response and log_{10} viral load. P values and r-values are based on Spearman's rank test. Solid lines represent the linear trend.

In summary, these data indicate that both the absolute and relative breadth of the Gag-specific CD8 T cell response are inversely correlated with viral load control and that this was shown to be mostly due to targeting of Gag p24. By contrast, the magnitude and breadth of the Env-specific CD8 T cell response, as well as the relative breadth of Nef-specific CD8 T cell response were associated with high levels of viraemia in this cohort.

4.4.3 The influence of CD4 T cell count on HIV-specific CD8 T cell responses

In order to explore the relationship between the pattern of HIV-specific CD8 T cell responses and declining CD4 T cell count, correlations between the cumulative breadth and magnitude of total HIV-specific CD8 T cell responses and CD4 T cell counts were initially explored. Within our data set, the absolute cumulative magnitude or breadth of the total HIV-specific CD8 T cell responses did not significantly associate with changes in CD4 T cell counts.

4.4.3.1 HIV-1-specific protein magnitude and breadth and CD4 T cell count

HIV-1-specific protein breadth was correlated with CD4 T cell count in order to analyse changing patterns of CD8 T cell targeting of HIV-1 proteins at different CD4 T cell counts. Gag breadth was positively correlated with the magnitude of CD4 T cell count and no other significant associations were observed (Gag-breadth: $r = 0.213$, $P = 0.0082$). To further explore this association, I compared the total Gag-breadth between the different CD4 count strata and found that there was a significant difference between the group with CD4 >350 cells/uL and those with CD4 counts <100 cells/uL (Mann-Whitney $p = 0.0043$, Fig. 4.6).

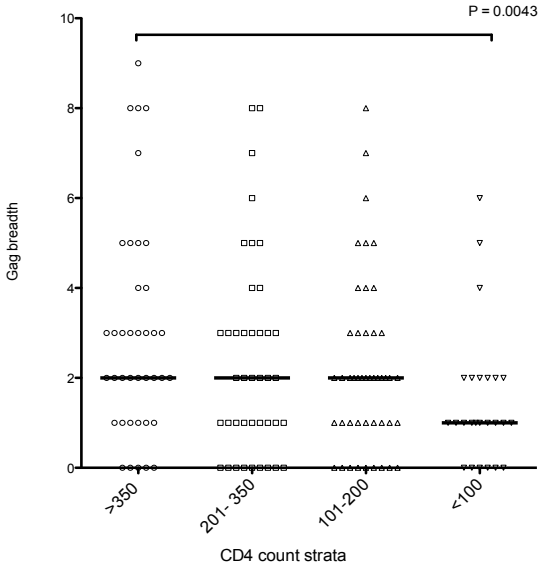


Figure 4.6 Total Gag breadth compared within each CD4 count stratum (no of Gag OLPs targeted) Horizontal bars show medians. P value is indicated for Mann-Whitney tests.

A narrowing in the overall breadth and the relative contribution of Gag to the overall breadth of responses has previously been shown with CD4 count decline to AIDS (Huang et al. 2011). Whilst the presented data supported this trend (Fig. 4.7 and 4.8), the trend was not significant (r = 0.1547, P = 0.0588).

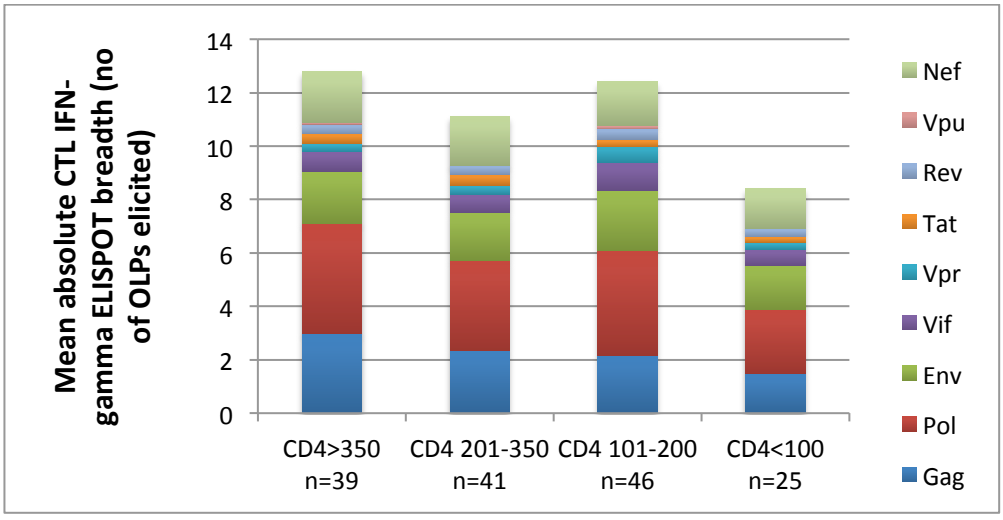


Figure 4.7 Mean absolute CD8 T cell breadth shown by CD4 count stratum. The mean contribution of each HIV-1 protein to overall HIV-specific breadth is shown.

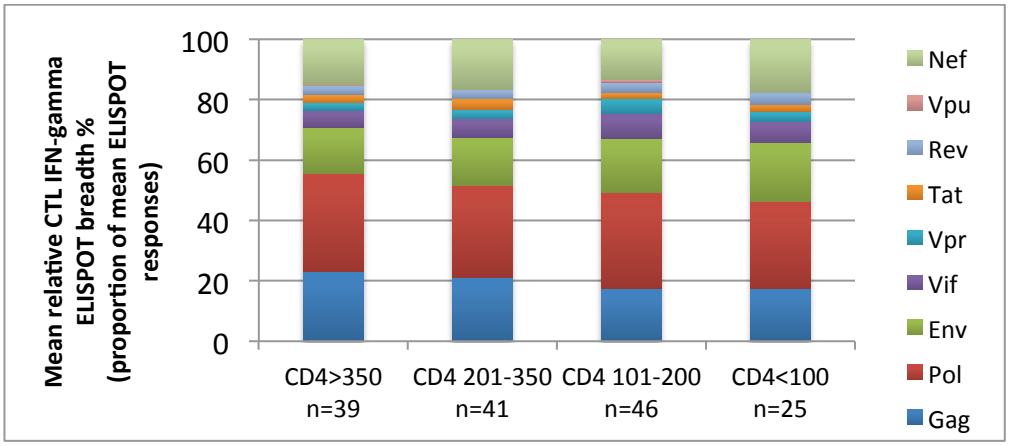


Figure 4.8 The relative contribution of each HIV-1 protein to overall breadth of HIV-1 specific CD8 T cell responses.

4.4.4 A comparison of individuals with CD4 T cell counts >350 and <100

In order to analyse the relationship between HIV-specific CD8 T cell response and CD4 T cell loss in detail, I selected the two groups of participants at both extremes of the CD4 cell count stratification (CD4 cell count >350/uL and CD4 cell count <100/uL). I initially determined whether there was a significant difference in distribution of CD8 T cell responses to particular OLPs and then examined the differences in magnitude and breadth of HIV-1 protein-specific CD8 T cell responses between the groups.

4.4.4.1 Targeting of Gag OLP AFSPEVIPMFTALSEGA associated with high CD4 count

414 single OLP-specific CD8 T cell responses were compared between the two groups using Fisher's exact tests and only one OLP was targeted significantly differently. Responses to 18mer OLP AFSPEVIPMFTALSEGA (Gag residues 167 – 179) were shown to be significantly better represented in the group with CD4 >350 ($P = 0.0367$), however this difference became insignificant when corrected for multiple comparisons. I can therefore find no evidence to suggest differential targeting of specific epitopes based on CD4 T cell count.

4.4.4.2 Breadth and magnitude of HIV-specific CD8+ responses

The breadth and magnitude of CD8 T cell responses to HIV-specific proteins were compared between the two groups using Mann-Whitney tests. The proteins measured were restricted to those previously shown to have a differential pattern of recognition in the earlier analyses. The group with CD4 counts >350 had greater total HIV-specific breadth, Gag-breadth and Pol-Breadth (Table 4.4). There were no associated differences as evaluated by total HIV-specific magnitude or by protein-specific relative magnitude and relative breadth between the two groups.

Table 4.4 The differences in HIV-1 protein-specific CD8 T cell responses: CD4 350 vs. CD4 <100

		CD4 >350	CD4 <100	P Value
CD8 T cell breadth	**Total**	12.79	8.4	**0.035***
(Mean no of OLPs	Gag	2.97	1.98	**0.004***
recognised)	Pol	4.12	2.4	**0.025***
	Env	1.95	1.64	0.865
	Nef	1.90	1.48	0.427
CD8 T cell magnitude	**Total**	2978.11	2072.75	0.093
(total SFCs/10^6)	Gag	1252.63	1145.25	0.075
	Pol	977.01	659.19	0.208
	Env	510.84	636.26	0.232
	Nef	638.47	672.85	0.308

P value is shown for Mann-Whitney tests. * P <0.05

4.4.5 Identification of HLA Class I linked polymorphisms in HIV-1 Gag at different extremes of HIV-1 disease is suggestive of changing selection pressure

In light of the dominant role of Gag-directed CD8 T cell responses in suppressing viral load, the influence of CD4 T cell decline on CD8 T cell control of HIV-1 was further investigated through examining whether CD4 T cell decline was associated with a loss of CD8 T cell-mediated selection pressure on the virus. Therefore HIV-1 Gag sequences from a second cohort of individuals that represent two extremes of HIV-1

disease progression were analysed. 100 Gag sequences from participants with low CD4 counts (CD4<50) and 92 Gag sequences from participants with high CD4 counts (CD4>350) were obtained and compared for the presence of escape mutations in cognate CD8 T cell epitopes. The characteristics of participants in this cohort are shown in Table 4.5.

Table 4.5 Characteristics of individuals included in the Gag sequence analysis

Characteristic		**Total (n=205)**	**CD4 >350 (n=103)**	**CD4 <50 (n=102)**
Gender	Female - Number (%)	133 (64.9)	60(58.2)	73(71.6)
	Male - Number (%)	72 (35.1)	43 (41.8)	29(28.4)
Mean age (years)		35.8	34.6	37
Prev OI or AIDS defining illness - Number (%)		19(9.3)	0	19(18.6)
Mean CD4 count (cells/uL)		263.7	489	24.8

HIV-1 Gag sequences were typed using REGA HIV-1 Subtyping Tool version 2.0 revealing 186 Clade C, 4 Clade B and 2 indeterminate sequences. Sequences were placed on a phylogenetic tree to test for PCR contamination for quality control assessment and to measure alignment with other Southern African consensus sequences. The non-Clade C sequences were removed from further analysis.

Due to the fact that the HLA information of these individuals is unknown, a restricted sequence analysis was carried out for the presence of known HLA class I linked polymorphisms associated with HLA Class I alleles that have previously shown to distribute differentially across extremes in CD4 count in a cohort of similar ethnic and geographic background (Kiepiela et al. 2007; Huang et al. 2009; Frater et al. 2007). The analysis was focused on polymorphisms in CD8 T cell epitopes associated with

HLA class I alleles previously shown to predominate with higher CD4 counts (HLA-B 5703 and HLA-B 8101).

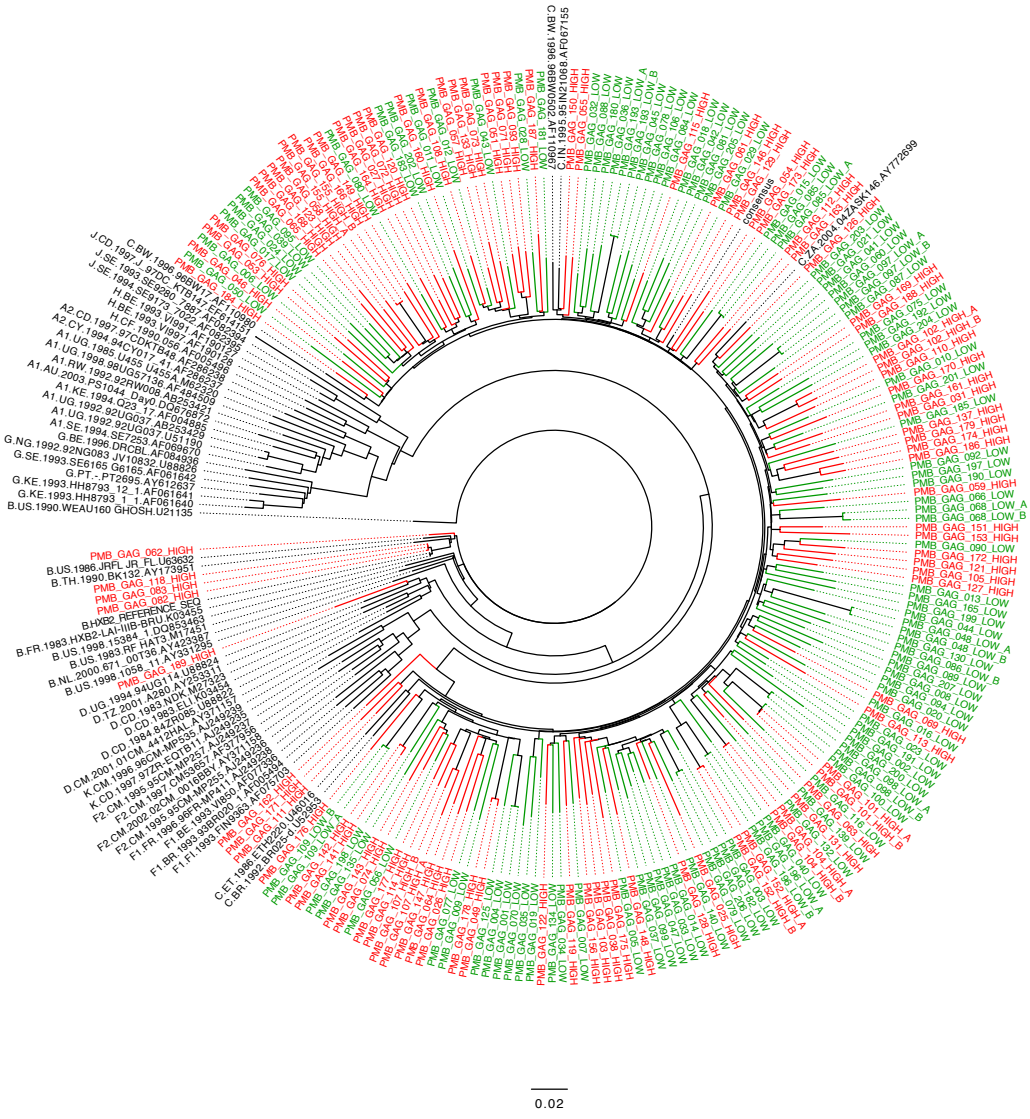


Figure 4.9 Phylogenetic tree showing HIV-1 Gag sequences from the study population. Sequences are shown from 100 individuals with CD4 counts <100 cells/uL and 92 individuals with CD4 counts >350 cells in the context of other Group M Clade consensus sequences

A significantly greater number of individuals with low CD4 counts were shown to have the A146P escape mutation in HLA-B 5703 restricted epitope ISPRTLNAW (ISW-9) (Fisher's exact P = 0.040). This is explained by the lack of an associated fitness cost with the mutation which precludes reversion back to the wild-type sequence (Draenert et al. 2004). By contrast individuals with high CD4 counts had a greater

number of T186S combined with Q182S escape mutations in the HLA-B 8101 restricted epitope TPQDLNTML (TL-9) (Fisher's exact P = 0.010). The T186S mutation in the HLA-B 8101 restricted TL-9 epitope is associated with a fitness cost which is not completely compensated for by the accompanying Q182S mutation (Wright et al. 2012). Therefore it appears that loss of CD4 T cells in the low CD4 T cell group is associated with a loss of CD8-mediated HIV-Gag-specific selection pressure and consequent reversion of the costly T186S mutation. These data are compatible with different strengths of selection within different CD4 T cell count strata.

Table 4.6 Mutations in Gag HIV-1 sequences at known HLA Class I CD8 T cell epitopes

	Epitope Abbreviation	**Mutation**	**CD4 > 350 n = 87**	**CD4 < 50 n = 99**	**P value**
HLA-B 5703					
	TSTLQEQIAW	T242N	10	14	0.151
	TW-10	T242S	2	1	0.451
	ISPRTLNAW	A146X	19	35	**0.017***
	ISW-9	A146P	16	28	**0.040***
		A146S	3	7	0.148
	KAFSPEVIPMF	A163X	8	14	0.107
	KF-11	A163N	4	7	0.194
		A163G	0	1	0.532
		A163S	3	1	0.263
		A163G and S165N	1	4	0.186
		A163G and S165K	0	1	0.532
		S165X	5	6	0.243
HLA-B 8101					
	TPQDLNTML	T186S	3	4	0.293
	TL-9	T186S and Q182S	6	0	**0.010***
		Q182X	19	12	0.057

Differences in frequencies of escape mutations are shown between individuals in Cohort 2 with CD4 cell counts >350/uL and <50/uL. P values are shown for Fisher's exact tests. * P <0.05

4.5 Discussion

HIV-specific CD8 T cell responses are believed to play a significant role in control of HIV-1 viraemia. The objectives of this study were to investigate the pattern of HIV-specific CD8 T cell responses in a cohort of African patients at varying stages of HIV-1 disease progression, to correlate them with HIV-1 viral control and to explore the effect of CD4 T cell loss on such responses.

By employing a HIV-1 whole-proteome IFNγ ELISPOT approach we were able to show that although all HIV-1 proteins are targeted by HLA-class 1 restricted CD8 T cells, when adjusted for protein size, Nef was targeted most frequently. Indeed the three most commonly recognised peptides were all Nef OLPs, a finding supported by other studies (Almeida et al. 2011; Addo et al. 2003). When correlating HIV-1 protein-specific CD8 T responses to HIV-1 viral load, targeting predominantly Gag was associated with viral control as has been shown in multiple previous studies (Zuñiga et al. 2006; Huang et al. 2011; Kiepiela et al. 2007; Jia et al. 2012). This effect was particularly evident for individuals with 3 or more OLP Gag responses and the superior role of Gag p24-specific CD8 T cell responses was also confirmed by these data (Zuñiga et al. 2006).

The positive correlation between the magnitude and breadth of Env-specific CD8 responses and HIV-1 viral load confirms the association of such Env responses with conditions of elevated viral load (Zhuang et al. 2008). Within this cohort relative-Nef breadth was also positively correlated with viral load. Both Huang et al. and Zuniga et al. have associated Nef-specific CD8 T cell responses to HIV-1 disease progression whilst Almeida et al showed that HIV-1 viral adaptation in chronic infection leads to

an inflation of Nef directed CD8 T cell responses relative to other proteins (Huang et al. 2011; Zuñiga et al. 2006; Almeida et al. 2011). The finding of this result within our cohort may suggest the relative advanced stage of HIV-1 infection of the majority of the cohort (mean CD4 T cell count 261 cells/uL).

In this study we used peptides from a 2006 HIV Clade C consensus, however HIV-1 has been shown to adapt at a population level to human HLA class I and as a result any given clade consensus is likely to change over time (Kawashima et al. 2009). An additional fact that may contribute to an underestimation of peptide specific response by our IFNγ ELISPOT assay is the discordance between an individual's autologous virus and the consensus peptide used, which may be particularly relevant for responses to highly variable HIV-1 proteins.

HIV-1 disease progression and susceptibility to opportunistic infection are associated with declining absolute CD4 T cell counts. Gag-breadth positively correlated with CD4 T cell count in this ELISPOT analysis indicating a reduction in Gag-breadth with declining CD4 T cell count. A comparison between individuals with CD4 >350 and CD4 <100 showed a narrowing in breadth of total HIV-specific CD8 responses in individuals with CD4 <100 and further analysis revealed this decrease to be predominantly due to a reduced breadth in Gag and Pol responses in individuals with CD4 cell counts <100. Huang et al. showed the same result in a similar cohort of individuals in Bloemfontein and Durban, South Africa thus confirming the association(Huang et al. 2011).

Whilst we were able to show that individuals with more extensive CD4 T cell loss had a narrowing of HIV-specific CD8 T cell responses this was not sufficient to infer

changes in CD8-mediated selection pressure on the virus. The analysis of HIV-1 Gag sequence data from Cohort 2, in the absence of ELISPOT data and HLA typing may show evidence for changing patterns of immune pressure at different stages of CD4 T cell loss. This analysis is limited by the absence of the details of HLA class I frequency distribution between the two groups as it relies on there being an equal distribution of the alleles for which epitope variations are tested.

Due to the cross-sectional nature of the study design and the obvious ethical limitations to study natural progression of HIV-1 infection, the presented analyses are inherently limited in their ability to precisely determine contributions of HIV-specific CD8 T cell responses to viral control. However, we extend existing knowledge of the importance of the Gag-specific CD8 T cell responses and the effect of declining CD4 T cell count on CD8 mediated HIV-1 control.

5 APPLICATION OF ELISPOT AND QUANTITATIVE PCR TO THE MEASUREMENT OF MTB-SPECIFIC IMMUNITY

5.1 Background

The immunological correlates of protection to Mtb remain largely unresolved, despite knowledge that the Mtb-specific Th_1 T cell response has been durably shown to be important (albeit not completely sufficient) in conferring host resistance (Kagina et al. 2010). IFNγ production by T cells in response to mycobacterial antigens can be detected through the use of IFNγ release assays (IGRAs), quantitative PCR and flow cytometry. IGRAs, such as the enzyme-linked immunosorbent assay (ELISA) and the enzyme-linked immunospot assay (ELISPOT), are extensively used in the measurement of Mtb-specific T cell responses and form the mainstay of diagnostics for LTBI in many parts of the world (Talati et al. 2009).

When used with antigens ESAT-6 and CFP-10, ELISPOT detects latent Mtb infection as both antigens are encoded by the RD1 genomic segment of Mtb (which is absent from all BCG strains and most environmental mycobacteria (Sørensen et al. 1995; M Harboe et al. 1996; Berthet et al. 1998)). The use of IFNγ ELISPOT in the diagnosis of LTBI has been thoroughly investigated, with some studies showing good sensitivity in HIV-1 infected populations and thereby demonstrating the presence of IFNγ-producing T lymphocytes well into advanced HIV-1 infection (Lawn et al. 2007; Chapman et al. 2002; Day et al. 2008). However, the degree to which the magnitude of Mtb-specific IFNγ T cell responses are affected by HIV-1 infection has produced conflicting results (Scarpellini et al. 2004; Chapman et al. 2002; Zhang et al. 1994). A cross-sectional study carried out by Day et al. in Durban, South Africa measured Mtb-specific CD4 and CD8 responses using Mtb over-lapping peptides (OLPs) and displayed

no relationship between breadth or magnitude of responses and HIV disease status as measured by VL and CD4 count (Day et al. 2008). However, Karam et al. found that although the sensitivity of a ESAT-6/CFP-10 ELISPOT assay to detect LTBI was retained in HIV infection, it was dependent on HIV progression with loss of sensitivity evident in severely immune-suppressed individuals (Karam et al. 2008). Interestingly, a study in The Gambia revealed that although the proportion of ELISPOT responders to ESAT-6 and CFP-10 was unaffected by CD4 T cell count, the proportion of responders to PPD decreased with a reduction in CD4 count (Hammond et al. 2008). This suggests that Mtb-specific T cell responses to different Mtb antigens, as measured by IFNγ ELISPOT may be differentially affected by HIV-1 infection. No studies thus far, have assessed the impact of HIV-1 infection on responses to potential "latency" associated antigens within the context of parameters of HIV-1 disease progression.

IFNγ ELISPOT has been shown to be particularly sensitive in detecting low-level immune responses, when compared to methods such as ELISA, flow cytometry and qPCR (Karlsson et al. 2003; Tassignon et al. 2005). However, as mentioned above, it does have limitations within the context of severe immune suppression and its sensitivity can be influenced by a variety of factors (Smith et al. 2009). A recent study using IFNγ ELISPOT to measure Mtb-specific T cell responses longitudinally in a South African HIV-infected population similar to that studied here, reported that only 52% of individuals maintained assay status for the entire period of follow up and a significant proportion of individuals had responses close to the assay cut-off (Mitchell et al. 2012). The recent development of monokine-amplified IFNγ release assays (MIGRAs) propose an alternative to IGRAs with the added benefit of possible increased sensitivity in the detection of Mtb-specific T cell responses (Kasprowicz et

al. 2012; Kasprowicz et al. 2011). MIGRAs are based on the fact that IFNγ produced by T cells acts on other cell types (including monocytes, neutrophils, endothelial cells, keratinocytes and fibroblasts) to produce a range of chemokines and cytokines (Berthoud et al. 2009; Abramo et al. 2006). The "amplified" chemokine signal may therefore provide a more sensitive method of detecting IFNγ production than measuring IFNγ itself. Two such chemokines are monokine-induced by IFNγ (MIG or CXCL9) and IFNγ-inducible protein-10 (IP10 or CXCL10). A novel MIGRA assay, measuring Mtb-specific MIG and IP10 production by qPCR, has been developed and validated for use in HIV-infected individuals in Durban, South Africa (Kasprowicz et al. 2011).

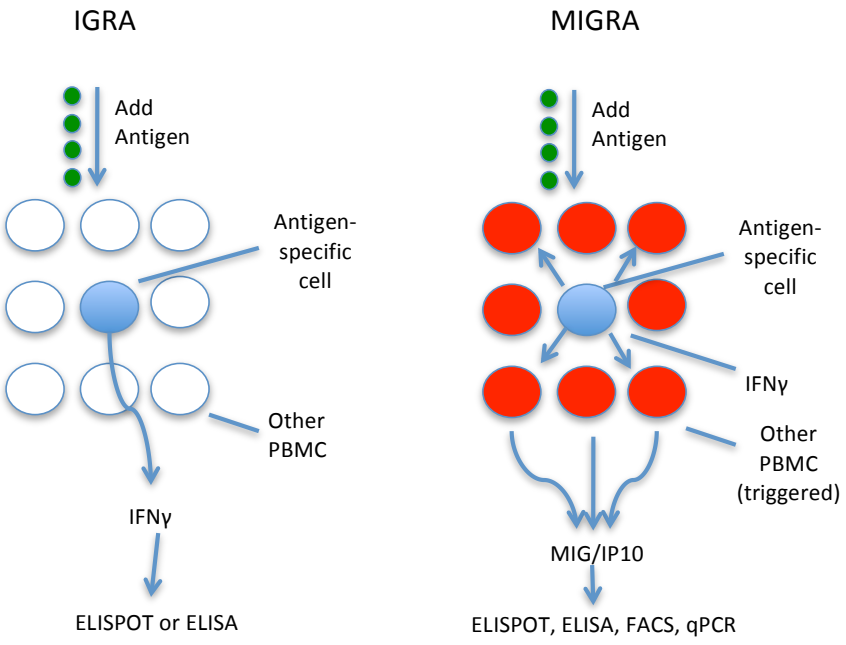


Figure 5.1 A comparison between IFNγ Release Assays (IGRAs) and Monokine-amplified IFNγ Assays (MIGRAs) IGRAs work on the principle of IFNγ secretion and direct detection of IFNγ (left-hand panel). MIGRAs work on the principle of chemokine (MIG and/or IP-10) production by IFNγ- responsive cells (approximately 1000-10,000 fold more numerous) (right-hand panel). Blue = Antigen-specific T cells. White= IFN-gamma responsive cells. Red = Activated IFNγ responsive cells. (Image adapted from Kasprowicz et al. (Kasprowicz et al. 2012))

In this chapter I describe an investigation into the impact of HIV-1 disease and ART treatment on Mtb-specific T cell responses. We used IFNγ ELISPOT to measure T cell responses to PPD, ESAT-6/CFP-10 (EC) and Rv2031c Mtb-antigens and adapted the assay developed by Kasprowicz et al. for concurrent use in our laboratory (Kasprowicz et al. 2011). For IFNγ ELISPOT responses, CMV lysate was used as a comparative antigen.

5.2 Hypothesis

Mtb-specific IFNγ T cell responses are preferentially depleted during HIV-1 infection. This depletion occurs early, and progressively, and is not completely restored on ART. Within the Mtb-specific T cell response, PPD-, EC- and Rv2031c-specific responses may be depleted at different rates and to different extents during HIV-1 infection. IFNγ T cell responses to latency-associated antigen Rv2031c may be preferentially depleted during HIV-1 infection and thereby contribute to the increase risk of active tuberculosis within HIV-1 infected individuals.

5.3 Experimental objectives

1. To investigate the impact of HIV-1 infection on the Mtb-specific T cell response, by comparing EC-, PPD- and Rv2031c-specific IFNγ ELISPOT responses in HIV-uninfected individuals to those of HIV-infected individuals.
2. To determine the effect of HIV-1 disease progression, as defined by CD4 T cell count and plasma HIV-1 viral load, on the Mtb-specific T cell response.
3. To evaluate the tempo and magnitude of reconstitution of Mtb-specific IFNγ ELISPOT responses over 12 months of ART.

4. To evaluate the impact of HIV-1 disease on the Mtb-specific T cell response as well as the reconstitution of this response on ART by means of a novel MIG/IP10 qPCR assay.
5. To compare the sensitivity of IFNγ ELISPOT to that of the MIG/IP10 qPCR assay in detecting IFNγ-producing T cell responses.

5.4 Results

The results in this chapter are presented with an initial cross-sectional baseline analysis of Mtb-specific IFNγ ELISPOT responses. Comparisons are made between HIV-uninfected and HIV-infected study participants within different CD4 T cell counts strata. This is followed by a longitudinal analysis of the impact of both ART initiation and HIV-1 disease progression on Mtb-specific IFNγ ELISPOT responses. Finally a novel MIG/IP10 qPCR assay is used to measure T cell responses at baseline and longitudinally during 12 months of follow-up.

5.4.1 Baseline IFN-γ ELISPOT analysis

5.4.1.1 *Characterisation of study participants included in the baseline IFN*γ ELISPOT analysis

IFN-γ ELISPOT assays were used to assess T cell responses to Mtb antigens and CMV lysate in 199 participants at the baseline visit. 14 participants were excluded from the analysis as they had indeterminate results due to high background in the negative wells. 10 participants died and 5 participants developed active TB within 2 months of study enrolment and were thus excluded. IFNγ ELISPOT responses in 137 HIV-infected participants with different CD4 cell counts and 33 HIV-uninfected participants were included in this analysis (Table 5.1). Mtb-specific antigens used included PPD, EC

(indicating LTBI) and Rv2031c. Responses were measured in duplicate and reported as the mean.

5.4.1.2 Proportion of IFN-γ ELISPOT responders across the entire cohort

The proportions of individuals with a positive response to each of the antigens used in the IFNγ ELISPOT assay are shown in Figure 5.2. 84.1% of individuals had a positive response to CMV lysate, illustrating the widespread nature of CMV infection in this population, whilst 70.0% responded to PPD. 45.3% of individuals had a positive response to EC reflecting the proportion of the cohort, as measured by this RD1-based ELISPOT assay, with LTBI. 33.5% of the cohort had a positive response to latency-associated antigen Rv2031c. The magnitude of IFNγ ELISPOT responses was highest for CMV-specific responses and lowest for Rv2031c-specific responses. The higher frequency and magnitude of EC responses in comparison to Rv2031c has previously been shown to be due to the relative immunodominance of ESAT-6 and CFP-10 antigens and the reduced antigenicity of Rv2031c (Arlehamn et al. 2012).

Table 5.1 Characteristics of study participants included in baseline IFNγ ELISPOT analysis

Characteristic	Total cohort	HIV-	HIV+				
	n= 170	n= 33	**Total HIV+** n= 137	**CD4 >350** n= 38	**CD4 201 -350** n= 39	**CD4 101 – 200** n= 42	**CD4 ≤100** n= 18
Gender							
Female - n (%)	101 (59)	11 (33)	90 (66)	28 (74)	26 (67)	22 (52)	14 (78)
Male - n (%)	69 (41)	22 (67)	47 (34)	10 (26)	13 (33)	20 (48)	4 (22)
Age (years)[a]	33 (27 – 42)	24 (20 – 32)	34 (29 – 44)	36 (28 – 44)	33 (28 – 43)	35 (29 – 44)	35 (20 – 40)
CD4 count (cells/uL)[a]	293 (170 – 503)	795 (603 – 1032)	232 (149 – 362)	462 (377 – 617)	259 (233 – 315)	160 (132 – 185)	68 (52 – 93)
Viral load (RNA copies/mL)[a]	N/A	N/A	63694 (12885 – 186496)	16451 (9548 – 78011)	58249 (11337 – 183293)	130012 (23232 – 352854)	166840 (66445 – 488469)
BMI (kg/m^2)	24.3 (22.3 – 27.6)	24.7 (22.7 – 26.8)	24.2 (22.1 – 27.6)	25.7 (22.5 – 31.9)	23.4 (21.0 – 27.1)	23.7 (22.0 – 26.8)	23.5 (22.1 – 32.6)
Prev TB - n (%)	12 (7)	0 (0)	12 (9)	5 (13)	3 (8)	3 (8)	1 (6)
BCG scar - n (%)	91 (54)	16 (48)	75 (55)	24 (63)	16 (41)	26 (62)	9 (50)
INH prophylaxis - n (%)	52 (31)	0 (0)	50 (36)	10 (26)	15 (38)	18 (43)	7 (40)
Time on INH prophylaxis[a b]	14 (8 – 56)	0 (0)	14 (8 – 56)	109 (42 – 173)	14 (7 – 40)	9 (7 – 14)	15 (9 – 16)

[a] Data represented as median (interquartile range) [b] Duration of INH prophylaxis in individuals on IPT at time of baseline sampling in days.

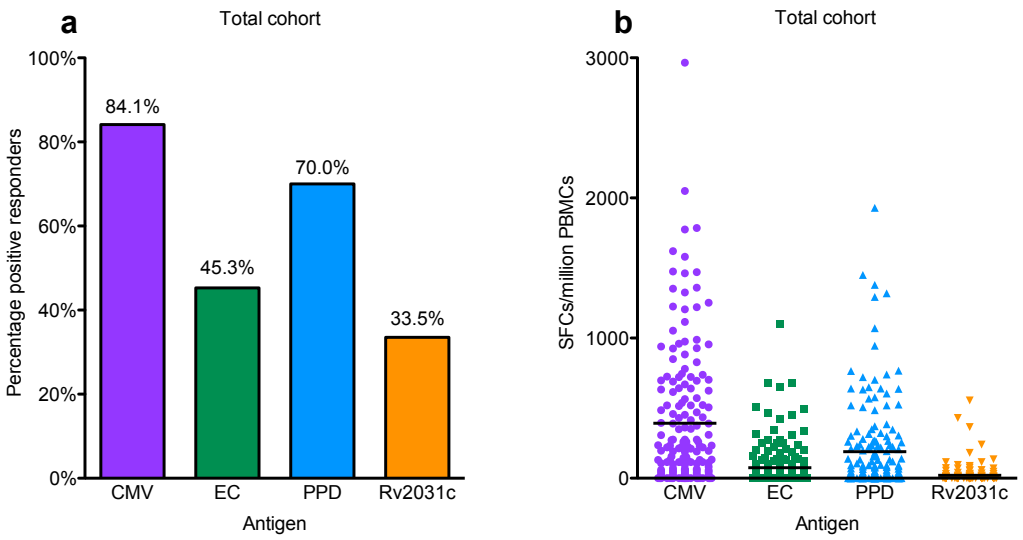


Figure 5.2 IFNγ ELISPOT responses in 170 study participants at baseline. a) Percentage of positive responders to each antigen tested. b) Magnitude of IFNγ ELISPOT responses (SFC/million PBMCs). EC=Esat-6/CFP-10 protein

5.4.1.3 The effect of HIV infection on antigen response

The influence of HIV-1 infection on the antigen-specific IFNγ ELISPOT response was assessed by a comparison of responses between HIV-infected and HIV-uninfected individuals. The proportions of responders to EC and PPD, but not to Rv2031c or CMV were significantly lower in HIV-infected individuals (Fig. 5.4a; EC, P = 0.007; PPD P<0.0001; Rv2031c, P=0.15; CMV, P = 0.61). When the magnitude of response was similarly compared, HIV-infected individuals had a lower median magnitude of response to EC, PPD and Rv2031c whilst not to CMV (Fig. 5.4b; EC, P = 0.008; PPD P <0.001; Rv2031c P=0.028; CMV, P = 0.23). The contrasting patterns of CMV- and Mtb-specific T cell depletion evident here, suggest an increased susceptibility of Mtb-specific T cells to HIV-1 associated depletion in vivo.

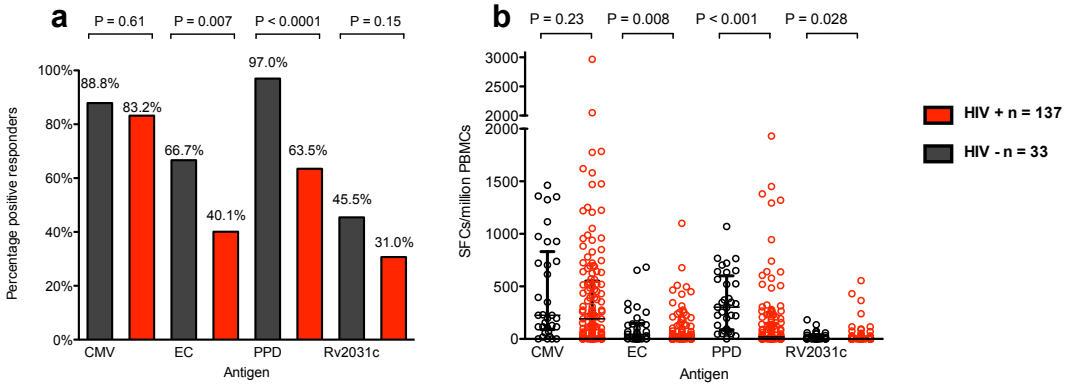


Figure 5.3 The effect of HIV-infection on CMV- and Mtb-specific IFNγ ELISPOT responses a) The difference in proportion of positive responders to CMV, EC, PPD and Rv2031c between HIV-infected and HIV-uninfected individuals. P values derived using Fisher's exact tests. b) The difference in magnitude of IFNγ ELISPOT responses between HIV-infected and HIV-uninfected individuals. P values derived using Mann-Whitney tests.

5.4.1.4 The effect of HIV-1 disease progression on the antigen-specific response

The stage of HIV-1 disease progression at which pathogen-specific immunity is significantly affected is important in correlating immunology with pathogenesis of disease as well as for informing optimal timing of ART initiation. This was evaluated in the current cohort by calculating the percentage of responders to each antigen in HIV negative individuals and comparing that to the percentage of responders in HIV positive individuals within progressively declining CD4 count strata (Fig. 5.4 a - h).

CMV responses did not differ significantly in proportion of responders or magnitude of response between HIV-uninfected and HIV-infected individuals, nor between HIV-infected individuals within any CD4 count stratum (Fig. 5.4 a-b, P >0.05 for all comparisons). Only individuals with CD4 T cell counts between 100 and 200 cells/uL had a reduced frequency of response to EC when compared to HIV-uninfected individuals, whilst individuals with CD4 T cell counts below 100 had a lower median IFNγ ELISPOT response than the HIV-uninfected group (Fig. 5.4 c, P=0.001; Fig. 5.4 d,

P=0.048). No other differences in EC-specific responses were evident. By contrast, the proportions of individuals responding to PPD as well as the median magnitudes of PPD-specific IFNγ ELISPOT responses were lower in HIV-infected individuals within all CD4 count strata than in HIV-uninfected individuals (Fig. 5.4 c-d, P<0.0001 for all comparisons). Interestingly, no differences were observed between HIV-infected individuals within different CD4 count strata (Fig. 5.4 c-d, P>0.05 for all comparisons). Rv2031c-specific IFNγ ELISPOT responses revealed an interesting pattern as the magnitude of responses to Rv2031c within the group with CD4 T cell counts 201-350 was lower than that of the HIV-uninfected group (Fig. 5.4 h, P=0.015), whilst there were no differences between the other CD4 count strata (Fig. 5.4 g-h, P>0.05 for all other comparisons).

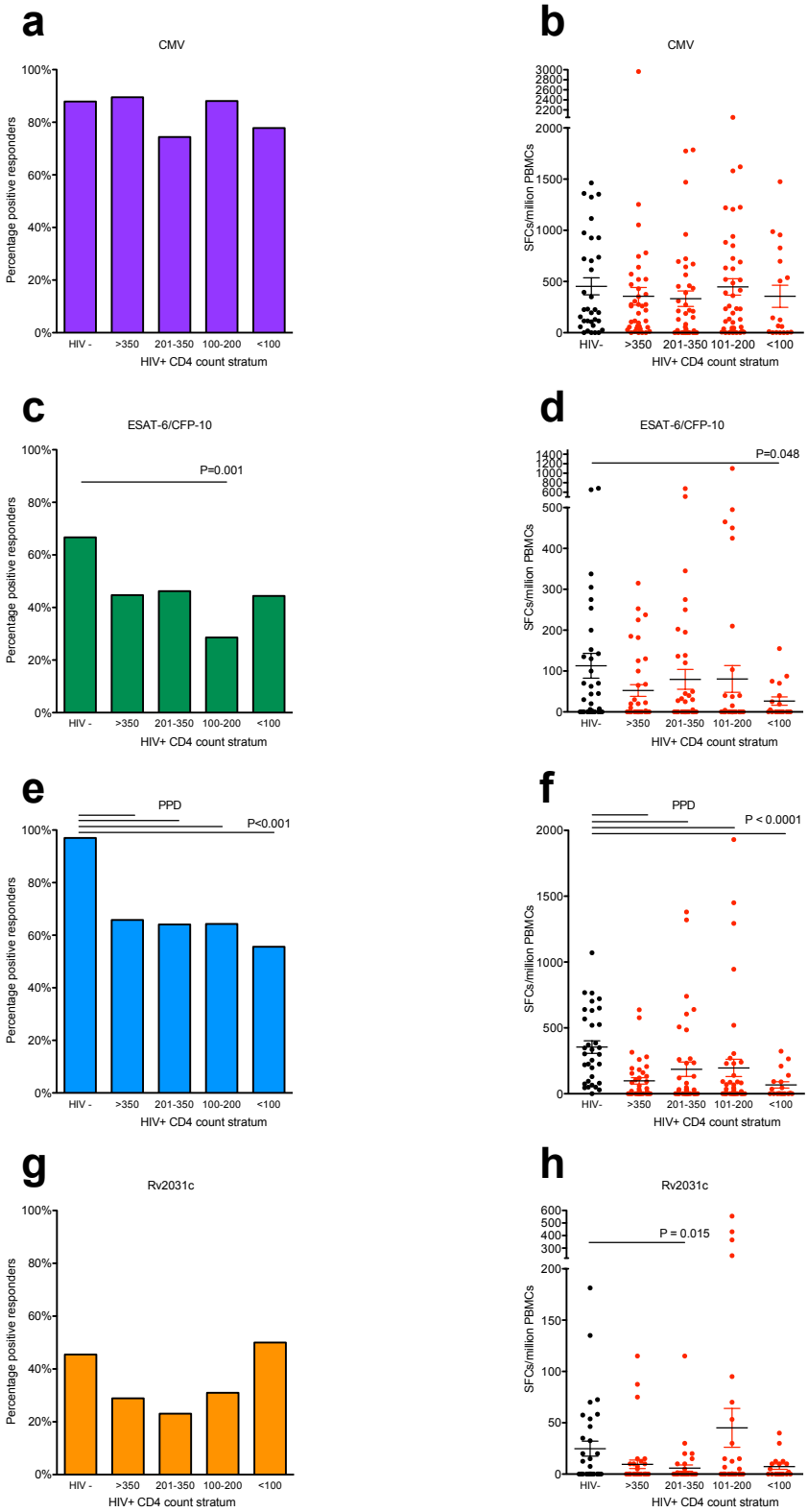


Figure 5.4 The effect of HIV-1 infection and CD4 stratification on CMV- and Mtb-specific IFNγ ELISPOT responses. The proportions of positive responders and magnitude of responses to CMV, EC, PPD and Rv2031c are compared between HIV-uninfected individuals and HIV-infected individuals at different CD4 counts. a) P values derived using Fisher's exact tests. b) P values derived using Mann-Whitney tests

5.4.1.5 An analysis of the factors associated with a positive IFNγ ELISPOT response to Mtb antigens at baseline

The factors that potentially contribute to the presence and detection of an IFNγ ELISPOT T cell response are multiple and complex. I aimed to use multivariate logistic regression analyses to determine the key demographic and clinical characteristics that impacted on the likelihood of an individual having a positive response to each of the Mtb-specific antigens. I tested across all individuals included in the baseline IFNγ ELISPOT analysis and within the HIV-infected cohort specifically. Odds ratios and the associated 95% confidence intervals were estimated initially through a series of univariate analyses (Tables 5.2 and 5.4). Variables that had a P value of < 0.25 in the univariate analyses were included in a multivariate logistic regression analyses as shown in Table 5.3 and Table 5.5.

Across the entire cohort, only the presence of HIV infection significantly altered the likelihood of having a positive response to PPD (Table 5.3 – PPD OR = 0.05, P = 0.01). Interestingly, none of the included factors was significantly associated with a positive IFNγ ELISPOT response to EC or Rv2031c within the total cohort (Table 5.3 – EC OR 0.66. P = 0.53; Rv2031c OR = 0.26, P = 0.02). Of particular note is the fact that being on INH at baseline did not influence the likelihood of having a T cell response to any of the Mtb-specific antigens tested at that time point.

Table 5.2 Univariate analysis of the determinants of a positive IFNγ ELISPOT response to Mtb antigens in all participants

Variable	Esat-6/CFP-10 positive response		PPD positive response		Rv2031c positive response	
	OR (95%CI)	*P*	*OR (95%CI)*	*P*	*OR (95%CI)*	*P*
Age	0.99 (0.97 – 1.02)	0.54	0.97 (0.94 – 0.99)	0.05	1.02 (0.96 – 1.02)	0.33
Sex	0.76 (0.41 – 1.41)	0.39	0.64 (0.33 – 1.28)	0.21	1.13 (0.59-2.17)	0.71
HIV status	0.34 (0.15 – 0.75)	0.007	0.05 (0.007 – 0.41)	**0.005**	0.53 (0.24 – 1.15)	0.11
CD4 count	1.00 (1.00 – 1.03)	0.006	1.00 (1.00 – 1.01)	0.03	1.00 (1.00-1.01)	0.99
BCG scar	0.81 (0.44 – 1.48)	0.51	0.83 (0.43 – 1.60)	0.57	0.67 (0.36 – 1.31)	0.25
Prev TB	0.58 (0.17 – 2.01)	0.39	0.40 (0.12 – 1.30)	0.13	0.64 (0.17 – 2.47)	0.52
INH	0.66 (0.33 – 1.29)	0.22	0.77 (0.38 – 1.57)	0.46	0.61 (0.29 – 1.26)	0.18
INH time	1.00 (0.99 – 1.00)	0.77	1.00 (0.99 – 1.01)	0.94	1.00 (0.99 – 1.01)	0.62
Mass	1.01 (0.99 – 1.03)	0.46	1.01 (0.98 – 1.03)	0.62	0.99 (0.97 – 1.02)	0.52
BMI	1.01 (0.96 – 1.06)	0.77	1.00 (0.94 – 1.06)	0.90	1.00 (0.94 – 1.05)	0.97

Prev TB = a history of previous active TB disease. CD4 count= cells/uL/100

Table 5.3 Multivariate analysis of the determinants of a positive IFNγ ELISPOT response to Mtb antigens in all participants

Variable	Esat-6/CFP-10 positive response		PPD positive response		Rv2031c positive response	
	OR (95%CI)	*P*	*OR (95%CI)*	*P*	*OR (95%CI)*	*P*
Age	1.01 (0.97 – 1.04)	0.77	0.99 (0.95 – 1.02)	0.42	0.99 (0.96 – 1.03)	0.71
Sex	0.84 (0.43 – 1.65)	0.61	0.84 (0.39 – 1.79)	0.65	1.39 (0.68 – 2.87)	0.37
HIV status	0.66 (0.20 – 2.29)	0.53	0.05 (0.004 – 0.49)	0.01	0.29 (0.08 – 1.05)	0.06
CD4 count	1.12 (0.97 – 1.30)	0.13	0.95 (0.79 – 1.14)	0.55	0.88 (0.75 – 1.04)	0.12
BCG scar	0.81 (0.43 – 1.53)	0.52	0.83 (0.40 – 1.70)	0.60	0.76 (0.39 – 1.48)	0.39
Prev TB	0.62 (0.17 – 2.28)	0.48	0.60 (0.18 – 2.02)	0.40	0.81 (0.21 – 3.39)	0.81
INH	0.89 (0.43 – 1.86)	0.76	1.19 (0.56 – 2.52)	0.65	0.70 (0.32 – 1.55)	0.38

Prev TB = a history of previous active TB disease. CD4 count= cells/uL/100

A similar analysis was carried out to evaluate factors within the HIV-infected group. However, no variable significantly influenced the likelihood of a positive response to any of the antigens tested (Table 5.3 $P > 0.05$ for all comparisons). Of particular importance is the fact that both HIV-1 viral load and CD4 T cell count (as markers of

HIV-1 disease progression) were included in this analysis and failed to be significantly associated with the likelihood of having a response to any Mtb-specific antigen.

Table 5.4 Univariate analysis of the determinants of a positive IFNγ ELISPOT response to Mtb antigens in HIV-infected participants

Variable	Esat-6/CFP-10 positive response		PPD positive response		Rv2031c positive response	
	OR (95%CI)	*P*	*OR (95%CI)*	*P*	*OR (95%CI)*	*P*
Age	0.99 (0.97 – 1.03)	0.95	0.99 (0.96 – 1.02)	0.44	0.99 (0.96 – 1.03)	0.67
Sex	0.98 (0.48 – 2.02)	0.96	0.98 (0.47 – 2.04)	0.95	1.72 (0.77 – 3.84)	0.19
HIV VL	1.00 (1.00 – 1.00)	0.08	1.00 (1.00 – 1.00)	0.69	1.00 (1.00 – 1.00)	0.18
CD4 count	1.00 (1.00 – 1.00)	0.98	1.00 (1.00 – 1.00)	0.91	1.00 (1.00-1.00)	0.09
BCG scar	0.99 (0.50 – 1.96)	0.97	0.92 (0.46 – 1.86)	0.82	0.66 (0.32 – 1.37)	0.27
Prev TB	0.73 (0.21 – 2.54)	0.62	0.54 (0.17 – 1.79)	0.32	0.74 (0.19 – 2.87)	0.66
INH	0.87 (0.43 – 1.77)	0.70	1.19 (0.57 – 2.46)	0.65	0.70 (0.32 – 1.52)	0.37
INH time	1.00 (0.99 – 1.01)	0.91	1.00 (0.99 – 1.01)	0.65	1.00 (1.00 – 1.01)	0.62
Mass	1.00 (0.98 – 1.03)	0.97	1.01 (0.98 – 1.04)	0.57	0.99 (0.96 – 1.02)	0.52
BMI	0.99 (0.94 – 1.05)	0.77	1.01 (0.95 – 1.07)	0.76	1.00 (0.94 – 1.06)	0.91
OI	1.38 (0.50 – 3.83)	0.54	1.06 (0.37 – 3.07)	0.91	1.70 (0.60 – 4.8)	0.32

Prev TB = a history of previous active TB disease. OI = opportunistic infection. CD4 count= cells/uL/100. Viral load = Log^{10} copies/mL. BMI= Body mass index (kg/m^2)

Table 5.5 Multivariate analysis of the determinants of a positive IFNγ ELISPOT response to Mtb antigens in HIV-infected participants

Variable	Esat-6/CFP-10 positive response		PPD positive response		Rv2031c positive response	
	OR (95%CI)	*P*	*OR (95%CI)*	*P*	*OR (95%CI)*	*P*
Sex	0.99 (0.48 – 2.05)	0.98	0.98 (0.47 – 2.05)	0.96	1.83 (0.81 – 4.14)	0.15
Viral load	1.37 (0.86 – 2.18)	0.19	1.16 (0.76 – 1.78)	0.50	0.97 (0.61 – 1.54)	0.90
CD4 count	1.06 (0.86 – 1.31)	0.58	1.02 (0.83 – 1.26)	0.76	0.81 (0.63 – 1.03)	0.09

Prev TB = a history of previous active TB disease. OI = opportunistic infection. CD4 count= cells/uL/100. Viral load = Log^{10} copies/mL. BMI= Body mass index (kg/m^2)

5.4.2 Longitudinal IFN-γ ELISPOT analysis

The rapid decline in susceptibility to active TB that is associated with ART initiation in HIV-infected individuals suggests a quantitative and/or qualitative restoration of Mtb-specific T cell immunity. I aimed to assess restoration of IFNγ ELISPOT responses in HIV-infected individuals that started ART over a 12 month period of therapy. A total of 70 HIV-infected individuals initiated ART at baseline, remained on ART for 12 months and had IFNγ ELISPOT assays carried out at all 3 time points. As a comparative group I analysed 23 HIV-infected individuals that did not meet criteria for ART initiation for the remainder of the study period. Details of all participants analysed longitudinally are described in Table 5.4.

Table 5.6 Characteristics of study participants included in longitudinal IFNγ ELISPOT analysis

		HIV+ on ART	HIV+ not on ART
Total number		**70**	**23**
	Female - n (%)	45 (64)	13 (56)
	Male - n (%)	25 (36)	10 (44)
Age (years) [a]		35 (28 – 44)	37 (27 – 44)
CD4 (cells/uL) [a]	**Baseline**	187 (128 – 259)	426 (365 – 608)
	6 months	341 (230 – 447)	396 (332 – 472)
	12 months	354 (289 – 492)	338 (310 – 481)
Viral load Baseline (RNA copies/ml) [a]	**Baseline**	122174 (22902 – 275658)	17235 (9724 – 69742)
	6 months	40 (40 – 67))	31095 (9325 – 57604)
	12 months	40 (40 –105)	21556 (12144 – 57534)

[a] Data represented as median (interquartile range)

5.4.2.1 The effect of ART on the proportion of responders and the magnitude of IFNγ ELISPOT responses

The proportion of individuals responding to each of the antigens was compared at each time point within the groups of HIV-infected individuals on and off ART. There

were no significant differences in the proportion of individuals responding to CMV, EC or PPD between any of the time points within either group (Fig. 5.5 a-b, P>0.05 for all comparisons). The proportion of individuals on ART that responded to Rv2031c was actually lower at both 6 and 12 months that it was at baseline (Fig. 5.5 a, BL vs. 6m, P=0.002, BL vs. 12m, P=0.0001) A similar trend was evident for the proportion of responders to Rv2031c within the non-ART group, although this did not reach statistical significance (Fig. 5.5 b, P>0.05 for all).

When the magnitudes of IFNγ ELISPOT responses were compared before and after 6 or 12 months of ART there were no significant changes for CMV, EC and PPD. However, magnitudes of Rv2031c-specific responses decreased at both 6 and 12 months when compared to baseline.

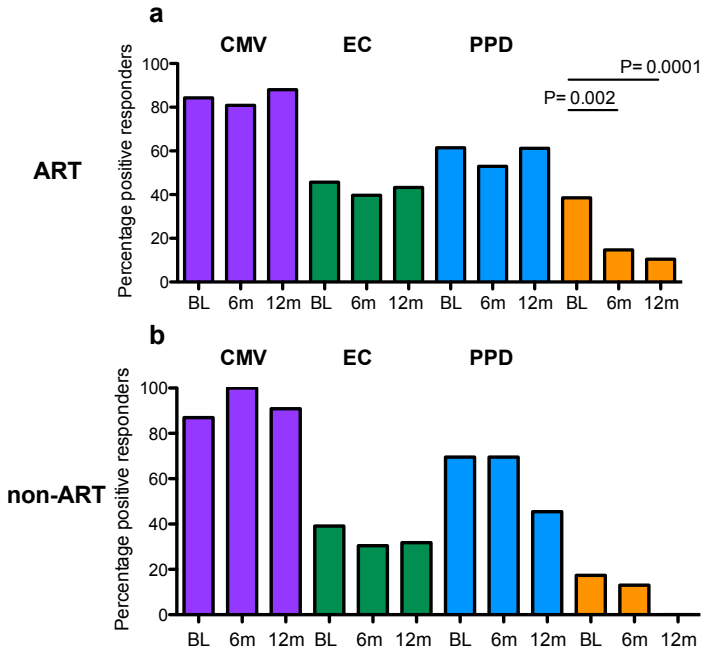


Figure 5.5 Changes in the proportions of IFNγ ELISPOT positive responders to CMV, EC, PPD and Rv2031c within HIV-infected individuals on and off ART over a period of 12months. a) Individuals initiating ART at baseline n = 70 b) Individuals not on ART n = 23. P values derived using Fisher's exact tests.

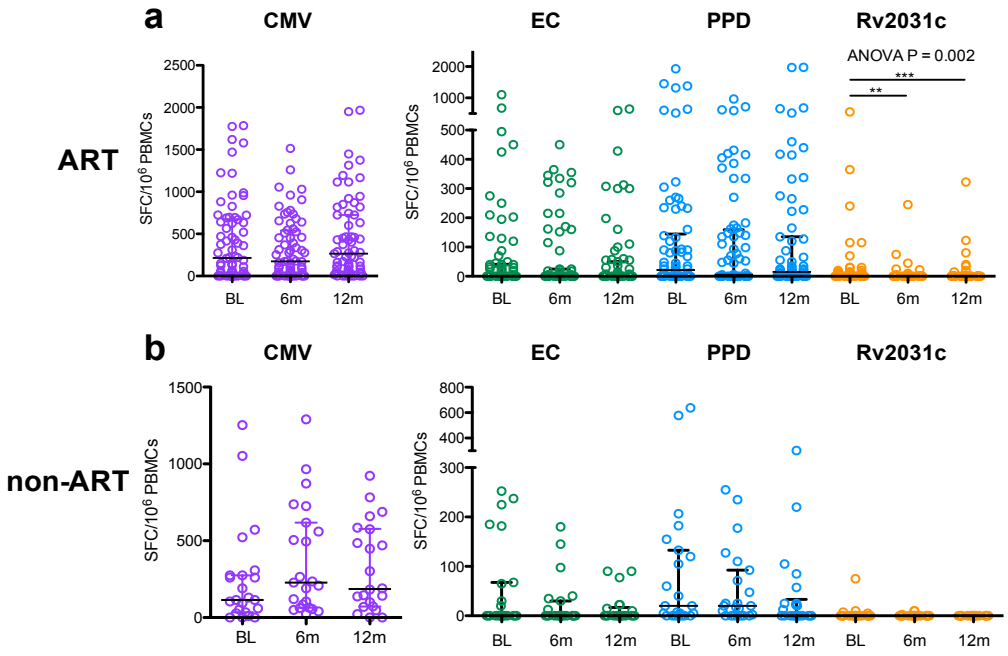


Figure 5.6 Changes in the magnitude of IFNγ ELISPOT responses to CMV, EC, PPD and Rv2031c within HIV-infected individuals on and off ART over a period of 12months. a) Individuals initiating ART at baseline n = 70 b) Individuals not on ART n = 23. Data within each antigen stimulation compared using Kruskal-Wallis ANOVA followed by Dunn's post-test comparison. (*, P <0.05,**, P <0.005, ***, P <0.0005)

5.4.2.2 The affect of IPT on the magnitude of IFNγ ELISPOT responses to Mtb-specific antigens.

As discussed in section 1.2.6, IPT has shown a suppressive effect on the level of IFNγ production in response to Mtb-specific antigens in previous studies(D. F. Johnson et al. 2014; O'Shea et al. 2014). 6 months of IPT is currently recommended for all HIV-infected individuals according to South African ART guidelines(South African National Department of Health 2013b). This, along with the confounding influence of ART on the Mtb-specific T cell responses, made it challenging to dissect the impact of IPT on such responses. A retrospective analysis revealed that a small proportion of the individuals initiating ART did not receive IPT as the standard of care. I therefore compared the change in Mtb-specific IFNγ ELISPOT responses after 6 and 12 months of ART between those individuals that did and did not receive IPT.

Of the 70 individuals involved in this analysis, 28 individuals were not on IPT at the point of baseline sampling and were therefore included in the sub-analysis. Of these individuals, 12 did not receive any IPT during the following 6 months and 6 patients did not receive IPT at any stage during the study. A comparison of the change in SFCs/10^6 in baseline responders to EC, PPD and Rv2031c between those that received IPT and those that did not, failed to reveal any significant differences at both the 6 month or 12 month time point ($P>0.05$ for all comparisons). However, this study was not ideally powered or structured to investigate the effect of IPT on the magnitude of Mtb-specific T cell responses.

5.4.3 Measuring T cell responses to ESAT-6/CFP10 using a novel MIG/IP10 qPCR assay

We adapted a novel MIG/IP10 qPCR assay to run under our own conditions (Kasprowicz et al. 2011). The assay was carried out for 67 individuals at baseline including 55 HIV-infected and 12 HIV-uninfected individuals. MIG/IP10 qPCR assays were completed for 43 individuals at all 3 time points including 38 HIV-infected and 5 HIV-uninfected individuals.

5.4.3.1 Additional method: Development of a cut-off value for qPCR assay positivity

In order to establish an assay cut-off, the assay was carried out for the 12 uninfected individuals at baseline. 4 of the 12 uninfected individuals were IFNγ ELISPOT negative in response to EC for all 3 study visits. The mean MIG and IP10 fold increases (stimulated/unstimulated) for these individuals + 3 standard deviations were used to

define an assay cut-off, which was then applied across the cohort for HIV-infected individuals (EC MIG = 3.64, EC IP10 = 2.76).

5.4.3.2 The effect of CD4 count stratification on MIG and IP10 expression in response to ESAT-6/CFP10 at baseline

I initially wanted to assess T cell responses to EC measured by this assay in HIV-infected individuals with different levels of immune suppression. Whilst both the median fold increase (Fig. 5.7 a-b) and proportion of individuals within each group (data not shown) increased with increasing CD4 T cell count, these differences did not reach statistical significance (P>0.05 for all comparisons).

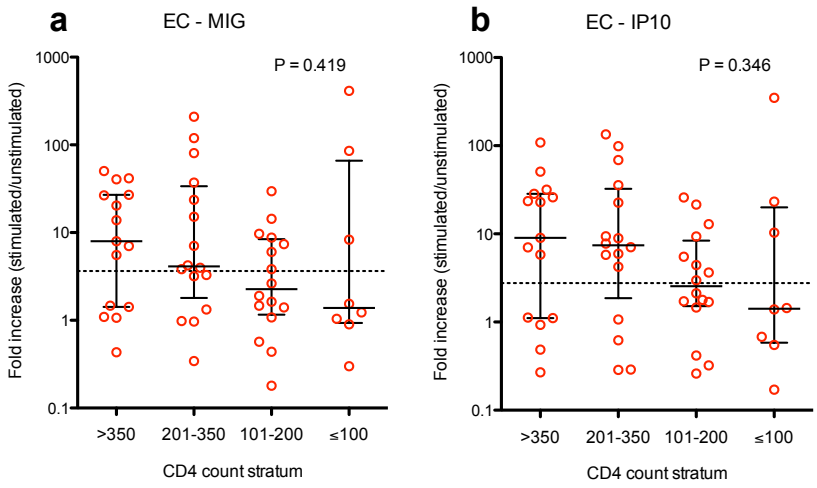


Figure 5.7 EC-specific MIG and IP10 fold increases in HIV-infected individuals at baseline. Patients are grouped according to CD4 T cell count: ≤100 n=8, 101-200 n= 16, 201-350 n= 16, >350 n=15. Medians and IQR are shown. Dotted lines represent cut-off level for assay positivity: MIG = 3.64 , IP10 = 2.76. Medians are compared and P values displayed for Kruskal-Wallis ANOVA.

5.4.3.3 The effect of ART on restoring MIG and IP10 responses to ESAT-6/CFP10

I next wanted to assess whether ART restores EC-specific T cell responses, as measured by our MIG/IP10 qPCR assay. 22 HIV-infected individuals initiated ART at baseline and had qPCR assay results at all 3 time points. Despite effective ART, no

difference in the proportion of MIG or IP10 responders or median fold increase in MIG or IP10 expression was observed at 6 or 12 months of ART when compared to baseline levels (Fig. 5.8 a-b, P >0.05 for both).

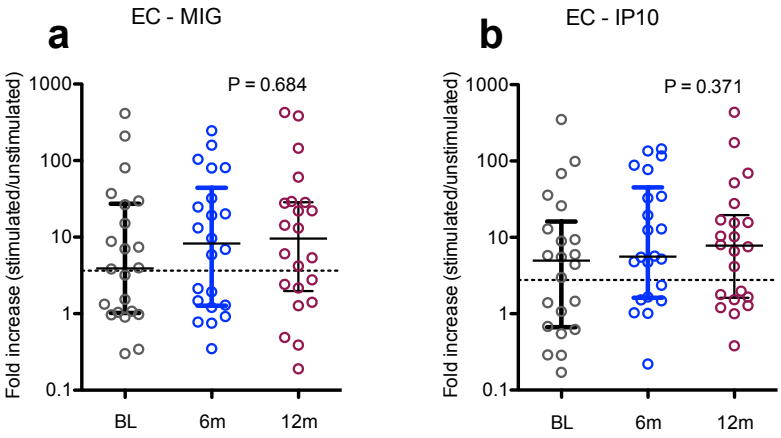


Figure 5.8 EC-specific MIG and IP10 fold increases in 22 HIV-infected individuals on ART. Fold increases are shown before and at 6 and 12 months after initiating ART. Medians and IQR are shown. Dotted lines represent cut-off level for assay positivity: MIG = 3.64, IP10 = 2.76. P values are displayed for Kruskal-Wallis ANOVA.

5.4.4 An assessment of MIG/IP10 qPCR vs. IFNγ ELISPOT in the detection of ESAT-6/CFP10-specific T cell responses

Finally I compared the ability of both assays to detect EC-specific T cell responses within the same individuals. For 134 individuals, distributed across the 3 study visits, both EC-IFNγ ELISPOT and EC-MIG/IP10 qPCR assays were carried out. Of all the EC-IFNγ ELISPOT positive assays (55/134), 43/55 (78%) and 45/55 (82%) were MIG and IP10 positive by qPCR respectively. Interestingly a higher sensitivity of the qPCR assay for the detection of LTBI is suggested by the fact that of the 79 EC-IFNγ ELISPOT negative assays, 39 (49%) and 45 (57%) were MIG and IP10 positive by qPCR respectively.

When a correlation of the magnitudes of IFNγ ELISPOT SFC/10^6 and EC-MIG/IP10 fold increase was performed for the 134 concurrent measurements, they were found to be strongly correlated (Fig. 5.7. EC-MIG, P <0.0001, R=0.544; EC-IP10, P<0.0001, R= 0.476). This indicates that the magnitudes of EC-specific T cell responses are similarly estimated by both assays.

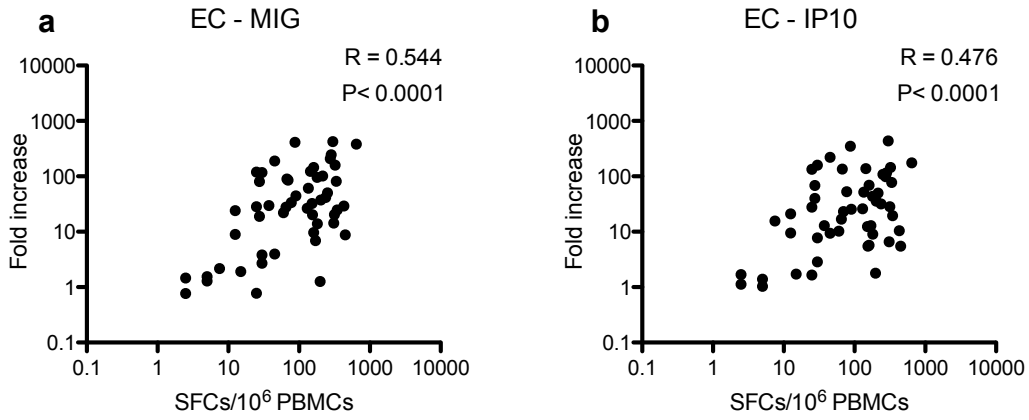


Figure 5.9 Quantitative correlation between IFNγ ELISPOT and MIG/IP10 qPCR EC-specific T cell responses. ELISPOT SFC/10^6PBMCs and qPCR fold increases of MIG and IP10 in response to EC were compared using Spearman's correlation.

5.5 Discussion

In this study, Mtb-specific T cell responses were preferentially depleted in HIV-infected study participants at the baseline visit. Use of the multivariate logistic regression analysis indicated that the observed difference occurs irrespective of CD4 count for PPD and Rv2031c and highlights the effect of HIV infection alone on responses to these antigens. This depletion appears to occur early in the progression of HIV-1 disease for PPD-specific responses with a significant decrease in proportion and magnitude of PPD-specific responses evident in HIV-infected individuals with CD4 T cell counts above 350 cells/uL, when compared to the HIV-uninfected group. The fact that there were no significant decreases between any of CD4 T cell count strata of HIV-infected individuals possibly indicates that the majority of this depletion occurs early within the course of HIV-1 disease progression. These findings are supported by data in other published studies in both acute and chronic HIV-1 infection (Sutherland et al. 2006; Geldmacher et al. 2008). Within my study, a corresponding depletion of EC- and Rv2031c-specific responses was not evident, suggesting that different Mtb antigen-specific T cell responses are affected at different rates and to different extents during HIV-1 infection (this particular concept is explored further in Chapter 6).

The ability to draw conclusions on the timing of Mtb-specific T cell depletion from these data, and the aforementioned studies, is limited by the cross-sectional nature of the analyses. A longitudinal study of HIV-infected individuals before and after HIV-seroconversion would give better insight into the tempo and character of antigen-specific T cell depletion during acute HIV-1 infection and consequent disease

progression. One such study of a small group of individuals by Geldmacher et al. revealed an early and profound depletion of Mtb -specific CD4 T cells with 6-12 months of HIV-1 seroconversion, as measured by flow cytometry (Geldmacher et al. 2010). The antigen used in this study was also PPD and poses the question as to whether a different dynamic may have been observed in that study had other Mtb antigen-specific T cell responses been evaluated.

Longitudinal analysis of antigen-specific T cell responses in individuals initiating ART, did not reveal any significant reconstitution of these responses as measured by IFNγ ELISPOT or MIG/IP10 qPCR at 6 or 12 months. ART has reliably been shown to restore CD4 T cell responses to PPD in several other studies (Autran et al. 1997; T. Li et al. 1998; Wilkinson et al. 2009; Wendland et al. 1999). A detailed study by Wilkinson et al. of 19 individuals with LTBI in Cape Town, South Africa demonstrated an increase in the magnitudes IFNγ ELISPOT responses to EC and PPD over 48 weeks of ART. It was therefore surprising to not observe similar results in our cohort. The former study longitudinally sampled a smaller number of individuals but with more frequent study visits thus having increased ability to track progressive changes in dynamic T cell responses. IFNγ ELISPOT assays were carried out from frozen PBMCs, thereby reducing inter-assay variability. The fact that we executed our IFNγ ELISPOT assays in real time from fresh PBMCs may have affected the consistency in results between time points. Despite being an advantage in terms of avoiding the influence of freeze/thawing PBMCs on assay outcomes, this method did introduce the possibility of more inter-assay variability.

We adapted the novel MIG/IP10 qPCR assay to detect EC-specific T cell responses within this cohort. Whilst we were unable to detect significant reconstitution of EC-specific T cell responses, the assay results were similar to those of the IFNγ ELISPOT assay. As described by Kasprowicz et al, we also found an increased sensitivity to detect EC-specific responses when using the qPCR assay in comparison to our IFNγ ELISPOT assay (Kasprowicz et al. 2011). Difficulties still exist as to how to best define a positive response in the assay as well as the utility of the use of the fold increase as a quantitative tool for longitudinal monitoring of antigen-specific T cell responses.

6 ANALYSING THE IMPACT OF HIV-1 INFECTION ON THE MTB-SPECIFIC T CELL RESPONSE

The work presented in this chapter forms the basis of an accepted abstract for poster presentation at Keystone Symposia Conference: Host Response in Tuberculosis, 22-27 Jan 2015.

6.1 Background

An in-depth understanding of the immune correlates of protection to TB is required for the development of future vaccine and therapeutic strategies. The complexity of the immune response to Mtb, however, is a major impediment to this as is the fact that the outcome of LTBI is dependent on the interplay of both host and pathogen.

To date most attention has been directed at the Th_1 CD4 Mtb-specific response with a particular focus on the cytokines IFNγ and TNFα. Defects in production of IFNγ and TNFα or their receptor function, have been reliably linked to susceptibility to active TB disease (Flynn et al. 1993; Harris & Keane 2010). IFNγ and TNFα are therefore essential in mediating protection to TB, however they are not sufficient for protection on their own (Gallegos et al. 2011). The role of polyfunctional Th_1 responses (which are characterised by concurrent expression of a combination of IFNγ, TNFα and IL-2) in conferring protection to Mtb and other pathogens is a source of debate. Some evidence points to a role for polyfunctional CD4/CD8 T cells in controlling viral infections including CMV and HIV-1 and in animal TB vaccination studies, polyfunctional responses have conferred a degree of protection (Pantaleo & Harari 2006; Betts et al. 2006; Aagaard et al. 2009). Human TB vaccine studies have shown that although able to induce polyfunctional vaccine responses, they have not as yet been able show an association of these responses with protection from TB

disease (Scriba et al. 2012; Tameris et al. 2013). When considering the effect that HIV-1 infection (as the most potent risk factor for the development of active TB) has on the polyfunctional Mtb-specific T cell response, the data are inconsistent – HIV-1 infection has been shown to both increase and decrease the polyfunctional Mtb-specific T cell response in different studies (Day et al. 2008; Sutherland et al. 2009).

As our understanding of the Mtb-specific immune response has expanded, it has become clear that non-Th_1 T cell responses play an important role in protection from TB disease. A Th_{17} Mtb-specific response has been described and postulated as an important component of the Mtb-specific T cell response (Scriba et al. 2008). HIV-1 infection results in preferential depletion of IL-17-producing CD4 T cells in the GALT and peripheral blood whilst IL-17 receptor expression as well as Th_{17} cell number have been inversely correlated with HIV-1 viral load (Brenchley et al. 2008; El Hed et al. 2010; Salgado et al. 2011). The latter suggests increased depletion of this subset with advancing HIV-1 disease. Although it has never been shown before within the context of Mtb-specific immunity, it is highly likely that the Th_{17} Mtb-specific T cell response is preferentially depleted in HIV-1 disease and may therefore play a significant role in increased susceptibility to active TB disease within this population.

CD8 T cells form a significant component of the Mtb-specific T cell response and contribute to a protective response to TB (Stenger et al. 1998). The importance of protection conferred by the Mtb-specific CD8 response in animal studies has been reliably demonstrated, however the role of CD8 T cells in human TB is unclear (Flynn et al. 1992; Sousa et al. 2000; Chen et al. 2009). Few studies have investigated the contribution of both Mtb-specific CD4 and CD8 responses in the context of healthy

individuals and those with progressive HIV-1 infection. One such study showed that with decreasing CD4 counts, the predominant response to PPD changed from a polyfunctional CD4 to a monofunctional CD8 response proposing an increased role for CD8 T cell responses in advanced HIV-1 infection (Sutherland et al. 2010).

Different pathogen-specific T cell responses are depleted during HIV-1 infection to different extents and at different rates (Geldmacher & Koup 2012). Geldmacher et al. found that PPD-specific CD4 T cells were depleted early and to a greater extent than CMV-specific CD4 T cells (Geldmacher et al. 2010). This was attributed to a greater proportion of IL-2 and a smaller proportion of MIP1-β expressing CD4 T cell components of the PPD response and the resultant susceptibility to direct HIV-1 infection.

When evaluating Mtb-specific responses it is important to consider the specific antigen concerned. Earlier studies of Mtb-specific T cell function used PPD, which is a non-specific mycobacterial antigen and when used in an IGRA reflects infection with multiple non-Mtb mycobacteria as well as BCG immunisation. Most recent studies have focused on responses to different Mtb-specific antigens that are expressed during the early phase of infection such as ESAT-6, CFP-10 and Ag85B. A study utilising IFNγ ELISPOT in The Gambia highlighted a difference in the extent to which the responses to different Mtb antigens are depleted during HIV-1 infection (Hammond et al. 2008). No studies thus far, to my knowledge, have investigated responses to Mtb-specific "latency" antigens such as Rv2031c within the context of HIV-1 infection.

In this chapter I use multiparameter flow cytometry, based on an on-site whole blood ICS protocol (WB ICS), to characterize the antigen-specific CD4 and CD8 T cell response in 44 healthy HIV-uninfected individuals to 3 different Mtb-antigens (ESAT-6/CFP-10, PPD and Rv2031c) as well as to CMV (as a comparative antigen). I proceed to investigate the effect of HIV-1 infection on the magnitude and character of these responses by comparing them to equivalent responses of 147 HIV-infected individuals at various stages of HIV-1 disease progression. The antigen-specific CD4 and CD8 response to each of the antigens is compared and evaluated alongside parameters of HIV-1 disease progression, thereby further elucidating the overall effect of HIV-1 disease on the host response to Mtb.

6.2 Hypothesis

I hypothesized that the observed increased risk of TB disease in individuals infected with HIV-1 is due to progressive loss of Mtb-specific CD4 T cells as well as the effect that loss of CD4 T cells has on the function of Mtb-specific CD8 T cells. I suspected that the Mtb antigen-specific CD4 responses would differ from each other in terms of cytokine expression profile and that these differences would translate into different dynamics of persistence or depletion during HIV-1 infection.

6.3 Experimental aims

1. To characterize the CD4 and CD8-specific response to each of the 4 antigens in healthy HIV-uninfected individuals by investigating:
    a. The magnitude of CD4 and CD8 T cell responses in terms of proportion of total CD4/CD8 T cells as well as absolute number/uL of whole blood.

b. The pattern of expression of Th_1 cytokines IFNγ, TNFα and IL-2 as well as the Th_{17} cytokine IL-17.

2. To determine the effect of HIV-1 infection on the Mtb- and CMV-specific CD4 and CD8 response.

6.4 Results

6.4.1 Characteristics of study participants included in the following analyses

Flow cytometric analysis was performed on baseline samples from 202 individuals. Criteria for inclusion of samples in the analysis were developed based on the number of CD3+ events after preliminary gating (Hanekom et al. 2004). A cut-off for inclusion in the analysis was a CD3+ event count of at least 50 000 and a CD4+ event count of at least 5000. On this basis, 9 samples were excluded from analysis. An additional 2 individuals were removed from the analysis due to developing TB within 2 months of sampling. The details of the 191 individuals are shown in Table 6.1.

6.4.2 Phenotype and function of CMV, EC, PPD and Rv2031c T cell responses in healthy HIV-uninfected individuals

In order to accurately define the impact of HIV-1 infection on host CD4 and CD8 responses to the Mtb and CMV, I elected to first examine these responses in detail in the control group of healthy HIV-uninfected individuals.

6.4.2.1 Magnitude of antigen-specific-CD4 responses to CMV, EC and PPD but not Rv2031c are greater than antigen-specific-CD8 responses to the same antigens in healthy HIV-uninfected individuals

Mtb- and CMV-specific CD4 responses in healthy individuals are generally of greater magnitude than CD8 responses responding to the same antigens. I measured the magnitude of IFNγ+ CD4/CD8 responses to each antigen in terms of percentage of total CD4/CD8 population and absolute number of cells/uL of whole blood (Fig. 6.1).

The frequency of IFNγ+ CD4 T cells after CMV, PPD and EC stimulation was significantly greater than that of IFNγ+ CD8 T cells ($p<0.001$ for CMV, PPD and Rv2031c, Fig. 6.1a). This finding was consistent when comparing the absolute number of IFNγ+ CD4 and CD8 T cells after CMV, PPD and EC stimulation ($P<0.001$ for CMV, PPD and EC, Fig. 6.1b). Interestingly this was not the case for the frequency and absolute magnitude of Rv2031c-specific IFNγ+ CD4 and CD8 responses ($p = 0.37$ and $p = 1.0$ respectively, Fig. 6.1 a-b).

The median non-specific background (as determined from the negative control) was less than 0.03% for single cytokines and negligible when more than one cytokine was examined. I therefore defined a positive cytokine response for a single cytokine as a response greater than 0.03% of total CD4/CD8 T cells (after subtraction of background), in order to ensure that only genuine responses were examined. The IFNγ response to antigenic stimulation was used to define an individual as a "responder" as it is most commonly the predominant CD4 and CD8 cytokine produced in response to the antigens used in this study and forms the basis of the currently used and standardized tests for LTBI (IGRAs) (Walzl et al. 2011).

97.6% and 95.5% of healthy individuals within our study had a positive CD4 response to CMV and PPD respectively (Table 6.2). Although the median frequency of the IFNγ+ CD4 response to Rv2031c was of smaller magnitude that that of EC (Fig 6.1 a-b), the proportion of the HIV-uninfected group with a positive response was actually higher for Rv2031c (79.5%) than for EC (65.9%) (Table 6.2).

When comparing the proportion of positive CD4 responses to that of the CD8 responses within the same antigen-specific T cell response to each antigen, the proportion of CD8 responses was significantly less for responses to EC and PPD (P = 0.005 and P = 0.0004 respectively, Table 6.2) but not for CMV and Rv2031c. This suggests a relative greater ability of CMV lysate and Rv2031c to generate HLA Class I restricted responses in healthy HIV-uninfected individuals.

Table 6.1 Characteristics of all individuals included in the flow cytometry analyses

Characteristic	Total cohort	HIV negative	Total HIV positive	HIV positive			
	n= 191	n= 44	n=147	**CD4 >350** n= 48	**CD4 201-350** n= 48	**CD4 101 – 200** n= 41	**CD4 ≤ 100** n= 10
Gender							
Female - n (%)	116 (61)	20 (45)	96 (65)	37 (77)	34 (71)	18 (44)	7 (70)
Male - n (%)	75 (39)	24 (55)	51 (35)	11 (23)	14 (29)	23 (56)	3 (30)
Age (years)[a]	34 (27 – 43)	24 (20 – 32)	36 (30 -44)	36 (31 – 44)	37 (29 – 46)	36 (31 – 44)	35 (32 – 44)
CD4 count (cells/μL)[a]	334 (192 – 597)	879 (686 – 1054)	259 (178 – 379)	473 (380 – 596)	260 (231 – 295)	168 (138 – 185)	78 (57 – 95)
Viral load (RNA copies/mL)[a]	N/A	N/A	48173 (11490 – 161087)	15306 (6914 – 64855)	58963 (16423 – 181851)	134018 (32275 – 387620)	177684 (80016 – 488469)
BMI (kg/m^2)[a]	24.8 (22.4 – 28.3)	24.3 (22.7 – 27.8)	24.9 (22.3 – 28.7)	26.5 (22.7 – 31.5)	24.9 (21.0 – 28.1)	23.4 (216 – 26.4)	27.6 (21.0 – 36.3)
Prev TB - n (%)	14 (7)	0 (0)	14 (10)	6 (13)	5 (10)	2 (5)	1 (10)
BCG scar - n (%)	109 (57)	20 (45)	89 (61)	31 (65)	25 (52)	28 (68)	5 (50)
INH prophylaxis - n (%)	51 (27)	0 (0)	51 (35)	12 (25)	19 (40)	15 (65)	5 (50)

[a] Data expressed as median (interquartile range)

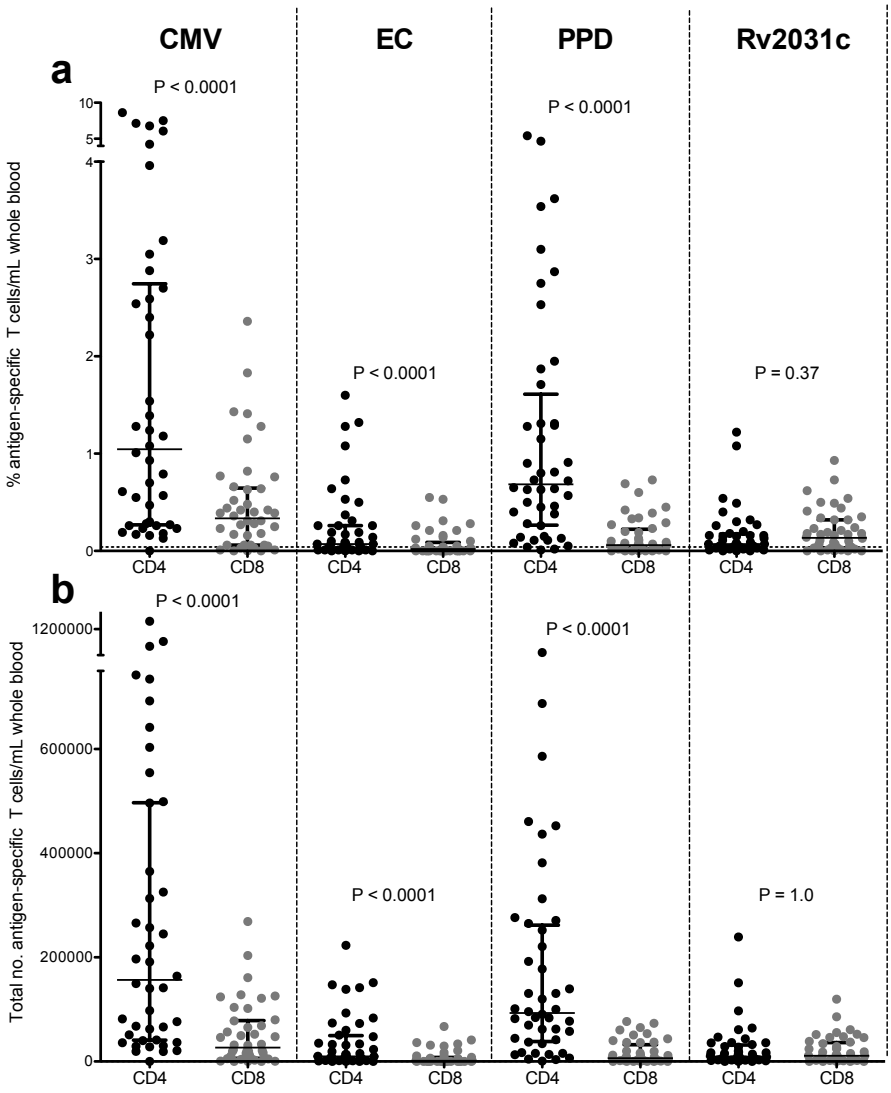


Figure 6.1 Comparison of CD4 and CD8 magnitude of responses to CMV, EC, PPD and Rv2031c. a) Percentage of total IFNγ+ CD4 and CD8 T cells. The dotted line represents the level of detection. b) Absolute number of IFNγ+ CD4 and CD8 T cells per mL of whole blood. Horizontal bars indicate the median and IQR. P values indicated for Wilcoxon signed rank test.

Table 6.2 The proportion of HIV-uninfected individuals with a positive IFNγ response.

Antigen	**Antigen-specific IFNγ positive response**		
	CD4 Number (%)	**CD8** Number (%)	**P value**
CMV (n = 42)	41 (97.6)	35 (83.3)	0.057
EC (n = 44)	29 (65.9)	15 (34.1)	**0.005**
PPD (n = 44)	42 (95.5)	28 (63.6)	**0.0004**
Rv2031c (n = 44)	35 (79.5)	33 (75.0)	0.800

P values are indicated for Fisher's exact tests.

6.4.2.2 Comparison of CD4/CD8 CMV- and Mtb-specific cytokine expression profiles

Geldmacher et al. found striking differences in depletion rates during HIV-1 infection between CD4 T cells specific for Mtb and CMV and attributed these differences, in part, to their differential production of cytokines IL-2 and MIP1-β (Geldmacher et al. 2010). I sought to compare and contrast the production of Th_1 cytokines IFNγ, TNFα and IL-2 as well as the Th_{17} cytokine IL-17 in CD4/CD8 responses to CMV and the Mtb antigens in healthy HIV-uninfected individuals. This analysis would form the basis of later comparisons with HIV-infected individuals.

a) Proportion of cytokine positive cells within total CD4 and CD8 T cells

I first compared the proportion of cytokine positive CD4/CD8 cells within the total number of CD4/CD8 T cells in individuals with a positive response to each of the antigens (Fig. 6.2 and 6.3).

CD4

IFNγ was the cytokine most frequently produced in response to stimulation with all 4 antigens followed by TNFα for CMV, EC and PPD (Fig. 6.2). Interestingly IL-17 was the second most frequency expressed cytokine in response to Rv2031c stimulation whilst both CMV-specific and Rv2031c-specific CD4 responses expressed markedly less IL-2 than the Mtb-responses of EC and PPD.

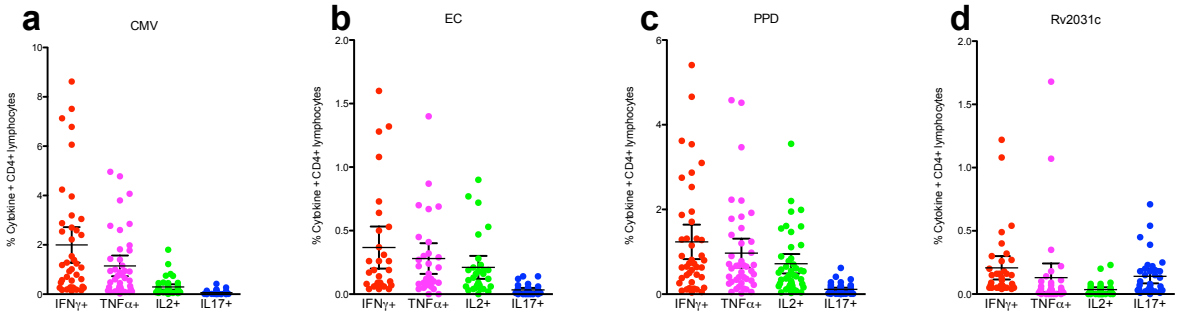


Figure 6.2 A comparison of the frequency of CD4 T cells expressing IFNγ, TNFα, IL-2 and IL-17. CD4 T cell responses to all antigens are shown. Horizontal bars show the median and whiskers the IQR.

CD8

IFNγ and TNFα were also the cytokines most frequently produced by antigen-specific CD8 T cells in response to stimulation with all the tested antigens with IL-2 expression again lowest amongst CMV- and Rv2031c-stimulated CD8 T cells. IL-17+ CD8 T cells were least frequent amongst the antigen-specific CD8 response to all antigens.

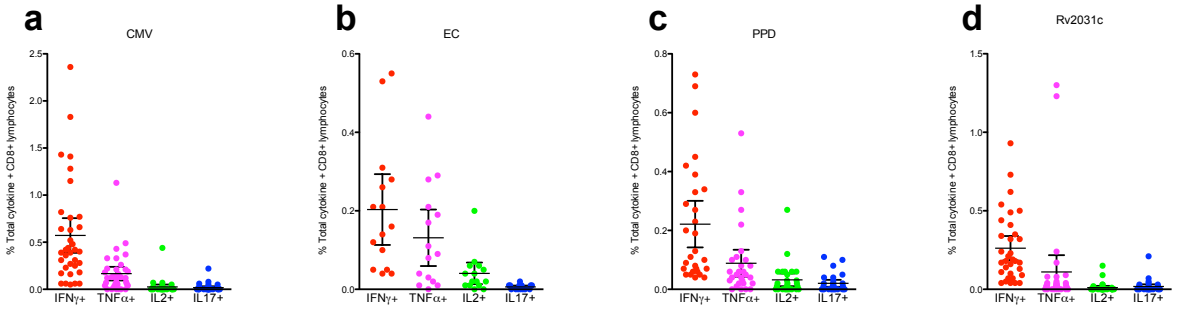


Figure 6.3 A comparison of the frequency of CD8 T cells expressing IFNγ, TNFα, IL-2 and IL-17. CD8 T cell responses to all antigens are shown. Horizontal bars show the median and whiskers the IQR

b) Proportion of cytokine-antigen-specific expression within total cytokine positive T cells

In order to compare the contribution of each cytokine expressing T cell population to the overall cytokine response to each antigen, I next looked at the contributions of

cytokine-expressing CD4/CD8 T cells as a fraction of the total the CD4/CD8-antigen specific cytokine-positive response.

CD4

The fraction of IFNγ+ CD4 T cells among total antigen-specific cytokine positive CD4 cells was greatest for CMV- and least for Rv2031c-stimulated CD4 T cells. PPD stimulated CD4 T cells expressed proportionally more IL-2 and TNFα than CMV-, EC- and Rv2031c-stimulated CD4 T cells. The fraction of total cytokine positive CD4 T cells expressing IL-17 was greatest for Rv2031c, highlighting the importance of a Th_{17} response to this "latency-associated" antigen.

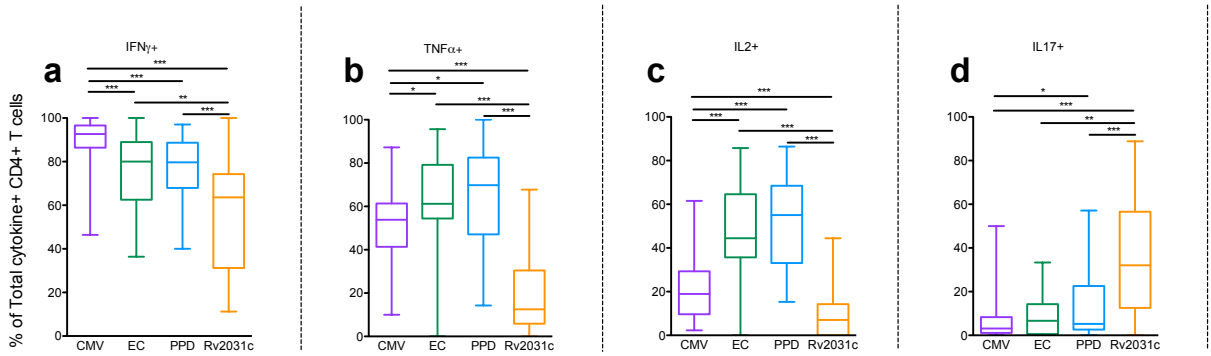


Figure 6.4 The fraction of IFNγ+, TNFα+, IL-2+ and IL-17+ CD4 T cells among total cytokine-positive CD4 T cells. CD4 T cell responses to all the antigens tested are shown. Statistical analyses were performed using Wilcoxon rank sum test (***, P<0.0005; **, P<0.005; * P<0.05).

CD8

IFNγ+ and TNFα+ CD8 T cells composed the largest fraction of the total cytokine positive CD8 response to all 4 antigens with IL-2 and IL-17 contributing minimally. The proportion of IFNγ+ CD8 T cells was higher for CMV than EC and PPD whilst EC

and PPD had a higher fraction of TNFα+ and IL-2+ CD8 T cells amongst the total cytokine positive CD8 response.

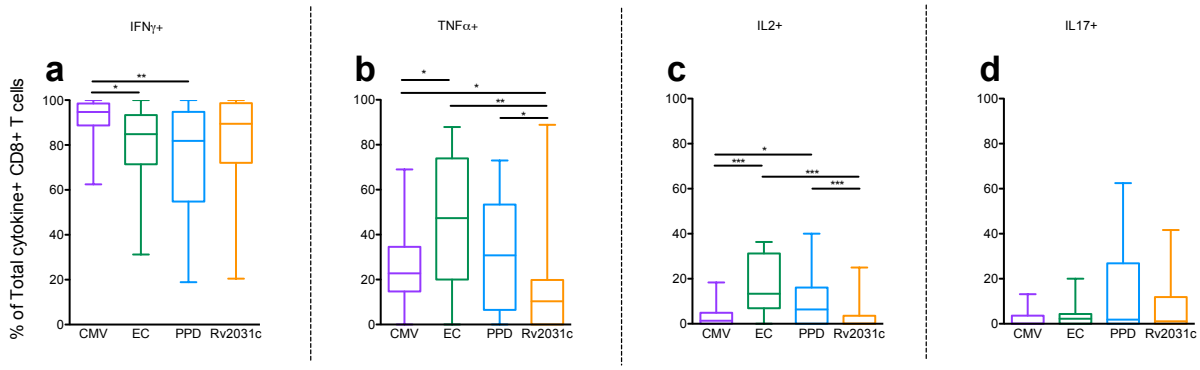


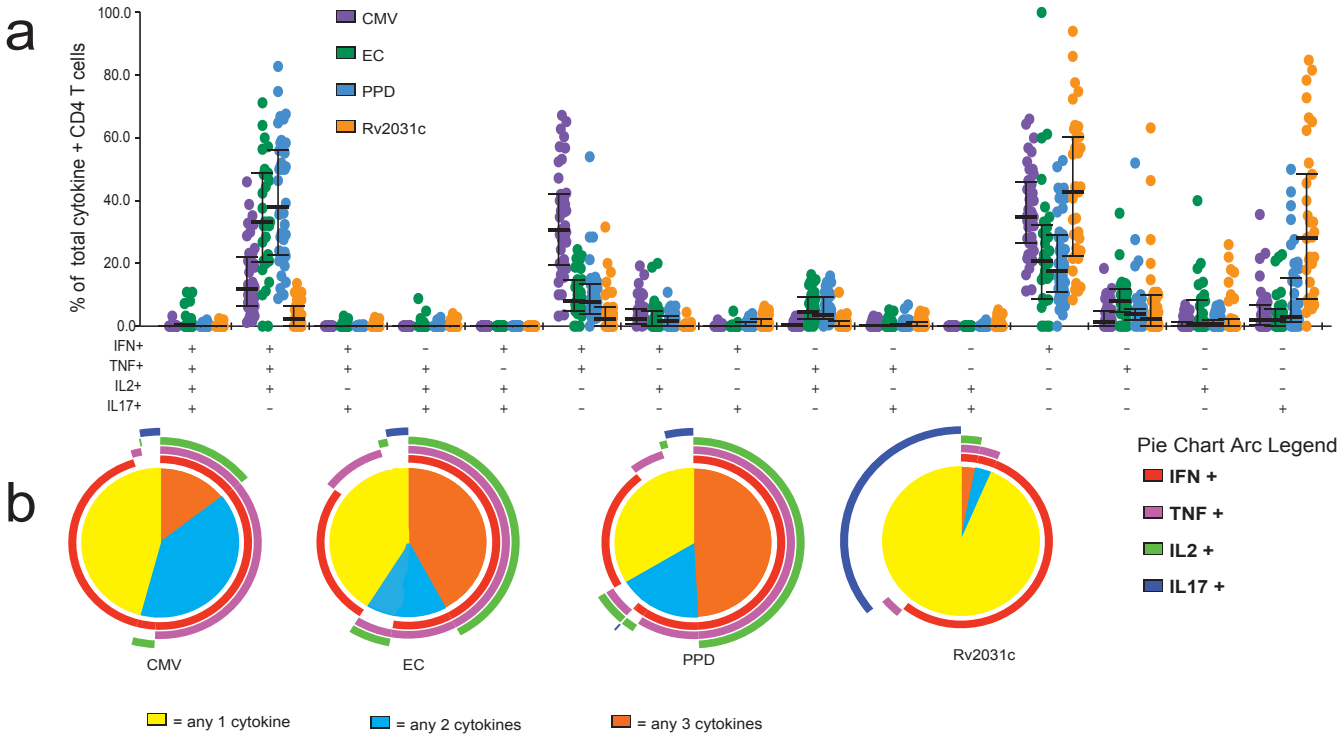
Figure 6.5 The fraction of IFNγ+, TNFα+, IL-2+ and IL-17+ CD8 T cells among total cytokine-positive CD8 T cells. CD8 T cell responses to all the antigens tested are shown. Statistical analyses were performed using Wilcoxon rank sum test (***, P<0.0005; **, P<0.005; * P<0.05).

c) Simultaneous cytokine expression on a single cell level

Through the use of a Boolean analysis technique, I was able to investigate CD4 and CD8 T cells simultaneously expressing any combination of the 4 cytokines measured and compare these cytokine expression profiles between different antigen-specific T cell populations.

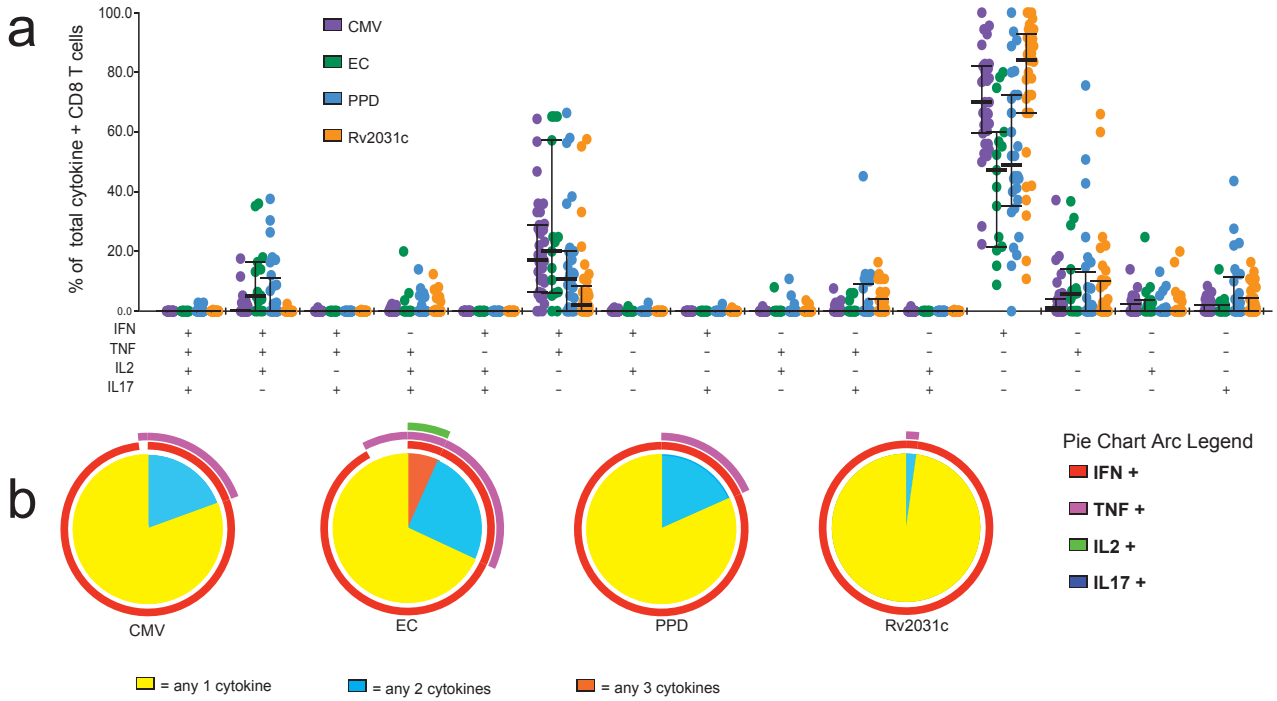
The proportions of cytokine-expressing subpopulations as a fraction of total cytokine-positive CD4 and CD8 T cells are shown for all antigens in Figure 6.6 and Figure 6.7 respectively. The highest proportion of cytokine-producing CD4 T cells after CMV stimulation was single-positive (1+) for IFNγ followed by double-positive (2+) for IFNγ+ and TNFα and triple-positive (3+) for IFNγ, TNFα and IL-2+ respectively. By contrast the PPD and EC cytokine responses were predominantly 3+ (IFNγ+TNFα+IL-2+) followed by 1+ (IFNγ+) and then 2+(IFNγ+TNFα and TNFα +IL-2+). The Rv2031c-specific CD4 response differed from the other Mtb-responses as it was

mainly composed of 1+ (IFNγ+) and 1+(IL-17+) CD4 populations. A very small proportion of each of the antigen specific responses was 4+. In fact IL-17 was rarely expressed in conjunction with any of the Th1 cytokines, confirming that CMV- and Mtb-specific Th_{17} CD4 T cells subsets are distinct from Th_1 cytokine-producing cells (Scriba et al. 2008).



Pie	CMV P value	EC P value	PPD P value	Rv2031c P value
CMV		< 0.0001	< 0.0001	< 0.0001
EC	< 0.0001		0.1410	< 0.0001
PPD	< 0.0001	0.1410		< 0.0001
Rv2031c	< 0.0001	< 0.0001	< 0.0001	

Figure 6.6 Flow cytometric analysis of IFNγ, TNFα, IL-2 and IL-17 production within pathogen-specific CD4 T cells stimulated with CMV and Mtb antigens. a) Values shown are the fraction of cells contributed by each cytokine-expressing population to the overall cytokine positive response to each antigen concerned. b) Pie charts show the fraction of cells expressing 1, 2, 3 or 4 cytokines. The arcs surrounding the pie charts indicate the proportion of the responses contributed by the single cytokines IFNγ, TNFα, IL-2 and IL-17. The statistics indicated compare the difference in cytokine expression profiles of the antigen-specific responses. P values are calculated using a partial permutation analysis (Roederer et al. 2011).



Pie	CMV P value	EC P value	PPD P value	Rv2031c P value
CMV		0.0008	0.0036	0.0177
EC	0.0008		0.2429	0.0004
PPD	0.0036	0.2429		0.0014
Rv2031c	0.0177	0.0004	0.0014	

Figure 6.7 Flow cytometric analysis of IFNγ, TNFα, IL-2 and IL-17 production within pathogen-specific CD8 T cells stimulated with CMV and Mtb antigens. a) Values shown are the fraction of cells contributed by each cytokine-expressing population to the overall cytokine positive response to each antigen concerned. b) Pie charts show the fraction of cells expressing 1, 2, 3 or 4 cytokines. The arcs surrounding the pie charts indicate the proportion of the responses contributed by the single cytokines IFNγ, TNFα, IL-2 and IL-17. The statistics indicated compare the difference in cytokine expression profiles of the antigen-specific responses. P values are calculated using a partial permutation analysis (Roederer et al. 2011).

6.4.3 The effect of HIV-1 infection on the Mtb- and CMV-specific T cell response

6.4.3.1 HIV-1 infection is associated with a decline in CD4:CD8 ratio as a result of CD4 and CD8 T cell depletion

Absolute CD4 and CD8 T cell counts were evaluated using flow cytometry in 44 HIV-uninfected and 147 HIV-infected adults in order to determine the effect of HIV-1 infection on the overall magnitude of these T cell populations. As was expected, the median CD4 T cell count was significantly lower in HIV-infected individuals (Fig. 6.8a, P<0.0001) whilst there was no significant difference in the absolute CD8 T cell counts between the two groups (Fig. 6.8a, P = 0.124). The median CD4:CD8 ratio of 1.52 in the HIV-uninfected group was as expected for healthy African individuals (Lugada et al. 2004; Tsegaye et al. 1999). By comparison, the CD4:CD8 ratio in the HIV-infected individuals was significantly reduced (Fig 6.8b, P <0.0001). This occurred despite a decline in both the CD4 and CD8 absolute numbers as a result of a decline in numbers of both CD4 and CD8 T cells associated with HIV-1 infection (Fig. 6.8c; P <0.0001, r = 0.354).

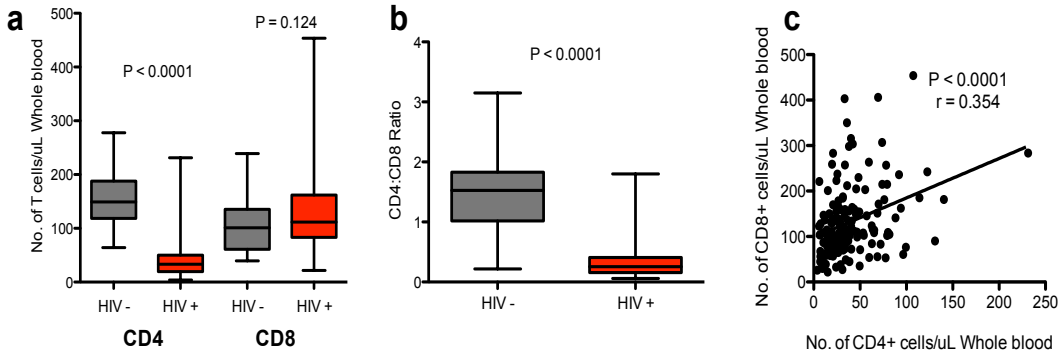


Figure 6.8 Comparisons of absolute CD4 and CD8 T cell numbers and CD4:CD8 ratios. HIV+ n= 147, HIV- n = 44 a) Absolute CD4 and CD8 T cell counts are compared between HIV+ and HIV- individuals. P values indicated for Mann-Whitney tests. b) CD4:CD8 ratios compared between HIV+ and HIV- individuals. P values indicated for Mann-Whitney tests. c) Spearman's correlation analysis for associations between absolute CD4 and CD8 T cell counts in HIV+ individuals

6.4.3.2 The effect of HIV-1 infection on the magnitude and proportion of positive responders to CMV and Mtb antigens

The magnitude of total CD4 and CD8 T cell numbers is clearly significantly affected by HIV-1 infection (6.8 a-c). I therefore wanted to investigate whether these effects were also evident within CMV- and Mtb-specific CD4 and CD8 T cell populations.

A comparison of overall median frequency of IFNγ+ CD4 T cells between HIV-uninfected and HIV-infected individuals revealed a significant decrease in magnitude of response to PPD and Rv2031c in individuals infected with HIV-1 (Fig. 6.9 c and d; PPD, P <0.0001; Rv2031c, P = 0.007). Whilst the median IFNγ+ CD4 response was higher in HIV-infected individuals for CMV and lower for EC, these differences were not however significant (Fig. 6.9 a and b; CMV, P = 0.068; EC, P = 0.055). Remarkably there was a similar trend for IFNγ+ CD8 T cell responses with higher median responses to PPD and Rv2031c amongst those uninfected with HIV-1 and no difference apparent between CD8 responses to CMV and EC (Fig. 6.9 e - h; PPD, P = 0.008; Rv2031c, P = 0.015,). It appears that the reduction in magnitude of the CD4 T cell response to these antigens was also associated with a reduction in the CD8 T cell response to the same antigen.

HIV-1 infection was unsurprisingly also associated with a significant decrease in the number of individuals with detectable IFNγ+ CD4 responses to PPD and Rv2031c (Table 6.3; PPD, P = 0.0004; Rv2031c, P = 0.012). Whilst 95.5% and 79.5% of the HIV-uninfected individuals responded to PPD and Rv2031c respectively, only 71.4% and 58.2% of HIV-infected individuals had a detectable CD4 response to these antigens. Although the proportion of PPD and Rv2031c CD8 responders was higher in the HIV-

uninfected group, this difference did not reach statistical significance (Table 6.3; PPD, P = 0.085; Rv2031c, P = 0.075).

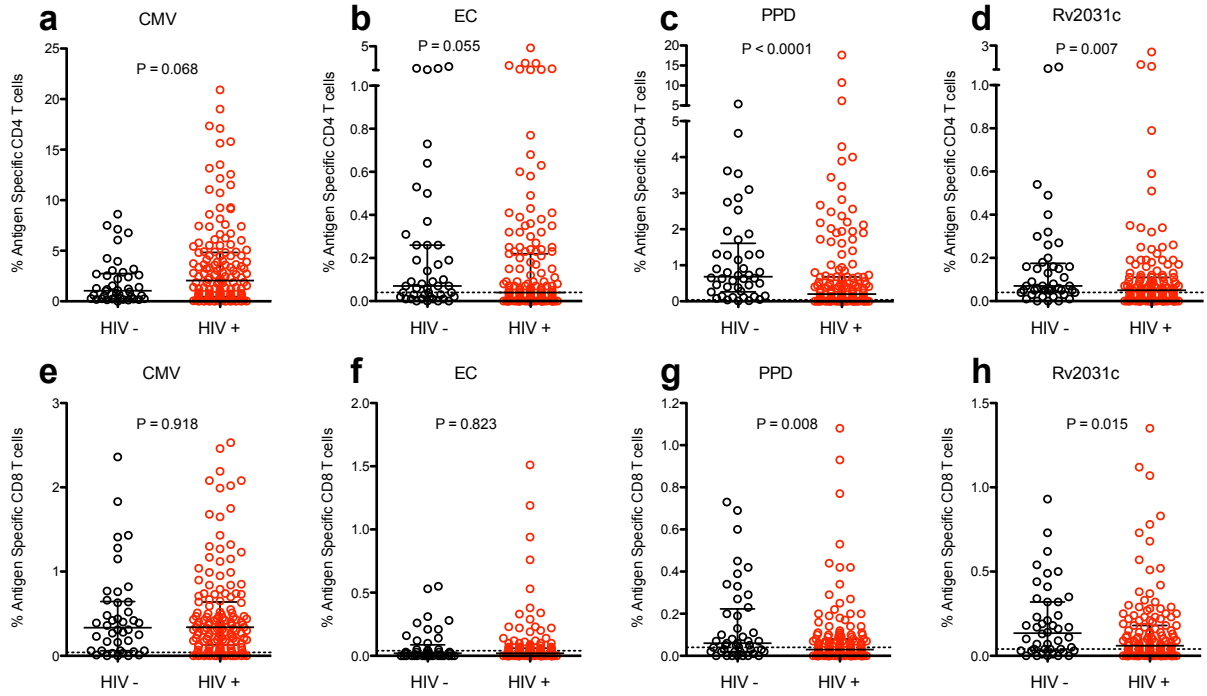


Figure 6.9 Comparison of IFNγ+ antigen-specific CD4 and CD8 T cell frequencies. a – d show CD4 frequencies and e – h show CD8 frequencies. P values indicated for Mann-Whitney tests.

Table 6.3 Percentages of IFNγ positive-responders compared between HIV+ and HIV- individuals

	Antigen	**Antigen-specific IFNγ positive response**		
		HIV - Number (%)	**HIV +** Number (%)	**P value**
CD4	CMV	41/42 (97.6)	137/147 (93.2)	0.461
	EC	29/44 (65.9)	79/147 (53.7)	0.169
	PPD	42/44 (95.5)	105/147 (71.4)	**0.0004** ***
	Rv2031c	35/44 (79.5)	85/146 (58.2)	**0.012** *
CD8	CMV	35/42 (83.3)	128/147 (87.1)	0.612
	EC	15/44 (34.1)	52/147 (35.4)	1.00
	PPD	28/44 (63.6)	70/147 (47.6)	0.085
	Rv2031c	33/44 (75.0)	87/146 (59.6)	0.075

P values are indicated for Fisher's exact tests. * P <0.05, ** P<0.005, ***P<0.0005

I next wanted to assess whether a comparison of the absolute number of T cells per mL of whole blood used in the WB ICS assay (as opposed to a percentage of total CD4/CD8 T cells) could provide a more sensitive method of evaluating HIV-1 associated perturbations in the antigen-specific T cell responses measured. A comparison of the absolute number of IFNγ+ CD4 T cells responding to each antigen, provided overwhelming evidence that HIV-1 infection is associated with depletion in the magnitude of all antigen-specific CD4 responses in this study (P <0.0001 for all antigens, data not shown). An identical analysis of absolute number of IFNγ+ CD8 T cells showed a reduction in magnitude of PPD-specific CD8 T cells only highlighting the predominant CD4 T cell depletion in HIV-1 infection (Fig. 6.10; P = 0.042).

Taken together these data indicate that HIV-1 infection results in the depletion of the total number of antigen-specific CD4 T cells responding to CMV, EC, PPD and Rv2031c. PPD- and Rv2031c-specific CD4 T cells appear to be preferentially depleted to a far greater extent, as the proportions of these antigen-specific CD4s of the total CD4 T cell population were significantly depleted in individuals with HIV-1 infection whilst that of CMV and EC were not.

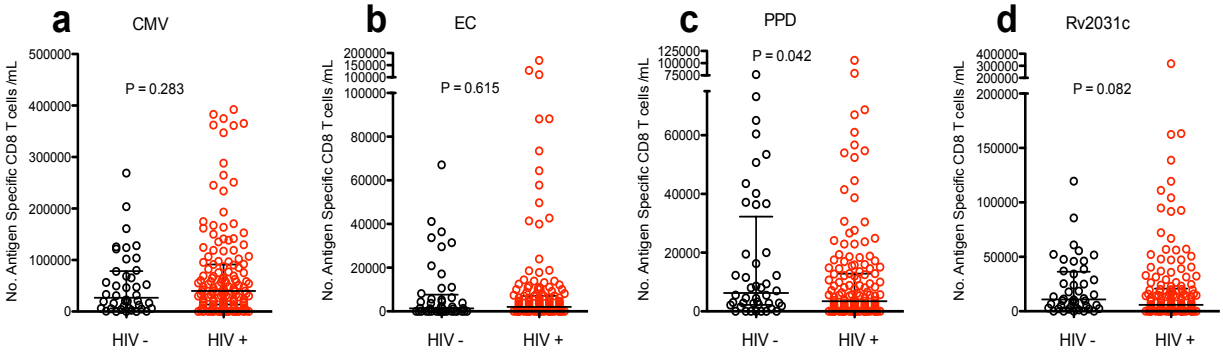


Figure 6.10 A comparison of the absolute magnitude of IFNγ+ antigen-specific CD8 T cells. Values are shown as number of cells per mL of whole blood. P values indicated are for Mann-Whitney tests.

6.4.3.3 The effect of HIV-1 infection on Th_1 and Th_{17} CD4 cytokine expression in responders to CMV, EC, PPD and Rv2031c

I next wanted to investigate whether the different dynamics of antigen-specific T cell perturbations noted above were associated with changes in particular CD4 cytokine-producing populations. To do this I compared the median proportion of cytokine-expressing CD4 T cells between the groups based on HIV-1 infection status (only in individuals with a clear IFNγ response as previously defined) (Table 6.4).

HIV-infected CMV-responders had a higher median frequency of TNF+ CD4 T cells (P = 0.045) than those uninfected with HIV-1 indicating that this cytokine expressing CD4 T cell population is not only maintained in chronic HIV-1 infection, but is inflated as a component of the total CD4 T cell population. In contrast the median frequency of IL-17+ CD4 T cells was significantly reduced in individuals within the same group when compared to those individuals uninfected with HIV-1 (Table 6.4, P = 0.005). As aforementioned, the Th_{17} response to CMV is however a minor fraction of the total CMV-specific CD4 response.

For EC-specific CD4 positive responses, the median frequencies of all cytokine-expressing CD4 T cells were lower in those infected with HIV-1, with this difference statistically significant for IL-2- and IL-17-expressing CD4 T cells only (Table 6.4; IL-2, P = 0.036; IL-17, P = 0.037). PPD-specific CD4 T cells expressing all the cytokines measured were significantly reduced within the HIV-infected group, highlighting the increased susceptibility to depletion of this antigen-specific CD4 response during HIV-1 disease. The frequency of total cytokine+ CD4 T cells responding to Rv2031c was reduced in those infected with HIV-1 (Table 6.4; P <0.001). As mentioned previously,

the Th_1 CD4 response to stimulation with Rv2031c was composed predominantly of IFNγ+ T cells whilst the Th_{17} response predominantly of IL-17+ T cells. The frequencies of both of these cytokine-expressing CD4 T cell subpopulations were reduced in the HIV-infected group, suggesting a susceptibility to HIV-1-associated depletion of these particular cytokine expressing components of the Rv2031c CD4 response (IFNγ, P = 0.007; IL-17, P <0.001).

Table 6.4 The effect of HIV-1 on antigen-specific cytokine expressing CD4 populations

	Cytokine	**Antigen-specific IFNγ positive response**		
		HIV - Median (IQR) (%)	**HIV +** Median (IQR) (%)	**P value**
CMV	Total cytokine+	1.14 (0.32 – 3.02)	2.23 (0.55 – 4.93)	0.061
	IFNγ+	1.05 (0.27 – 2.75)	2.05 (0.46 – 4.83)	0.068
	TNFα+	0.63 (0.18 – 1.52)	1.10 (0.29 – 3.02)	**0.045**
	IL2+	0.16 (0.09 – 0.33)	0.18 (0.08 – 0.44)	0.799
	IL17+	0.04 (0.01 – 0.07)	0.02 (0.00 – 0.05)	**0.005**
EC	Total cytokine+	0.13 (0.06 – 0.39)	0.09 (0.03 – 0.29)	0.155
	IFNγ+	0.07 (0.02 – 0.26)	0.04 (0.01 – 0.22)	0.055
	TNFα+	0.09 (0.03 – 0.27)	0.05 (0.01 – 0.17)	0.178
	IL2+	0.06 (0.02 – 0.19)	0.04 (0.01 – 0.10)	**0.036**
	IL17+	0.01 (0.10 – 0.22)	0.01 (0.00 – 0.02)	**0.037**
PPD	Total cytokine+	0.87 (0.38 – 1.94)	0.31 (0.07 – 0.90)	**< 0.001**
	IFNγ+	0.69 (0.27 – 1.61)	0.20 (0.03 – 0.68)	**< 0.001**
	TNFα+	0.58 (0.26 – 1.23)	0.23 (0.03 – 0.72)	**0.001**
	IL2+	0.45 (0.19 – 0.88)	0.17 (0.04 – 0.50)	**< 0.001**
	IL17+	0.05 (0.02 – 0.18)	0.03 (0.01 – 0.07)	**< 0.001**
Rv2031c	Total cytokine+	0.21 (0.08 – 0.38)	0.09 (0.03 – 0.17)	**< 0.001**
	IFNγ+	0.07 (0.04 – 0.18)	0.05 (0.01 – 0.11)	**0.007**
	TNFα+	0.03 (0.00 – 0.06)	0.02 (0.00 – 0.05)	0.276
	IL2+	0.01 (0.00 – 0.04)	0.01 (0.00 – 0.03)	0.148
	IL17+	0.05 (0.02 – 0.18)	0.01 (0.00 – 0.03)	**< 0.001**

P values indicated are for Mann-Whitney tests.

6.4.3.4 The effect of HIV-1 on "polyfunctional" CD4 and CD8 responses to CMV, EC, PPD and Rv2031c

The effect of HIV-1 infection on the polyfunctional Mtb-specific CD4 and CD8 response is, as yet, unclear. I compared the proportion of 1+, 2+ and 3+ Th_1 cytokine expressing CD4 and CD8 T cells, within the total CD4/CD8 cytokine-positive response, between those infected and uninfected with HIV-1.

Within the CMV-specific CD4 T cell response, HIV-1 infection was associated with a decrease in the proportion of the 3+(IFNγ+TNFα+IL-2+) response and an associated increase in the 2+ proportion (IFNγ+TNFα+ and TNFα+IL-2+) (Fig. 6.11 a and b; P=0.017). Whilst the median proportion of polyfunctional 3+ CD4 T cells specific for EC was higher and for PPD was lower in those uninfected with HIV-1, these differences did not reach statistical significance. There was however a higher proportion of 1+ (IFNγ+, P <0.05) and lower proportion of the 2+(TNFα+IL-2+, P <0.05) response within the PPD-cytokine+ CD4 response. The Rv2031c-specific cytokine profile showed a significant reduction in the proportion of IL-17+ CD4 expressing response but no changes with respect to expression of Th_1 cytokines.

Due to the almost negligible expression of IL-17 in healthy CD8 responses in this study, the focus of the CD8 polyfunctional analysis was on Th_1 cytokines only. There was very little difference evident in the overall patterns of CD8 antigen-specific responses. PPD-specific responses in HIV-infected individuals were associated with a greater proportion of the 1+ (IFNγ+) response (Fig. 6.12 e and f; P = 0.041).

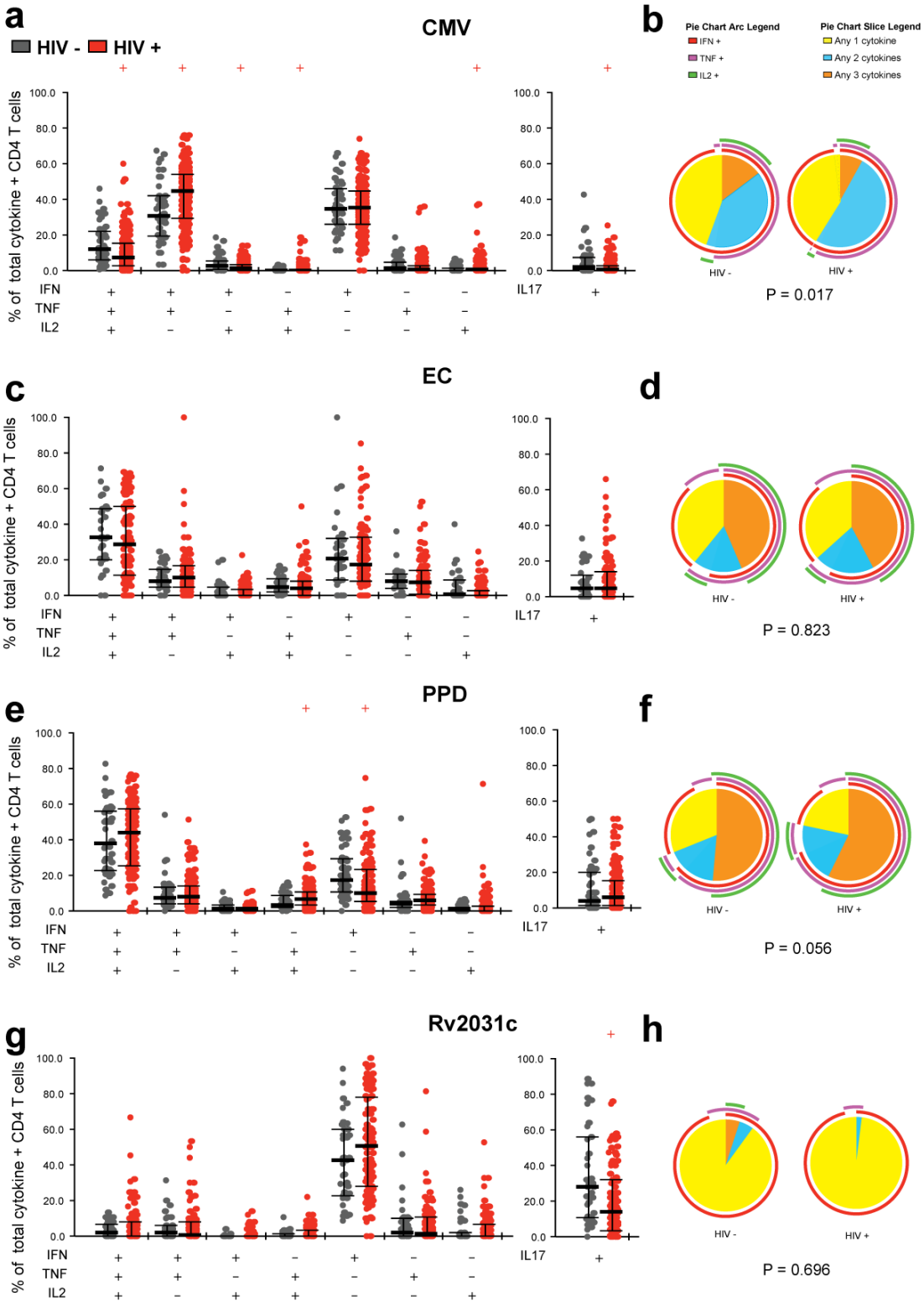


Figure 6.11 A comparison of Th_1 cytokine production by antigen-specific CD4 T cells stimulated with CMV and Mtb antigens. a,c,e and g - values shown are the fraction of cells contributed by each cytokine-expressing population to the overall cytokine positive response to each antigen concerned. Red x indicates $P < 0.05$ for Student's T test. b, d, f and h - pie charts show the fraction of cells expressing 1, 2, or 3 cytokines. The arcs surrounding the pie charts indicate the proportion of the responses contributed by the single cytokines. P values are calculated using a partial permutation analysis (Roederer et al. 2011).

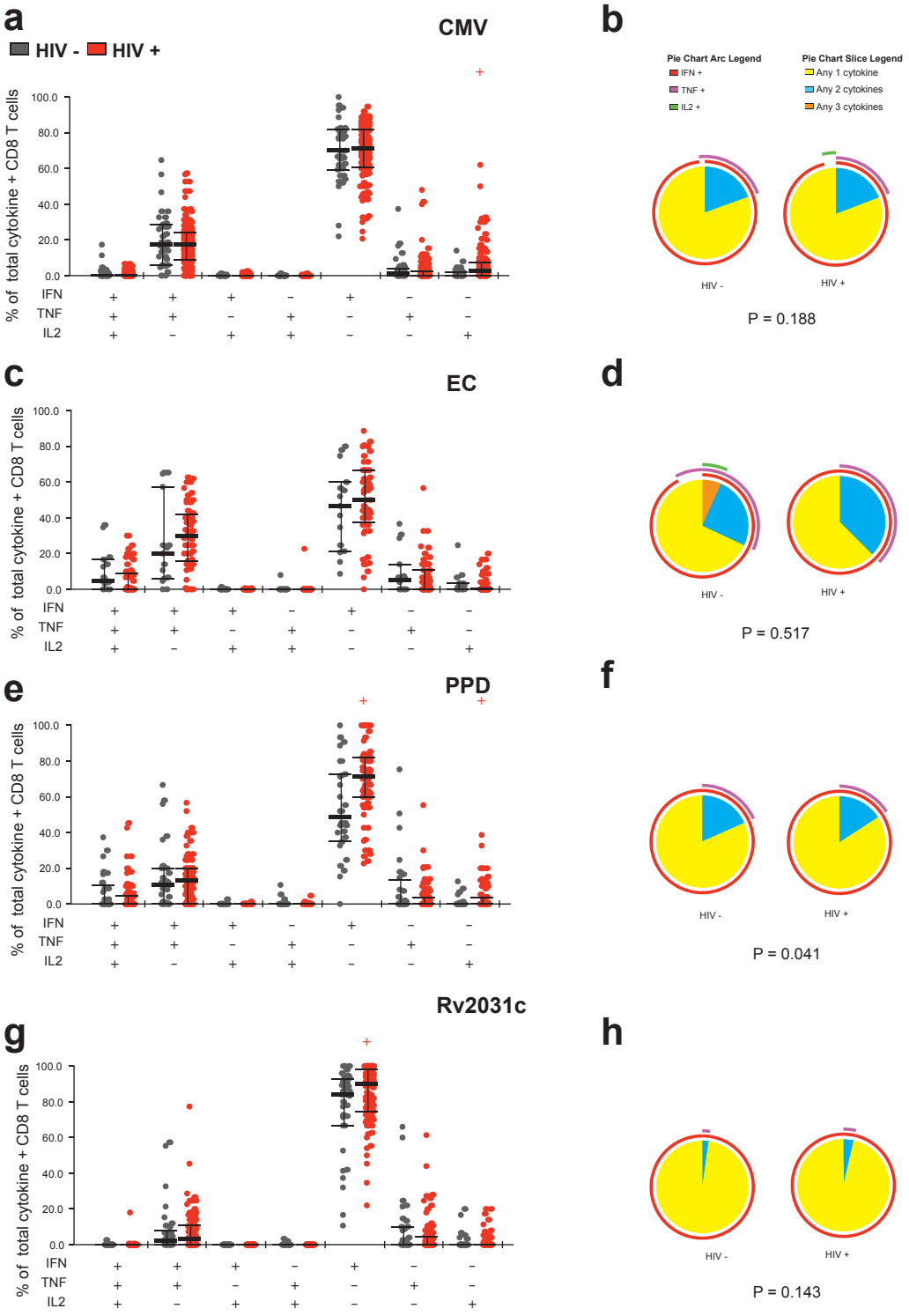


Figure 6.12 A comparison of Th_1 cytokine production by antigen-specific CD8 T cells stimulated with CMV and Mtb antigens. a,c,e and g - values shown are the fraction of cells contributed by each cytokine-expressing population to the overall cytokine positive response to each antigen concerned. Red x indicates $P < 0.05$ for Student's T test. b, d, f and h - pie charts show the fraction of cells expressing 1, 2, or 3 cytokines. The arcs surrounding the pie charts indicate the proportion of the responses contributed by the single cytokines. P values are calculated using a partial permutation analysis (Roederer et al. 2011).

6.4.4 The relationship between functionality of Mtb-specific T cell responses and parameters of HIV-1 disease progression

To further investigate the impact of HIV-1 infection on CMV and Mtb-specific T cell responses, I analysed the relationship between the magnitude of responses (in terms of frequency and absolute number of IFNγ+ CD4 T cells) and parameters of HIV-1 disease progression, including CD4 T cell count and HIV-1 log viral load.

I found no correlations between the frequency of IFNγ+ CD4 responses to any of the antigens and CD4 count, however the frequency of CMV-specific IFNγ+ CD4 T cells was inversely correlated with HIV-1 viral load (Table 6.5. Spearman's r = -0.227, P = 0.008). The absolute number of responding IFNγ+ CD4 T cells to all antigens was strongly correlated with absolute CD4 T cell count indicating that the number of IFNγ+ CD4 T cells specific for all of the antigens tested in this study decline with total CD4 T cell count (Table 6.5; P <0.005 for all). The inverse correlation between CMV- and Rv2031c-specific absolute IFNγ+ CD4 T cell counts and HIV-1 viral load, possibly indicates steady and progressive depletion of these antigen-specific CD4 responses (Table 6.5. CMV: Spearman's r = -0.420, P < 0.0001; Rv2013c: Spearman's r = -0.290, P = 0.007). By contrast the lack of a significant correlation between EC- and PPD-specific absolute IFNγ+ CD4 T cell counts and viral load could lead us to hypothesize that these particular antigen-specific CD4 T cell populations are depleted early in HIV-1 infection.

Table 6.5 Correlations between antigen-specific CD4 T cell populations and markers of HIV-1 disease progression

		Correlation with CD4 counts		Correlation with Viral Load	
	Antigen	Spearman's R value	P	Spearman's R value	P
CD4 %	CMV	0.019	0.822	**-0.227**	**0.008**
	EC	-0.102	0.373	0.134	0.238
	PPD	-0.145	0.139	0.010	0.311
	Rv2031c	-0.154	0.160	0.083	0.453
CD4 abs mag	CMV	**0.383**	**<0.0001**	**-0.420**	**<0.0001**
	EC	**0.406**	**0.0002**	-0.173	0.127
	PPD	**0.253**	**0.009**	-0.168	0.087
	Rv2031c	**0.499**	**<0.0001**	**-0.290**	**0.007**

Both % and absolute number of IFNγ+ CD4 T cells responding to each antigen are correlated with total CD4 T cell count and HIV-1 viral load. P values are generated using Spearman's correlation analysis.

By exploring the association of the specific-cytokine expressing proportion of the total cytokine + CD4 T cell response to each of the antigens, with markers of HIV-1 disease progression the effect of HIV-1 infection on each cytokine-expressing CD4 T cell population can be evaluated.

The fact that the proportions of IL-17+ CD4 T cells of the total cytokine+ CD4 T cell response to EC, PPD and Rv2031c were positively correlated with absolute CD4 T cell count and negatively correlated with HIV-1 viral load confirms that IL-17+ CD4 T cells are preferentially and progressively depleted during HIV-1 disease progression. The proportion of TNFα expressing CD4 T cells responding to PPD increases with decreasing CD4 count and rising HIV-1 viral load, suggesting a preservation of this cytokine-producing subset of the PPD CD4 response during HIV-1 disease progression. The absence of any correlation between the proportion of IL-2+ CD4 T cells and markers of disease progression is surprising and may possibly be explained by early

depletion during the course of HIV-1 disease (Geldmacher et al. 2010; Geldmacher et al. 2008).

Table 6.6 Correlations between cytokine-expressing CD4 T cell populations and markers of HIV-1 disease progression.

		Correlation with CD4 counts		**Correlation with Viral Load**	
	Antigen	Spearman's R value	P	Spearman's R value	P
CMV	IFNγ+	0.062	0.472	**-0.220**	**0.010**
	TNFα+	**0.173**	**0.044**	-0.14	0.104
	IL2+	-0.006	0.947	0.042	0.625
	IL17+	0.003	0.977	-0.077	0.367
EC	IFNγ+	-0.015	0.894	0.031	0.788
	TNFα+	-0.049	0.67	-0.078	0.495
	IL2+	0.158	0.164	-0.164	0.150
	IL17+	**0.248**	**0.028**	**-0.248**	**0.027**
PPD	IFNγ+	-0.109	0.271	0.039	0.695
	TNFα+	**-0.261**	**0.0071**	**0.193**	**0.049**
	IL2+	-0.076	0.444	0.002	0.986
	IL17+	**0.283**	**0.004**	**-0.276**	**0.0043**
Rv2031c	IFNγ+	-0.158	0.149	**0.423**	**<0.0001**
	TNFα+	-0.136	0.215	-0.070	0.523
	IL2+	-0.106	0.333	-0.085	0.438
	IL17+	**0.376**	**0.0004**	**-0.493**	**<0.0001**

The proportion of the total cytokine+ CD4 T cell response to each antigen contributed to by each cytokine measure is correlated with total CD4 T cell count and HIV-1 viral load. P values are generated using Spearman's correlation analysis.

6.5 Discussion

This study provides a detailed analysis of Th_1 and Th_{17} Mtb-specific CD4 and CD8 responses at various stages of HIV-1 disease progression. This is the first study to compare the commonly explored PPD and ESAT-6/CFP-10 Mtb T cell responses to those of a latency associated antigen Rv2031c and CMV within the presence and absence of HIV-1 infection.

We were able to detect IFNγ+ CD4 and CD8 responses to all of the antigens tested in both healthy HIV-uninfected and HIV-infected individuals. CMV-specific CD4 and CD8 responses were more frequent and of higher magnitude than responses to the other antigens tested, reflecting the higher prevalence of CMV infection within the population in Bloemfontein as well as the unique ability of CMV to generate high frequency CD4 and CD8 responses (Sylwester et al. 2005). CMV seropositivity in South Africa is almost ubiquitous and therefore the high prevalence of infection in our cohort was as expected (Schaftenaar et al. 2014). A positive IFNγ+ CD4 response to EC was present in 29/44 (65.9%) of HIV-uninfected individuals reflecting the proportion of the cohort with LTBI. A similar prevalence of LTBI was found in other studies in sub-Saharan African populations as well as within our own study when using our in-house RD1 IFNγ-ELISPOT assay (Mitchell et al. 2012; Day et al. 2008).

The high proportion of CD4 and CD8 responders to Rv2031c, as measured by this assay, was interesting as the frequency of responders was lower in other studies and when using IFNγ-ELISPOT within the same individuals (data not shown)(Arlehamn et al. 2012; Sutherland et al. 2013). This is likely related to the improved efficiency of the

WB ICS assay used, as well as the combination of co-stimulatory antibodies during antigen incubation with unprocessed whole blood (Hanekom et al. 2008).

Through analysis of the cytokine profiles of CD4 and CD8 T cells responding to each of the antigens tested in healthy individuals, we were able to gain insight into the different character of the stimulated antigen-specific responses prior to HIV-1 infection. Most notable was the increased IL-17 expression on Rv2031c-specific CD4 responses and the relatively increased IL-2 expression of EC- and PPD-specific T cell responses. Later analysis of the effect of HIV-1 infection on these antigen-specific responses links the IL-2 expressing phenotype to HIV-1 infection and depletion, resulting in greater depletion of Mtb-specific CD4 T cells in comparison to the CMV-specific CD4 response (Geldmacher & Koup 2012). We showed too that the IL-17 expressing components of the antigen-specific CD4 T cell responses were also preferentially depleted. Past studies have indicated that Th_{17} CD4 T cells are preferentially depleted in HIV-1 infection, although this has never been confirmed for Mtb-specific Th_{17} CD4 responses before (Prendergast et al. 2010; El Hed et al. 2010). This preferential depletion has been linked to the increased expression of CD4, CXCR4 and CCR6 of this specific CD4 T cell population, resulting in enhanced permissiveness to HIV-1 infection (Alvarez et al. 2013).

Some previous studies have failed to measure a difference in the magnitude of antigen-specific CD4 T cell responses in HIV-infected individuals when compared to those uninfected with the virus (Rangaka et al. 2007; Geldmacher et al. 2010). When we compared the frequency of CD4 T cells (as a proportion of the total CD4 T cell pool) responding to each antigen between the groups in our cohort, as is done in

most other flow cytometry studies, the only significant differences we noted were for PPD- and Rv2031c-specific responses (Fig. 6.9). However, if the absolute number of CD4 T cells was compared, there was a significantly lower magnitude of antigen-specific CD4 T cells responding to all the antigens within the HIV-infected group ($P<0.0001$ for all). I therefore propose that by using the former method, we may gain insight into the relative change of different antigen specific T cell populations but may also lose sensitivity to detect real changes in magnitude of immune response. The measurement of absolute number of responding T cells has been used in another study of Mtb-specific immunity within the context of HIV-1 infection (where T cell numbers are dynamic)(Sutherland et al. 2010). The use of the WB ICS stimulation protocol in our study (where 1 mL of whole blood was stimulated with antigen and the entire sample run on the flow cytometer) facilitates a direct comparison of CD4 and CD8 T cell numbers. This approach is particularly useful in the analysis of fluctuating T cell number, especially in individuals with different degrees of HIV-associated immune suppression or in individuals on ART.

7 RECONSTITUTION OF MTB-SPECIFIC T CELL FUNCTION ON ANTIRETROVIRAL THERAPY

7.1 Background

HIV-1 is the greatest risk factor for the development of tuberculosis (TB) and has resulted in the resurgence of TB in sub-Saharan Africa (Harries et al. 2010). HIV-associated TB is a major contributor to worldwide TB prevalence, however sub-Saharan Africa is the region worst affected and harbours 80% of global HIV/TB cases (WHO 2013b). In South Africa 73% of all incident TB cases are co-infected with HIV-1 making the management of HIV/TB co-infection a national public health priority (South African National Department of Health 2013a).

Considering the critical impact of HIV-1 infection as a driver of the tuberculosis epidemic, antiretroviral therapy (ART) has proven the most effective intervention in reducing HIV-related TB across all CD4 count strata (Suthar et al. 2012). The reduction of TB risk associated with ART has been shown to be as high as 67%, despite the apparent increase in incidence of TB during the first 3 months of ART (Lawn et al. 2011). The clear benefit associated with ART in reducing TB risk, indicates the restoration of some essential components of the Mtb-specific T cell response.

In Chapter 6 I showed that the absolute loss of CD4 T cells, along with the loss of particular Mtb-specific T cell subsets, (and their associated decline in functionality) were associated with HIV-1 infection. Proliferation as well as IFNγ secretion in response to Mtb antigens, is improved by ART which also results in reconstitution of naïve and central memory CD4 T cells (T. S. Li et al. 1998; Schluger et al. 2002; Wilkinson et al. 2009). Most studies evaluating immune reconstitution on ART have

been cross-sectional in nature and used only IFNγ production as measured by ELISPOT as the primary measure of the Mtb-specific T cell response. This approach excludes the concurrent analysis of other T cell subsets as well as the analysis of additional cytokines such as TNFα, IL-2 and IL-17 that are postulated to play a protective role in the Mtb-specific T cell response.

Only two detailed studies have examined production of cytokines by flow cytometry before and after initiation of ART (Sutherland et al. 2010; Wilkinson et al. 2009). Wilkinson et al. showed that despite a decline in the proportion of CD4 T cells producing both IFNγ and IL-2 in response to PPD during 48 weeks of ART (as measured by flow cytometry), the actual number of IFNγ+ PPD-specific T cells measured by ELISPOT significantly increased (and could be assumed to be similar for IL-2+ CD4 T cells). T cells producing IFNγ in response to EC also increased when measured by ELISPOT in the same cohort. Sutherland et al. found that immune reconstitution on ART resulted in a return of an IFNγ+/TNFα+ PPD-specific CD4 response with retention of the CD8 response that was present prior to ART initiation (Sutherland et al. 2010). The analysis of polyfunctional CD4 T cell responses to EC and PPD in the same cohort, showed a significant increase by 6 months of ART. However, the absolute number of CD4 T cells expressing IFNγ, TNFα or IL-2 was not significantly different in response to EC after 12 months of ART although there was an increase in the magnitude of the PPD-specific CD4 TNFα+ response.

In summary these data indicate that immune reconstitution associated with initiation of ART includes restoration of the Mtb-specific T cell response both in terms of magnitude and functionality. The extent of this restoration, however, appears to be

limited as multiple studies have shown that despite long term ART, responses to Mtb-antigens remain suppressed when compared to HIV-uninfected individuals (Mendonça et al. 2007; Sutherland et al. 2006; Wilkinson et al. 2009). This is most likely a contributing factor to the increased TB incidence seen in ART-treated individuals when compared with HIV-uninfected individuals living in the same community (Girardi et al. 2005; Lawn, Badri, et al. 2005; Gupta et al. 2012).

In this chapter I aim to further elucidate the impact of ART on the reconstitution of the Mtb-specific CD4 and CD8 T cell response through the use of multiparameter flow cytometry. I analyse both Th_1 and Th_{17} T cell responses to 3 different mycobacterial antigens (EC, PPD and Rv2031c) and to CMV in HIV-infected individuals before initiation of ART and at 6 and 12 months thereafter. By doing so and by comparing "reconstituted" responses to those of healthy HIV-uninfected individuals, I hope to gain valuable insight into the character and tempo of Mtb-specific immune reconstitution with implications for better understanding protective T cell responses to TB.

7.2 Hypothesis

ART results in only partial restoration of Mtb-specific T cell responses, resulting in increased TB risk in HIV-infected individuals on ART as compared to HIV-uninfected individuals.

7.3 Experimental aims

I aimed to evaluate the Mtb- and CMV-specific T cell response in HIV-1 infected individuals before and after initiating ART and to compare these responses to those of HIV-uninfected individuals.

7.4 Results

7.4.1 Characteristics of individuals included in the evaluation of Mtb-specific T cell reconstitution on ART

In order to evaluate the effect of ART on the reconstitution of Mtb-specific T cell function over a 12 month period, we selected all HIV-1 infected individuals that had been initiated on ART within 2 weeks of their baseline visit and had attended both subsequent follow-up visits. ART-associated suppression of HIV-1 replication was assessed by viral load quantification at all time points. The analysis of immune reconstitution was restricted to individuals that completely suppressed viral replication at both of the follow-up visits.

261 individuals were recruited for baseline sampling and 157 individuals were followed-up at both the 6 and 12 month time points. Of these individuals, 98 were initiated on ART at baseline and were on treatment at the time of sampling at both 6 and 12 months. 71 individuals that suppressed viral load to below 150 RNA copies/uL at both follow-up time points and had suitable whole blood flow cytometry assays carried out were included in this analysis (see criteria outlined in 6.4.1). A viral load threshold of 150 HIV-1 RNA copies/mL was employed, as some of the assays performed in the laboratory in Bloemfontein were restricted to this limit of quantification.

Table 7.1 Characteristics of all individuals included in the analysis

Characteristic	HIV negative	HIV positive		
	n= 44	**All** n= 71	**CD4 < 201** n= 35	**CD4 201 – 350** n= 36
Gender				
Female - n (%)	20 (45)	46 (65)	17 (49)	29 (81)
Male - n (%)	24 (55)	25 (35)	18 (51)	7 (19)
Age (years)[a]	24 (20 – 32)	36 (31 – 45)	36 (32 – 44)	36 (28 – 46)
CD4 count (cells/uL)[a]	879 (686 – 1054)	201 (155 – 279)	155 (100 – 184)	272 (237 – 314)
CD4:CD8 Ratio[a]	1.54 (1.04 – 1.98)	0.21 (0.14 – 0.28)	0.16 (0.11 – 0.22)	0.26 (0.17 – 0.32)
Viral load (RNA copies/ml)[a]	N/A	77839 (24736 – 266629)	132740 (34425 – 365740)	55507 (21382 – 181851)
BMI (kg/m^2)[a]	24.3 (22.7 – 27.8)	24.1 (22.4 – 27.5)	24.1 (22.4 – 27.6)	24.2 (21.5 – 27.2)
Prev TB - n (%)	0 (0)	6 (8)	3 (9)	3 (8)
BCG scar - n (%)	20 (45)	47 (66)	27 (77)	20 (56)
Duration of IPT (days)[a]**:**				
BL	0 (0)	0 (0 – 9)	0 (0 – 9)	0 (0 – 9)
6m	0 (0)	174 (29 – 183)	168 (29 – 183)	175 (23 – 184)
12m	0 (0)	183 (181 – 184)	183 (181 – 184)	183 (181 – 184)

[a] Data expressed as median (interquartile range)

7.4.2 Response to ART at 6 months and 12 months

All of the HIV-infected individuals included in this analysis achieved virological suppression on ART at the 6 month and 12 month time points as required by the inclusion criteria for this analysis (Fig 7.1 a). Immune reconstitution was evident with robust increases in CD4 T cell count, CD8 T cell count and CD4:CD8 ratio associated with ART (Fig. 7.1 c – e). CD4 T cell count increased across the cohort by 6 and 12 months (Fig 7.1 b; $P<0.005$ for all comparisons). Understandably CD8 T cell count did not increase to the same extent but nevertheless an increase above baseline levels

was evident by 12 months of ART (Fig 7.1 d; P = 0.021). The increases in CD4:CD8 ratio at 6 and 12 months can be attributed primarily to the increase in the proportion of CD4 T cells (Fig 7.1 e; P<0.0001 for all comparisons). Body mass index (BMI) was also shown to increase after initiation of ART at both time points (Fig 7.1 d).

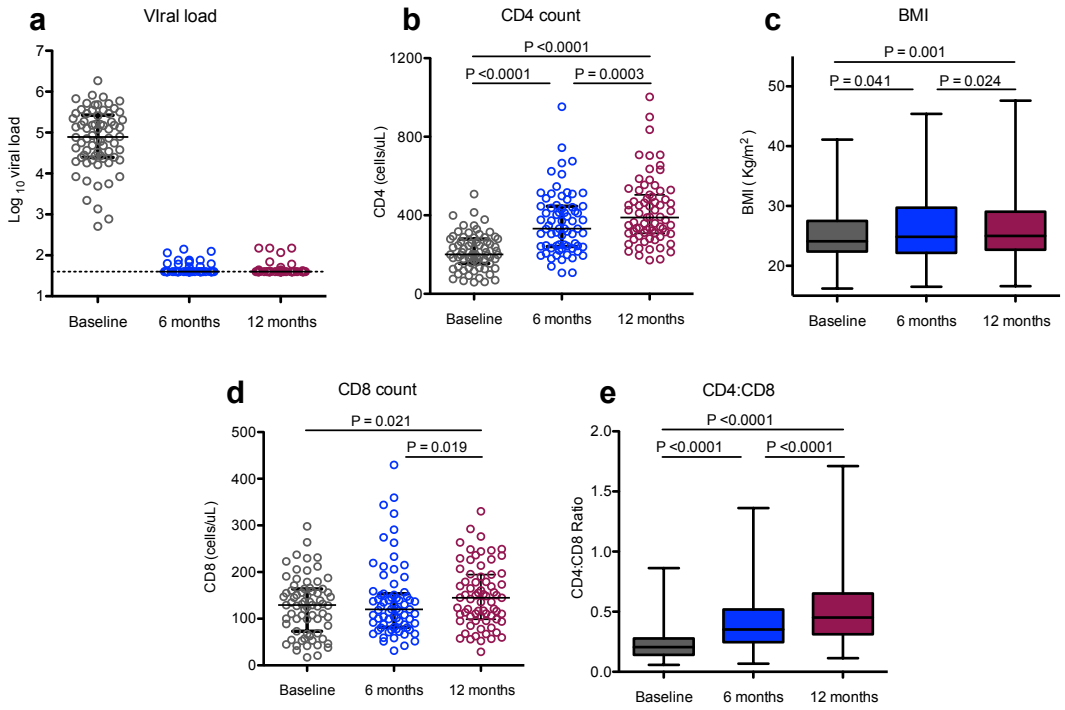


Figure 7.1 Changes in HIV-1 viral load, CD4 and CD8 T cell counts and BMI. a) Viral suppression was achieved in all individuals included in this analysis. Dotted line represents pVL of 40 copies/mL. b) Changes in CD4 T cell counts. c) Changes in body mass index (BMI). d) Changes in CD8 T cell counts e) Changes in CD4:CD8 ratios. All P values generated using Wilcoxon matched pair analyses

7.4.3 The proportion of positive responders to EC, PPD and Rv2031c is unaffected by 12 months of ART

In Chapter 6 I showed that the proportion of individuals with a positive CD4 IFNγ response to Mtb- antigens (PPD and Rv2031c) was significantly affected by HIV-1 infection. I therefore investigated whether ART would restore pathogen-specific CD4 and CD8 responses after 6 and 12 months of therapy.

The proportion of CD4 and CD8 CMV responders increased by 12 months of ART however these differences were not significant for responses to EC, PPD or Rv2031c (Fig. 7.2). Based on these data there was no clear evidence to suggest reconstitution of Mtb-specific CD4 or CD8 IFNγ responses over 12 months of ART.

IPT is a possible confounding factor in evaluating the effect of ART on the proportion of responders to Mtb-specific antigens. Only 6 individuals (8.5%) did not receive IPT at any stage during the study, and therefore provided little power for robust assessment of the influence of IPT on the measured Mtb-specific responses. Despite this fact, there were no significant differences in the incidence of conversion or reversion of responders to Mtb-specific antigens at either 6 or 12 months of ART, between individuals that did and did not receive IPT (Fisher's exact test, $P>0.05$ for all comparisons). Moreover, despite the majority of individuals receiving IPT during the course of the study, there were no significant changes in the proportions of responders to any of the Mtb-specific antigens. These findings are consistent with those of studies using alternative forms of IGRA (Takenami et al. 2012; D. F. Johnson et al. 2014).

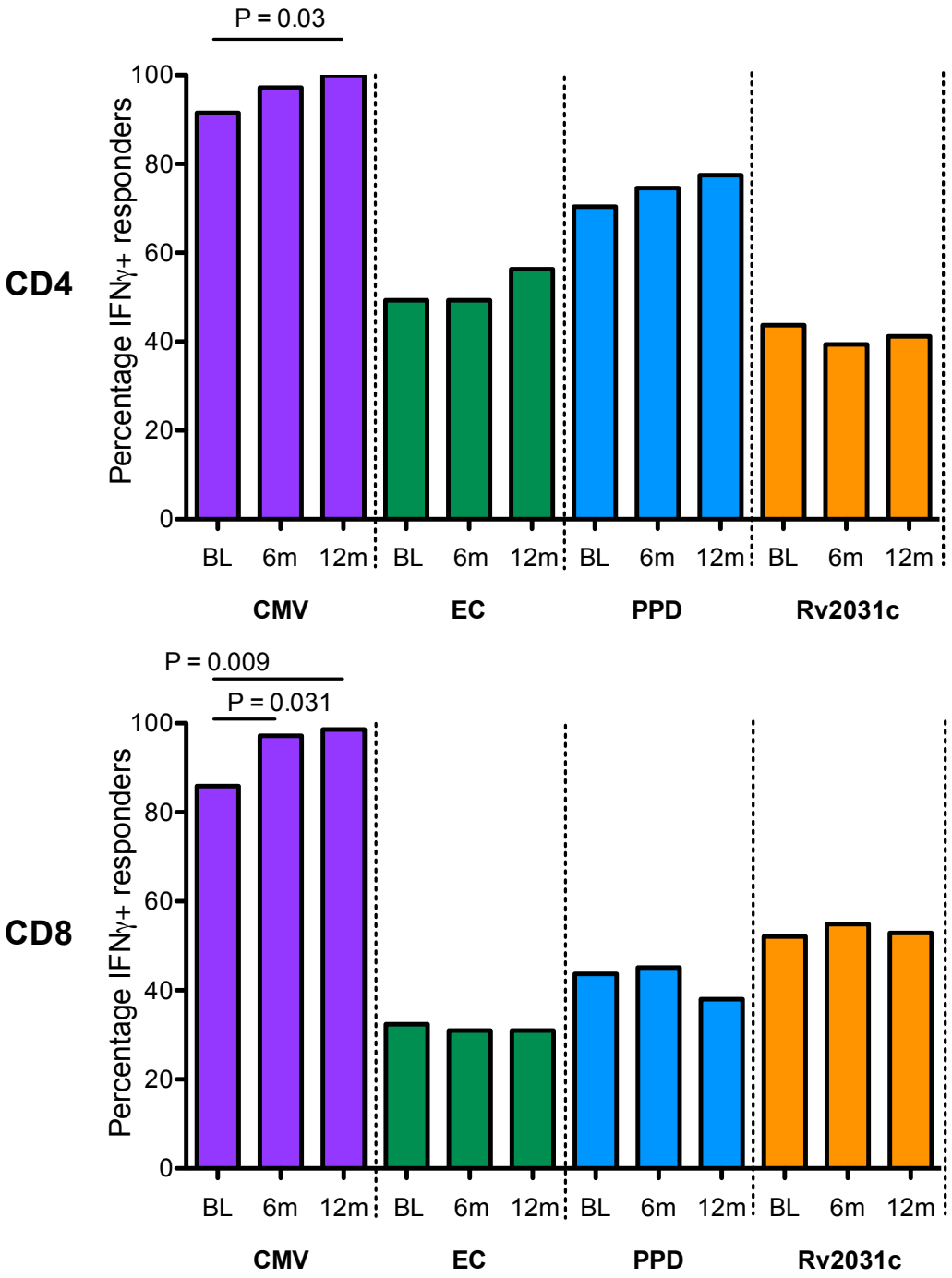


Figure 7.2 The impact of ART on proportion of positive responders. The proportion of positive responders to CMV, EC, PPD and Rv2031c are compared between 3 time points. BL = baseline, 6m = 6 months, 12m = 12 months. Significant P values <0.05 are shown for Fisher's exact tests.

7.4.4 The frequency of IFNγ+ T cell responses to EC, PPD and Rv2031c is unaffected by 12 months of ART

The median percent of IFNγ+ CD4 and CD8 T cells, responding to each of the antigens was evaluated before and after 6 and 12 months of ART. Within the CD4 T cell responses, only the frequency of CMV-specific CD4 T cells changed significantly with a reduction evident at 6 months of ART (Fig 7.3a, P= 0.015). By contrast the frequency of CMV-specific CD8 responses increased at 6 months (Fig 7.3e, P<0.0001) before decreasing between 6 and 12 months (Fig 7.3e, 6m vs. 12m P = 0.002). However, it did remain higher than that recorded at baseline (Baseline vs. 12m P<0.0001). There were no detected fluctuations in the median percentages of Mtb-specific CD4 or CD8 IFNγ+ T cells at any time point (CD4, Fig 7.3 b-d; CD8, Fig 7.3 f-h).

I evaluated the possible influence that IPT had on the change in the magnitude of Mtb-specific T cell responses on ART at 6 and 12 months by comparing the median change in frequency of IFNγ+ CD4 and CD8 T cells, responding to each of the antigens, between individuals that did or did not receive IPT. There were no significant differences detected at either 6 or 12 months of ART (Mann-Whitney tests, P > 0.05 for all comparisons). Finally, the magnitude of the change in the frequency of Mtb-specific IFNγ+ CD4 and CD8 T cells at 6 and 12 months was correlated with the duration of IPT received by each individual. The only significant correlation detected was the inverse correlation between the magnitude of change between baseline and 6 months of Rv2031c-specific IFNγ+ CD4 T cell responses and the duration of IPT received (Data not shown. Spearman's r = -0.256, P = 0.035). This suggests that IPT may reduce the effect of ART in the reconstitution of Rv2031c-specific CD4 T cell responses.

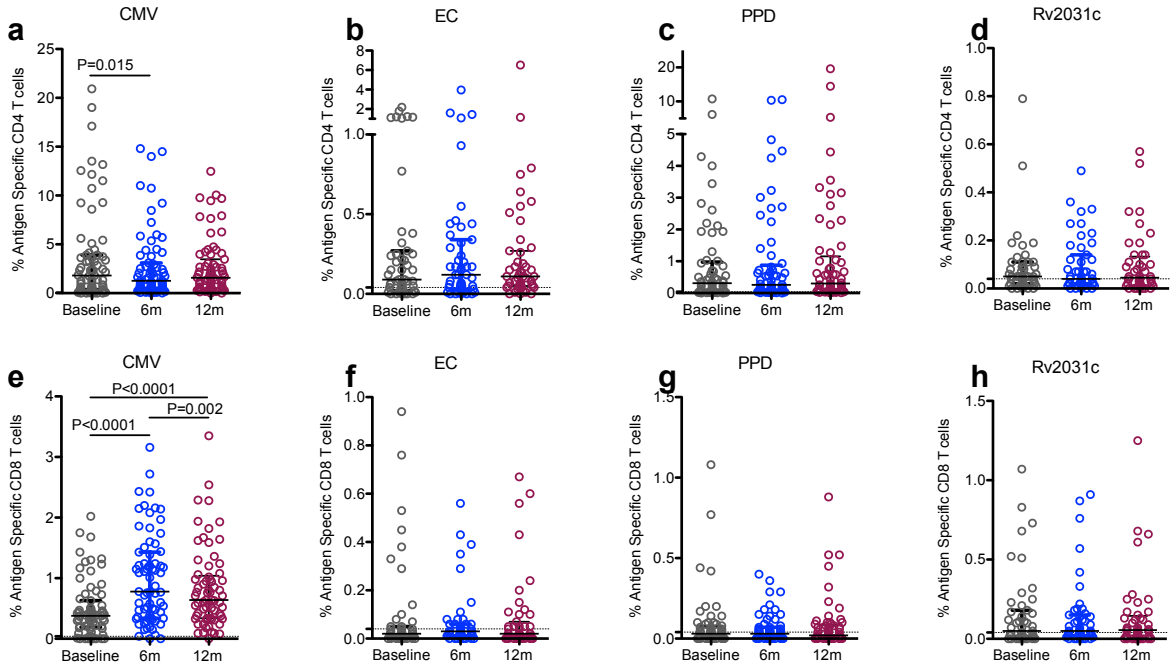


Figure 7.3 IFNγ-producing CD4 and CD8 T cells responding to CMV, PPD, EC and Rv2031c over 12 months of ART. Horizontal lines represent the median percent, and the whiskers the interquartile range. Significant P values <0.05 are shown for Wilcoxon matched pair analyses.

7.4.5 An analysis of the absolute numbers of Th_1 and Th_{17} antigen-specific CD4 T cells

The data presented above in section 7.4.4 are based on an evaluation of antigen-specific IFNγ+ T cell responses that are represented as a percentage of the total CD4 or CD8 T cell population within each individual and show little evidence of immune reconstitution on ART. Due to the dynamic change in the absolute number of CD4 and CD8 T cells during HIV-1 disease and ART, a change in proportion of antigen specific T cells may not be evident. In addition, considering only IFNγ expression in such antigen specific responses over-simplifies the complexity of the pathogen-specific T cell response. I therefore investigated the absolute number of CD4 and CD8 T cells expressing IFNγ, TNFα, IL-2 and IL-17 in response to CMV, EC, PPD and Rv2031c

before and after ART. All individuals that had a positive CD4 IFNγ response at any of the time points were considered sensitized to the antigen concerned and were included in the analysis at all time points.

7.4.5.1 Increases in the absolute number of cytokine-positive CD4 T cells reveals evidence of immune reconstitution

The absolute numbers of IFNγ+, TNFα+, IL-2+ and IL-17+ CD4 T cells responding to CMV were increased at both 6 and 12 month time points when compared to baseline indicating restoration of these cytokine specific CD4 populations (Fig 7.4 a-d, $P < 0.05$ for all comparisons). EC-specific CD4 responses showed similar increases for IFNγ+, TNFα+ and IL-2+ responses, however there was no increase in absolute number of IL17+ CD4 T cells responding to EC (Fig 7.4 e-h). PPD-specific responses showed a similar pattern, except that there was a small increase in IL17+ CD4 T cells at 12 months (Fig 6.4 k, $P=0.02$). CD4 responses to Rv2031c increased at both 6 and 12 months for IFNγ+, TNFα+, IL-2+ and IL-17+ responses (Fig 6.4 m-p, $P < 0.05$ for all comparisons).

A similar comparison of CD8 T cell responses to the antigens tested showed an increase in absolute number of IFNγ+ CMV-specific CD8 T cells at 6 months and 12 months (Data not shown, $P < 0.005$ for both comparisons). There was no increase in the absolute number of CD8 T cells responding to any of the Mtb antigens tested, despite such restoration being evident with corresponding CD4 responses.

These data indicate that the absolute number of cytokine-producing CMV- and Mtb-specific CD4 T cells are restored by ART within 6 months of therapy but appear to be hampered for IL-17+ responses to EC and PPD.

7.4.5.2 Absolute antigen-specific CD4 T cell numbers remain suppressed compared to HIV-uninfected individuals despite 12 months of ART

Having noted increases in the magnitude of the absolute number of cytokine-positive CD4 T cells responding to all antigens, I evaluated the extent of this reconstitution by comparing these values after 12 months of ART to HIV-uninfected individuals from the same community (Fig. 7.5). In almost every case (except TNFα+ and IL-2+ CD4 responses to CMV and Rv2031c) the absolute number of cytokine positive CD4 T cells responding to all antigens remained significantly lower in HIV-infected individuals after 12 months of ART than HIV-uninfected individuals (Fig 7.5 $P<0.05$ for all comparisons). Of note was that IL-17+ CD4 T cell responses to all antigens were uniformly significantly lower in HIV-infected individuals on ART (Fig 7.5 d,h,i and p, $P<0.0001$ for all)

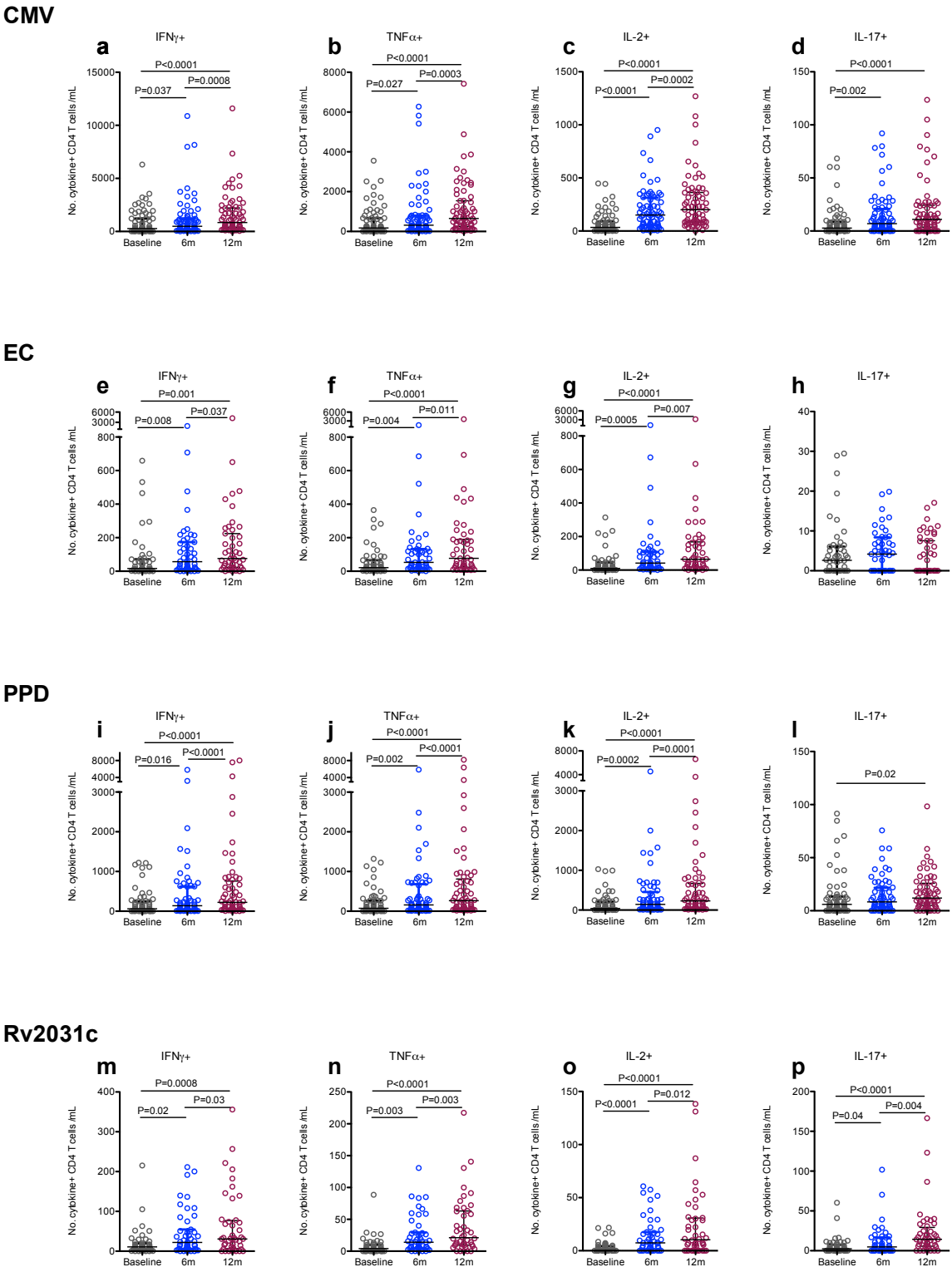


Figure 7.4 The effect of ART on absolute numbers of CD4 T cells expressing each cytokine in response to each antigen. Values are expressed as cells/mL whole blood and are compared between each time point. P values <0.05 are indicated for Wilcoxon matched pair analyses.

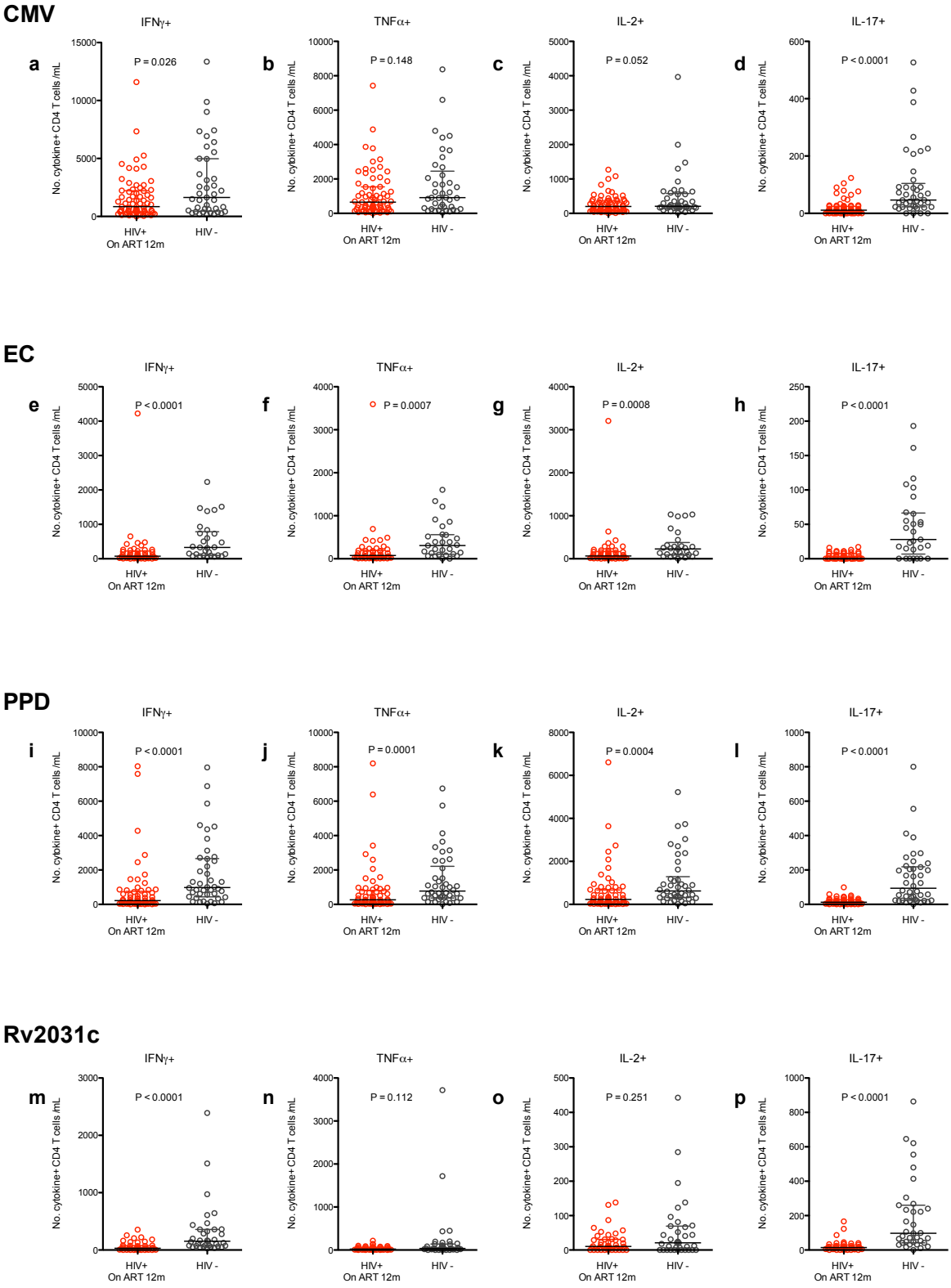


Figure 7.5 Comparison of 71 HIV-infected individuals on ART for 12 months and 44 HIV-uninfected individuals from the same community. The absolute CD4 T cell numbers expressing each cytokine in response to stimulation with each antigen is shown. P values are indicated for Mann-Whitney tests.

7.4.6 Preferential restoration of IL-2 and TNFα expressing CD4 T cells

The preferential depletion of IL-2+ and IL-17+ CD4 T cells shown to be associated with HIV-1 infection in Chapter 6, along with the lack of restoration of IL17+ CD4 T cells responding to EC and PPD suggested that cytokine-specific CD4 T cells may restore to differing degrees and tempos on ART. I therefore investigated whether the proportional contribution of each cytokine-producing CD4 subset to the overall CD4 cytokine-positive response to each antigen changed after initiation of ART (Fig. 7.6)

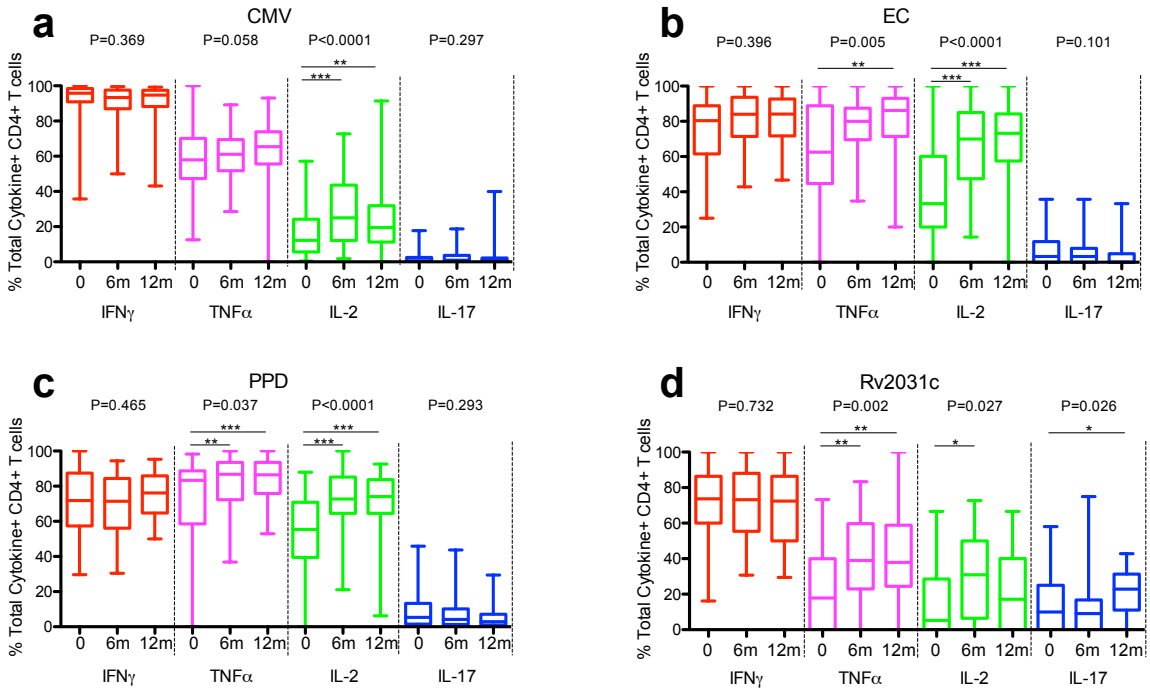


Figure 7.6 The proportions of IFNγ+, TNFα+, IL-2+ and IL-17+ among total cytokine positive CD4 T cells over 12 months of ART. Changes in proportions are evaluated across the 3 time points. P values across the top of each panel indicate Kruskal-Wallis P values across each cytokine+ group. Significant differences were confirmed between time points using Mann-Whitney tests. * P <0.05, ** P <0.005, * P <0.0005

The proportion of IL-2+ CD4 T cells within the total cytokine-positive CD4 T cell response to CMV, EC, PPD and Rv2031c increased by 6 months for CMV, EC, PPD and

Rv2031c (Fig 7.6 a-d, P<0.05 for all). This increase was sustained at 12 months for CMV, EC, and PPD (Fig 7.6 a-d, P<0.005 for all). The proportion of TNFα+ CD4 T cells increased for EC at 12 months (Fig 7.6 b, P<0.005), and for PPD and Rv2031c at both 6 and 12 months (Fig 7.6 c-d, P<0.005 for both). Interestingly, the contribution of IL-17+ CD4 T cells to the EC and PPD cytokine-positive response appeared to decrease at both 6 and 12 months, however these differences were not significant (Fig 7.6 b and c, Kruskal-Wallis P = 0.101 and P=0.293 respectively). The contribution of IL-17+ CD4 T cells to the Rv2031c response, however increased by 12 months suggesting some significant restoration of the IL17+ response to this antigen (Fig 6.5 d, P<0.05).

7.4.7 The effect of ART on the polyfunctionality of the antigen-specific T cell response

Through a "Boolean" analysis of cytokine co-expressing T cell populations, the effect of ART on restoration of particular cytokine-expressing subsets of CD4 and CD8 T cells was evaluated. The absolute number of CD4 and CD8 T cells expressing different combinations of the cytokines was measured at baseline and over 12 months of ART. ART-associated restoration of polyfunctionality to PPD- and EC specific CD4 responses in a small cohort has been shown before (Sutherland et al. 2010). I investigated whether this was true for both CD4 and CD8 responses to CMV- and Mtb-specific antigens within my larger cohort (Fig. 7.7 and 7.8)

The analysis of CMV-specific CD4 responses showed an increase in absolute number of CD4 T cells in all cytokine expressing subpopulations by 12 months (Fig. 7.7 a, P<0.05 for all at 12 months). Of particular note was the increase in the 3+ (IFNγ+TNFα+IL-2+) response at both 6 and 12 months and the greatest magnitude

response, 2+ (IFNγ+TNFα+) at 12 months. Analysis of the proportion of 1+, 2+ and 3+ cytokine expressing subpopulations within the overall cytokine+ CMV response highlights the increase in the proportion of polyfunctional CMV-responses with initiation of ART (Fig. 7.7 b)

Restoration of the EC-specific CD4 response was evident only within the 3+ (IFNγ+TNFα+IL-2+) and 2+(TNFα+IL-2+) cytokine expressing populations (Fig. 7.7 c and d). PPD also showed an increase within the 3+ (IFNγ+TNFα+IL-2+) and 2+(TNFα+IL-2+) cytokine expressing populations with the addition of an increase in the 1+ TNFα+ and IL-2+ at 6 and 12 months and of the 1+ IFNγ+ population at 12 months (Fig. 7.7 e and f). The Rv2031c-specific CD4 response showed an increase in the 3+(IFNγ+TNFα+IL-2+) and 2+(IFNγ+TNFα+) at 6 and 12 months whilst an increase in the 2+(TNFα+IL-2+) population was only evident at 12 months (Fig. 7.7 g and h).

When analysing the CD8 responses there were only significant differences in absolute number of CMV-specific CD4 T cells at 6 and 12 months after initiation of ART. These differences manifest as an increase in 3+(IFNγ+TNFα+IL-2+) CD8 T cells as well as an increase in 2+(IFNγ+TNFα+) and 2+(IFNγ+IL-2+) at 6 and 12 months. Increases in the magnitude 1+(IFNγ+) and 1+(IL-2+) were evident at both 6 and 12 months whilst an increase in 1+(TNFα+) was evident at 12 months only.

In summary, ART was associated with an increase in polyfunctional CD4 responses to all of the antigens tested.

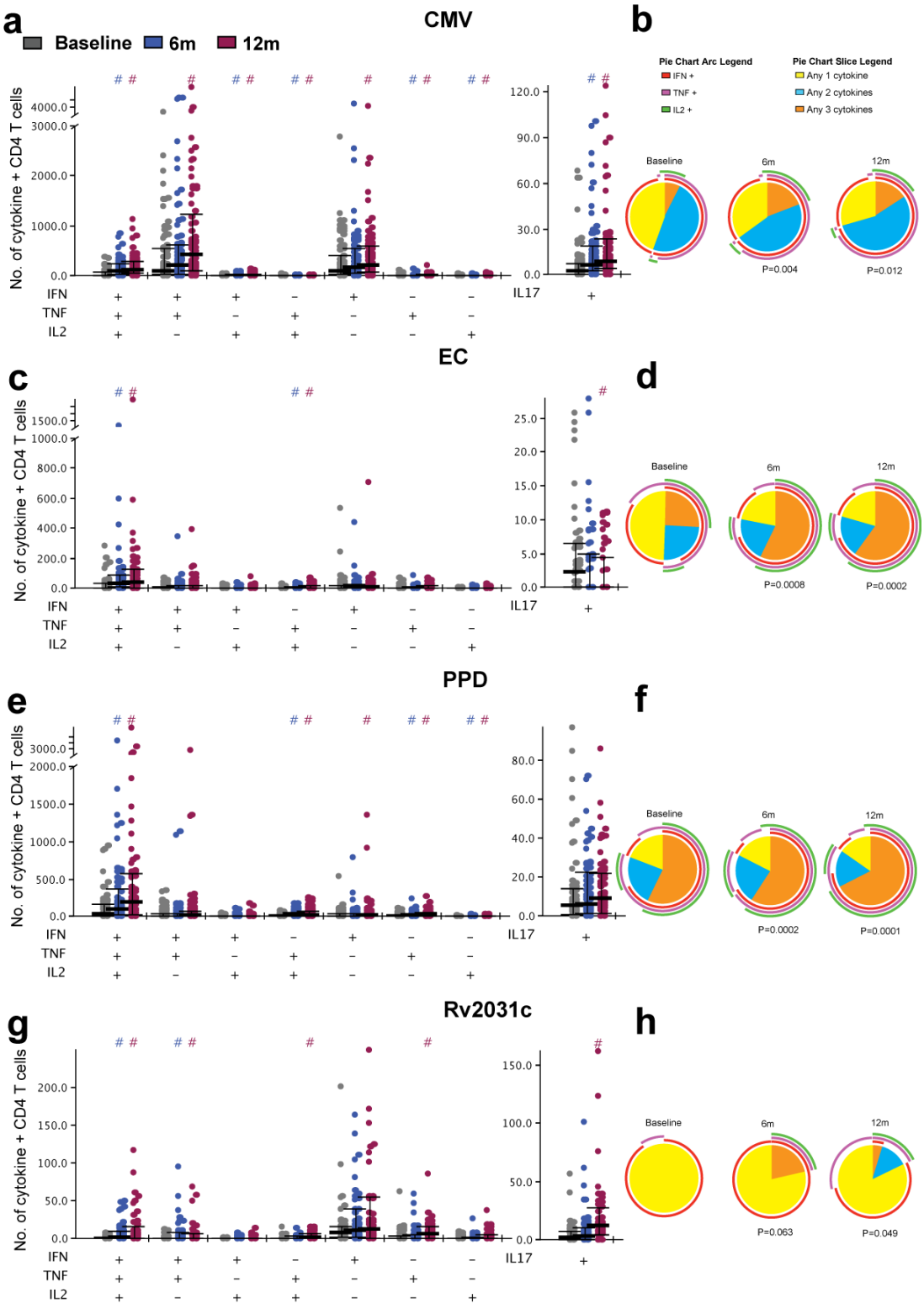


Figure 7.7 Changes in the absolute numbers of antigen-specific cytokine+ CD4 T cells over 12 months of ART. a,c,e and g - values shown are the absolute number of cells expressing each combination of cytokines. Colour-coded # indicates P < 0.05 for Wilcoxon matched pairs analyses. b, d, f and h - pie charts show the fraction of cells expressing 1, 2, or 3 cytokines. The arcs surrounding the pie charts indicate the proportion of the responses contributed by the single cytokines. P values indicate comparisons to baseline and are calculated using a partial permutation analysis (Roederer et al. 2011).

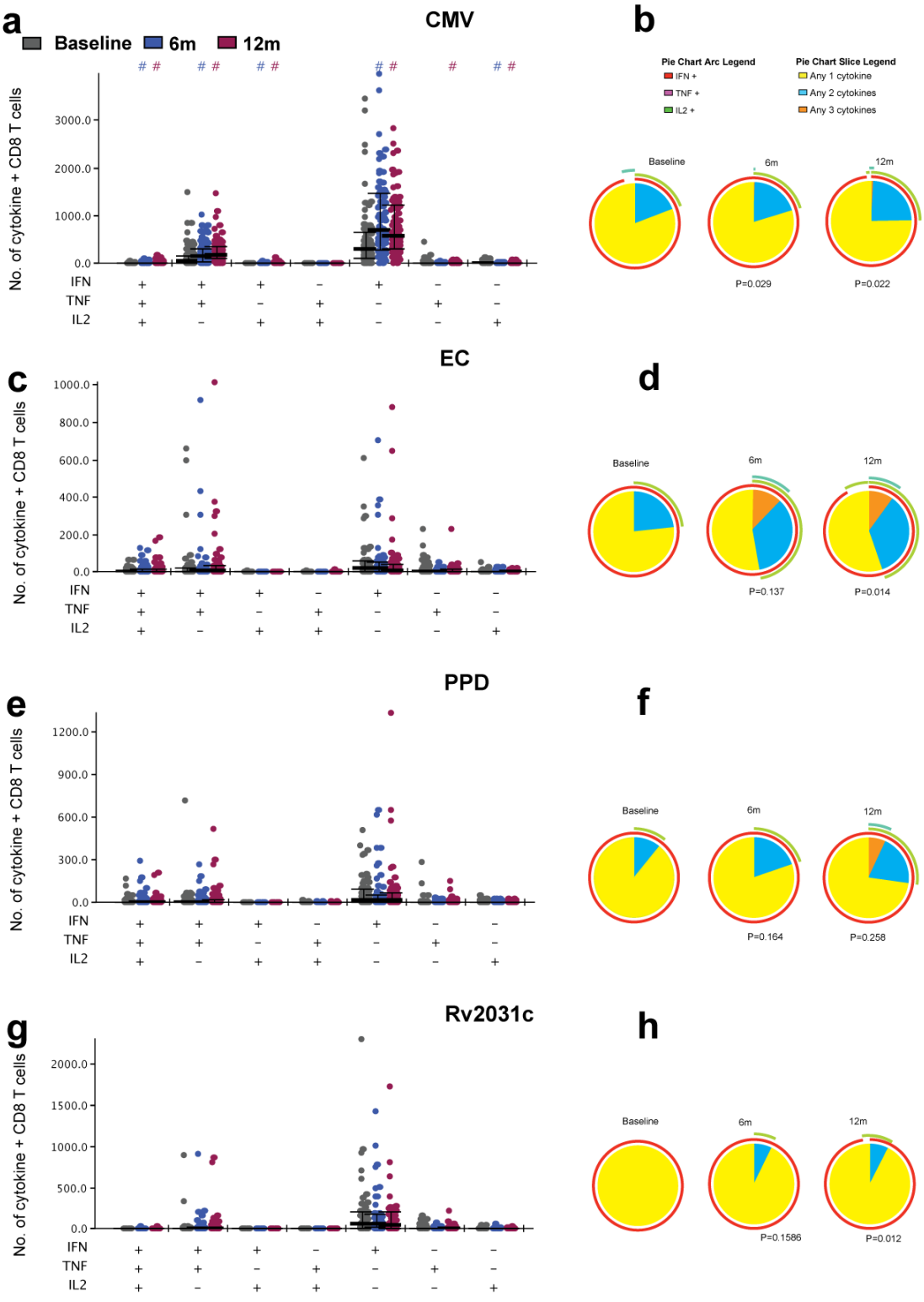


Figure 7.8 Changes in the absolute numbers of antigen-specific cytokine+ CD8 T cells over 12 months of ART. a,c,e and g - values shown are the absolute number of cells expressing each combination of cytokines. Colour-coded # indicates P < 0.05 for Wilcoxon matched pairs analyses. b, d, f and h - pie charts show the fraction of cells expressing 1, 2, or 3 cytokines. The arcs surrounding the pie charts indicate the proportion of the responses contributed by the single cytokines. P values indicate comparisons to baseline and are calculated using a partial permutation analysis (Roederer et al. 2011).

7.5 Discussion

In this chapter I present an extensive study of ART-mediated Mtb-specific CD4 and CD8 T cell reconstitution. The inclusion criteria aimed to identify individuals that had effective ART-mediated virological suppression, thus providing a framework from which to accurately assess ART-mediated immune reconstitution. The results were presented with an initial assessment of the change in numbers of individuals with an IFNγ CD4 or CD8 response on ART. The changes in the magnitude of antigen-specific CD4 and CD8 responses were then evaluated by looking both at the percentage of cytokine positive cells as well as the absolute number. The magnitudes, after 12 months of ART, were compared to those of HIV-uninfected individuals to assess the full extent of reconstitution. Finally the fluctuations in absolute numbers of antigen-specific polyfunctional T cells were assessed over 6 and 12 months of ART.

CD4 T cell counts increased significantly on ART, broadly reflecting immunological recovery known to be associated with ART. CD8 T cell counts by contrast, were unchanged after 6 months of ART but did increase after 12 months. CD4 T cell count increases that are associated with ART initially occur as a result of redistribution of CD4 T cells from sequestered sites in the body, expansion of the existing pool of memory and naïve CD4 T cells as well as an increase in thymic output (Wolthers et al. 1996; Autran et al. 1997; Fernandez et al. 2006). The latter is also likely to account for the increase in absolute number of CD8 T lymphocytes observed in this cohort after 12 months of ART. A low CD4:CD8 T cell ratio is a common feature of HIV-1 infection and has also been associated with increased mortality in the general population (Wikby et al. 1998). Unsurprisingly the CD4:CD8 ratio increased after 6 and 12 months

of ART and reflected the higher proportional increase of the CD4 T cell population over that of the CD8 T cell population.

The sustained increases in body mass index (BMI) on ART is likely to reflect a reversal of the wasting effects of HIV-1, although it is difficult to exclude confounding effects such as a change in lean mass and fat composition attributed to particular ART regimens (despite most of the individuals in this study being initiated on PI-free regimens)(McDermott et al. 2001; Ferrando et al. 2005).

I initially evaluated the effect of ART on the antigen-specific T cell responses by measuring the proportion of IFNγ-producing CD4 and CD8 T cells after stimulation with each of the antigens used. There were no changes in the number of individuals with a CD4 or CD8 responses to any of the Mtb antigens or in the median proportion of IFNγ+ CD4 or CD8 responses. Other studies, using in vitro proliferation in response to PPD, have shown an increase in the proportion of responders after the initiation of ART. However, the numbers of participants in these studies were relatively small, the participants in a more advanced stage of HIV-1 immune suppression and the methods less sensitive than those used in our study (T. Li et al. 1998; Autran et al. 1997; Wendland et al. 1999).

Not withstanding the above, the absence of measurable change in the median proportion of Mtb-specific CD4 and CD8 responses was surprising as Wilkinson et al. actually found a steady decrease in proportion of PPD-specific IFNγ+ CD4 T cells over a 1 year period of ART (Wilkinson et al. 2009). In the same study however, the number of PBMCs producing IFNγ (as enumerated by ELISPOT) in response to PPD and EC increased over 48 weeks of ART and therefore suggested a reconstituting

Mtb-specific host response, despite a proportional decline in Mtb-specific IFNγ+ CD4 T cells within the total expanding CD4 T cell population. In this study it was also difficult to separate the influence of Isoniazid prophylaxis from the effect of ART on fluctuating Mtb-specific T cells, as most individuals receiving ART also received IPT (Wilkinson et al. 2006).

Due to difficulties associated with PBMC separation, storage and transport to Oxford, we used a WB ICS assay in this study that has previously been described by Hanekom et al.(Hanekom et al. 2004). Through the use of a standard 1mL volume of whole blood from each subject per antigen stimulation, we were able to accurately evaluate the change in the absolute number of CD4 and CD8 antigen-specific cells after initiation of ART. I also chose to investigate the absolute numbers of the different cytokine expressing subpopulations of the antigen-specific T cell responses. There was significant evidence for a sustained increase in the absolute number of IFNγ+, TNFα+ and IL-2+ CD4 T cells responding to all of the antigens tested over 12 months of ART. The IL-17 CD4 response however did not reconstitute to the same degree. Despite increases in absolute CD4 T cell numbers, most of the responses remained lower after 12 months of ART than HIV-uninfected individuals. These data taken together indicate a clear yet suboptimal restoration of the Th1 CMV- and Mtb-specific CD4 response evident as early as 6 months and continuing through 12 months of ART. The Th_{17} CD4 response, however, did not restore to the same degree for EC-specific and PPD-specific responses indicating that Th_{17} responses to these Mtb-antigens are restored to a lesser extent or at a slower rate.

An identical analysis of the change in median magnitude of absolute numbers of Th_1 cytokine-expressing antigen-specific CD8 T cell counts revealed an increase in IFNγ+ and TNFα+ CMV-specific CD8 T cells after 6 and 12 months of ART. There were however, no changes in the absolute number of Mtb-specific CD8 T cells. Other studies have also shown an increase in frequency of CMV-specific CD8 T cells in ART-treated individuals (Naeger et al. 2010; Hsu et al. 2013). The contrasting patterns of CMV- and Mtb-specific CD8 T cell restoration are intriguing. In the case of CMV, this may be due to on-going low-level CMV replication and/or oligoclonal expansion of CMV-specific CD8 T cell populations present prior to the initiation of ART (Stone et al. 2005). In addition, Komanduri et al. demonstrated that individuals with robust CD4 T cell responses to CMV are more likely to have higher frequencies of CMV-specific CD8 T cells (Komanduri et al. 2001). Therefore within the context of ART-associated immune reconstitution, the increase in CMV-specific CD8 T cell number could possibly be attributed to an associated increase in CMV-specific CD4 T cell number. The lack of restoration in Mtb-specific CD8 T cell number may therefore reflect a relatively inadequate restoration of Mtb-specific CD4 T cell numbers as well as a lower antigen load in LTBI.

Considering antigen-specific CD4 and CD8 T cells expressing a single cytokine provides a useful method for assessing the impact of ART on broadly reconstituting cytokine expressing CD4 populations. However, as described in Chapter 6, the majority of CD4 T cells express a combination of cytokines. I therefore assessed the impact of ART on reconstituting the absolute numbers of particular CD4 populations expressing a combination of Th_1 cytokines. As the IL-17-expressing CD4 response to all of the antigens tested was distinct from the Th_1 response, these were assessed

independently. An increase in the number of “polyfunctional” 3+(IFNγ+TNFα+IL-2+) T cells responding to all of the antigens was evident at 6 and 12 months after initiating ART. In addition, the increase in the number of 2+(IFNγ+IL-2+) CD4 T cells was consistent across the responses to CMV and all of the Mtb-antigens. The changing pattern of cytokine expression for all of the antigens revealed an increase in the proportion of polyfunctional CD4 T cells, particularly of the 3+(IFNγ+TNFα+IL-2+) subgroup. The increase was significantly associated with IL2+ CD4 expression in combination with other cytokines. This finding is consistent with other studies that show a particular restoration of IL-2 expression on ART (Sutherland et al. 2010; Younes et al. 2003). Sutherland et al. also noted an increase in polyfunctional EC and PPD CD4 responses after a year of ART and found that these CD4 T cells had an effector memory phenotype (Sutherland et al. 2010).

When considering the effect of ART on CD8 T cell numbers, there were no changes in Mtb-specific CD8 T cell number at all, although the composition of the cytokine positive CD8 response to CMV, EC and Rv2031c became more “polyfunctional” after 12 months of ART. Within cytokine positive responses to EC and Rv2031c, these changes were not attributable to any particular cytokine-expressing subset although there was a trend towards an increase in 2+ (IFNγ+TNFα+) CD8 T cells with ART. These data suggest that despite failing to increase the number of Mtb-specific CD8 T cells, ART does result in an improvement in the quality of the Mtb- and CMV-specific CD8 T cell response.

8 CONCLUSION

The convergence of the TB and HIV-1 epidemics over the past 30 years has magnified the burden of both diseases with devastating consequences in sub-Saharan Africa and other parts of the developing world. The host T cell response is critical for control of both diseases and dual infection synergistically weakens these responses (Kaufmann & McMichael 2005). ART for HIV-1 has been the single most effective TB preventative intervention implemented on a large scale thus far, resulting in a significant reduction in HIV-associated TB in affected communities, although not abrogating risk completely (Gupta et al. 2012; Suthar et al. 2012).

This thesis studies a cohort of individuals from a community with high HIV-1 and TB prevalence in Bloemfontein, South Africa. I explored parameters of HIV-specific CD8 T cell function associated with HIV-1 viral control and the consequent effect of disease progression on Mtb-specific immunity. Finally the effect of 12 months of ART on restoration of Mtb-specific T cell function was examined in detail, providing a basis to understand the persistent TB risk associated with individuals on ART.

8.1 Summary of findings

Through longitudinal follow-up of our cohort we were able to confirm the commonly recognised deleterious effects of chronic HIV-1 infection. The rates of previous TB, and incident TB during the study follow-up period, were significantly higher in the HIV-infected group and almost 10% of HIV-infected individuals died within a year of study enrolment (Chapter 3) (Harries et al. 2010). This number may have been higher, had we been able to trace all study participants that were lost to follow up, but does

show the continuous impact of HIV-1 infection on this community despite the availability of ART.

Analysis of HIV-specific CD8 T cell responses in Chapter 4, confirmed the superior role of HIV-1 Gag-specific HLA class I restricted CD8 responses in controlling HIV-1 viraemia (Kiepiela et al. 2007; Jia et al. 2012; Zuñiga et al. 2006; Miura et al. 2009). CD4 T cell loss in this cross-sectional analysis was associated with a decrease in overall HIV-specific CD8 T cell breadth, mostly apparent within the Gag- and Pol-specific responses. I argued that a loss of CD4 T cells associated with a narrowing of Gag-specific T cell responses contributes to loss of viraemic control, HIV-1 disease progression and increased susceptibility to opportunistic infections such as TB.

Through the use of IFNγ ELISPOT, MIG/IP10 qPCR and flow cytometry assays, I was able to highlight the different effects of HIV-1 on the Mtb-specific T cell responses. CD4 T cell responses to all antigens tested were reduced in HIV-infected individuals when the absolute number of cells was considered (Chapter 6). Overall, PPD and Rv2031c CD4 T cell responses were depleted to a greater extent than EC and CMV-specific responses (Chapter 5 and Chapter 6) and appeared to be partly due to expression of IL-2 and IL-17 on these antigen-specific CD4 T cell populations (Hammond et al. 2008; Geldmacher et al. 2010). Indeed, IL-17 expressing CD4 T cell populations responding to all of the antigens tested were significantly reduced in HIV-1 infected individuals suggesting a particular susceptibility to depletion in the context of HIV-1 infection.

For the first time in studies investigating Mtb-specific immunity in humans, we were also able to determine that the magnitude of Mtb-specific CD8 T cell responses to

PPD and Rv2031c is affected by HIV-1 infection (Chapter 6). The relevance of this is uncertain, but does however add to an understanding of the summative effect of HIV-infection on the Mtb-specific T cell response.

A detailed investigation into the ability of 12 months of ART to restore aspects of the Mtb-specific T cell response in Chapter 7, gives insight into the persistent defects remaining despite evidence of immune reconstitution. The absolute numbers of Th_1 cytokine-expressing CD4 T cells increased over 12 months of ART, however this increase was not consistent for IL-17 expressing responses. It appears that Th_{17} CD4 T cell responses to certain antigens may restore to a lesser extent or possibly at a slower rate than Th_1 responses. Despite evidence of reconstitution of all antigen-specific CD4 populations, responses over 12 months of ART were still significantly lower than those in HIV-uninfected individuals (Wilkinson et al. 2009; Sutherland et al. 2006).

8.2 Discussion and future priorities

There is an urgent need to develop our understanding of the correlates of protective immune responses to HIV-1 and Mtb in order to design better vaccines and inform the development of new interventions (Kaufmann 2013; Schiffner et al. 2013). As HIV-1 infection is the most potent risk factor for the development of TB, studying the character and tempo of fluctuations in Mtb-specific immunity during acute HIV-1 infection, disease progression and reconstitution on ART, provides valuable insights into correlates of protection against Mtb.

It is difficult to investigate the longitudinal effect of HIV-1 disease progression on Mtb-specific immunity, due to the obvious need to initiate ART in HIV-infected

individuals at higher CD4 T cell counts (WHO 2013a). In this thesis, I therefore limited my evaluation of the effect of HIV-1 disease progression to a cross-sectional analysis of ART naïve individuals, based on CD4 T cell count. This approach is limited as it is unable to assess progressive changes within the same individual. However, the possibility exists within this data set to analyse those individuals that maintained CD4 T cell counts above the threshold for ART initiation and could contribute further to the findings herein.

Most current immunological methods used to detect Mtb-specific CD4 T cell responses rely mainly on the detection of IFNγ production. Whilst IFNγ and other Th_1 cytokines have been reliably linked to a protective role in Mtb-specific T cell responses they clearly are not sufficient (O'Garra et al. 2013). Indeed a recent vaccine study failed to show vaccine-mediated protection in infants during the first two years of life, despite CD4 T cell co-expression of Th_1 cytokines evident within 10 weeks of BCG vaccination (Kagina et al. 2010). Another study investigating immune responses to BCG in humans also failed to find any correlation between IFNγ responses and protection (Hoft et al. 2002). In addition, studies in mice have shown an IFNγ-independent mechanism for CD4 T cell-mediated control of Mtb supporting the fact that other CD4 functions in addition to Th_1 cytokine production are important in mediating Mtb-specific immunity, justifying our approach to examining other CD4 cytokines (Cowley & Elkins 2003; Gallegos et al. 2011).

Scriba et al. identified a distinct Mtb-specific Th_{17} CD4 T cell response in healthy individuals (Scriba et al. 2008). This, along with the fact Th_{17} CD4 T cells have been shown to be preferentially depleted in HIV-1 infection, helps to explain our finding

that Mtb-specific IL-17+ CD4 T cells are depleted in HIV-infected individuals in our cohort. The role of the Mtb-specific Th_{17} response is somewhat controversial with both protective and destructive roles described in the literature (O'Garra et al. 2013). Further analysis of Th_{17} responses detected in this cohort is required as well as further experiments to determine their influence on the Th_1 Mtb-specific T cell responses.

The role of CD8 T cells within the Mtb-specific T cell response in not well described in humans, despite a beneficial role having been demonstrated in murine and non-human primate models (Flynn et al. 1992; Sousa et al. 2000; Chen et al. 2009). Of particular interest therefore, was the observation of a lower frequency of IFNγ+ CD8 T cells responding to PPD and Rv2031c in HIV-infected individuals compared to HIV-uninfected. These differences also corresponded to differences in IFNγ+ CD4 T cell responses to the same antigens and it could be argued that a loss of CD4 T cells was associated with a corresponding decline in CD8 T cells specific for the same antigen. Although not shown in human Mtb-specific immunology before, this is not surprising as a murine study recently demonstrated that IFNγ produced by CD4 T cells enhances the CD8 T cell function in Mtb infection (Green et al. 2013). The associations between Mtb-specific CD4 and CD8 T cell responses within my cohort require further elucidation, as does the importance of the role of CD8 T cells in Mtb-specific immunity.

To my knowledge, this is the first study to investigate the effects of HIV-1 disease progression on "latency" associated antigens and compare them to immunodominant antigens such as ESAT-6 and CFP-10. The hypothesis that loss of T cell responses

directed towards mycobacterial antigens expressed during latency contributes to reactivation of LTBI, is difficult to test within this cohort as we were not powered to detect factors associated with the development of active TB. However, the preferential depletion of CD4 T cell responses to Rv2031c in contrast to the relative sparing of the EC-specific response does provide some evidence to support it. Recent vaccine strategies have included "latency" antigens, however their efficacy in human disease is yet to be determined (Aagaard et al. 2011; Dey et al. 2011).

The persistently elevated TB risk in HIV-infected individuals, compared with those uninfected with HIV-1, indicates a persistent defect in the Mtb-specific T cell response despite ART. We determined that defect to be an inability to restore both Th_1 and Th_{17} Mtb-specific CD4 responses as well as Mtb-specific CD8 T cell responses. A study by Rönsholt et al. found persistently reduced CD4 and CD8 T cell counts after 12 years of ART whilst Gupta et al. showed that despite over 8 years of ART, TB risk remains elevated when compared to HIV-uninfected individuals (Rönsholt et al. 2012; Gupta et al. 2012). It is highly unlikely therefore that 12 months of ART observed in this study would achieve full reconstitution. We of course did not expect this to occur and chose 12 months, as it is the period during which TB risk reduces most rapidly and is most clinically relevant.

Priorities for future work would include a longer period of observation on ART as well as a closer focus on acute changes in Mtb-specific T cell function in early HIV-1 infection, not able to be detected in this study. The findings in this thesis suggest that future vaccine efforts may benefit from a focus on the generation of Mtb-specific CD8

responses, Th_{17} CD4 T cell responses as well T cell responses directed at “latency” associated antigens.

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