

**CHARACTERIZATION OF CD4+
AND CD8+ T CELL RESPONSES
IN HIV-1 C-CLADE INFECTION**

BY

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Plagiarism declaration

I, Dhanwanthie Ramduth declare that:

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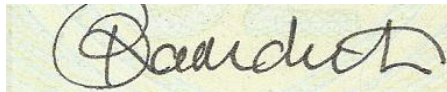


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DECLARATION

I, Dhanwanthie Ramduth, declare that this is my own unaided work. All experiments were conducted at the HIV Pathogenesis Programme Laboratory, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal. The work was supervised by Dr. Photini Kiepiela, Prof. Thumbi Ndung'u (University of KwaZulu-Natal) and Prof Bruce D. Walker (Ragon Institute of MGH, MIT and Harvard).

Signed:

A handwritten signature in black ink, appearing to read 'Dhanwanthie Ramduth', is written over a light green rectangular background.

Date: 04 March 2012

DEDICATION

This work is dedicated to my family, especially My Mother, who has taught me that anything is possible when you are loved. It is dedicated to my friends, who have given their energy in trying to understand and fight this disease. It is dedicated to all the men, women and children who give so much of themselves every day in humanity's war against HIV. Your courage and commitment are humbling and inspiring.

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ETHICS

The Faculty of Medicine at the Nelson R. Mandela School of Medicine, University of KwaZulu-Natal granted ethical approval for the study; Ref: H006/03.

Permission was granted by the Higher Degrees Committee at the Faculty of Medicine to upgrade this project from a Masters to a Doctoral degree.

“AIDS isn’t just a disease. It’s a symptom of something deeper which has gone wrong within the global family. It reveals our broken relationship, between individuals, communities and nations...

AIDS insists that it is time for us to sit down and address all the things we have kept quiet about – sexuality, poverty, and the way we handle our relationships from the family level to the global level/”

Reverend Gideon Byamugisha, a Ugandan priest living positively with HIV

ABSTRACT

HIV-1 specific CD4⁺ T cell activity in clade C infected subjects has not been studied. CD4⁺ T cells play a vital role in controlling infectious diseases and there is a need to augment our knowledge of HIV immunology to aid vaccine design. We therefore embarked on a study to characterize HIV-1 specific CD4⁺ T cell activity in both adults and infants; assess the relationship between CD4⁺ and CD8⁺ immune responses; and the relationship between CD4⁺ T cell activity and markers of disease progression (viral loads and CD4 counts). Our study revealed that the magnitude of CD8⁺ T cell responses correlated significantly with CD4⁺ T cell responses, but that the percentage of CD8⁺ T cells directed against HIV-1 was always greater than that of CD4⁺ T cells. Gag was the frequently targeted HIV-1 protein by CD4⁺ T cells and had the highest density of epitopes targeted by CD4⁺ T cells. Patients with either a dominant CD4 or CD8 T cell response against Gag had significantly lower viral loads than patients in whom non-Gag proteins were the main target ($p < 0.0001$ for CD4 activity and $p = 0.007$ for CD8 responses). Single IFN- γ producing CD4⁺ T cells were present in significantly higher numbers than cells producing both IFN- γ and IL-2 simultaneously ($p = 0.009$). Gag also dominated the CD4⁺ T cell response in acutely infected infants with IFN- γ production detected more frequently than IL-2 or TNF- α . Longitudinal analysis of infants receiving early ARV treatment and then ceasing after 12 months revealed that early treatment conferred no protection against increasing viremia and disease progression. CD4⁺ T cell responses were detected sporadically in untreated infants indicating a dysfunctional immune

response in the face of constant exposure to high levels of viremia. Taken together, the data reveal that a vaccine inducing Gag specific CD4⁺ T cell responses has the potential to confer some degree of protection, but other immunological parameters need to be investigated especially in infants.

ABBREVIATIONS

APCs	Antigen Presenting Cells
ARV	Antiretroviral
ELISPOT	Enzyme-Linked Immunosorbent Spot
BTLA-4	B and T Lymphocyte Attenuator
CTLA-4	Cytotoxic T-lymphocyte Antigen 4
CTL	Cytotoxic T-lymphocyte
HAART	Highly Active Antiretroviral Therapy
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
HVEM	Herpes Virus Entry Mediator
ICAM-1	Inter-Cellular Adhesion Molecule-1
ICOS	Inducible T Cell CoStimulator
ICOSL	Inducible T Cell CoStimulator Ligand
ICS	Intracellular Cytokine Staining
IFN-γ	Inteferon-gamma
IL-2	Interleukin-2
IL-21	Interleukin-21
LAG-3	Lymphocyte Activation Gene-3
LIGHT	Lymphotoxin-like, exhibits Inducible expression, and competes with herpes simplex virus Glycoprotein D for HVEM, a receptor expressed by T lymphocytes
LFA-1	Lymphocyte Function Associated Antigen-1

LTNP	Long Term Non-Progressor
MHC	Major Histocompatibility Complex
mRNA	Messenger RiboNucleic Acid
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NRTVVV	Nef, Tat, Rev, Vpr, Vpu and Vif (pool of HIV-1 peptides)
PD-1	Programmed Death-1
PD-L1	Programmed Death-Ligand 1
SIGLECS	Sialic acid-binding Immunoglobulin superfamily Lectins
SIV	Simian Immunodeficiency Virus type 1
TGF-β	Tumour Growth Factor beta
Th	T helper cell
TIM	T-cell Immunoglobulin Mucin
TNF-α	Tumour Necrosis Factor alpha
TRIM5α	TRIMpartite Motif 5 alpha
TNF:TNFR	Tumour Necrosis Factor:Tumour Necrosis Factor Receptor

CHAPTER ONE

GENERAL INTRODUCTION

CD4+ and CD8+ T cells and HIV-1 Infection

Chapter 1

1. HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1)

In the 1980s doctors in the USA identified a lentivirus, a subclass of the retrovirus family, as the causative agent of a new disease where patients presented with lymphadenopathy, opportunistic infections and a marked decrease in the number of CD4⁺ T lymphocytes (CDC, 1981, Coffin et al., 1986). This pathogen was subsequently named human immunodeficiency virus-1 (HIV-1) (Barre-Sinoussi et al., 1983, Gallo et al., 1983). A related but less pathogenic and immunologically different human retrovirus was later discovered and called HIV-2 (Levy, 1993b, Levy, 1993a, Gallo, 2002, Clavel et al., 1986). Both HIV-1 and HIV-2 are believed to have crossed over from simian immunodeficiency virus (SIV) infected chimpanzees to humans in the 20th century in Central West Africa (Gao et al., 1999, Sharp et al., 2001).

Phylogenetic analyses of the HIV-1 sequences from various regions across the world revealed three groups of HIV-1: M (major), O (outlier) and N (non-M or O). The M group contains more than 95% of the circulating viruses and consists of eight clades (A, B, C, D, F, G, H and J) (Gallo, 2006, Korber et al., 2000, Hoffmann et al., 2005). Currently HIV-1 clade C accounts for 48% of infections across the globe, and is most prevalent in Southern Africa, with 5.7 to 6.6 million people in South Africa living with HIV-1 (UNAIDS, 2008). Clade B viruses are concentrated mainly in North America and Europe and is the most extensively studied clade (UNAIDS, 2008).

HIV-1 Structure

HIV-1 has an envelope glycoprotein composed of two molecules - gp41 (located within the viral membrane) and gp120 (protruding from the virus surface) (Burton, 2006, Ganser-Pornillos et al., 2008, Turner and Summers, 1999, Gelderblom et al., 1989). The matrix protein p17 is anchored to the inside of the viral membrane and encloses the conical shaped p24 Gag (group-specific antigen) capsid protein, which in turn encloses two single stranded RNA molecules and the following viral enzymes - reverse transcriptase, integrase and protease collectively known as polymerase (Burton, 2006, Ganser-Pornillos et al., 2008, Turner and Summers, 1999). In addition, HIV also has six accessory and regulatory proteins- nef, vif, vpr, vpu, rev, and tat (Freed, 1998, Gallo, 1988, Hoffmann et al., 2005).

Nef downregulates the expression of CD4 co-receptors and MHC Class I molecules, and interferes with T cell activation, thereby providing an escape mechanism from virus-specific CD8⁺ cytotoxic T lymphocytes (CTLs) (Hoffmann et al., 2005, Turner and Summers, 1999, Aiken et al., 1994, Collins et al., 1998, Geyer et al., 2001). Tat, rev, vif, vpr and vpu all play key roles in the viral replication cycle (Calnan et al., 1991, Hoffmann et al., 2005, Rosen and Pavlakis, 1990, Turner and Summers, 1999, Cullen, 1999, Miller and Sarver, 1997).

An important feature of Vif is that it attaches to a cellular inhibitor called APOBEC3G (apolipoprotein B editing enzyme catalytic polypeptide-like 3G),

which acts specifically on HIV-1 to prevent the synthesis of proviral DNA. By binding to APOBEC3G, Vif inhibits its function and therefore promotes viral replication (Hoffmann et al., 2005, Sheehy et al., 2003).

(Barouch et al., 2012, Calattini et al., 2011, Hansen and Caspi, 2009, Hansen et al., 2009, Sacha et al., 2009, Seder and Ahmed, 2003, Virgin et al., 2009)

HIV replication cycle

The gp120 envelope molecules on the viral surface bind to the CD4 receptor and to a chemokine co-receptor. The most common chemokine co-receptors utilized by HIV-1 are CXCR4 and CCR5, which are expressed predominantly on macrophages, dendritic cells and T cells (Hoffmann et al., 2005, Turner and Summers, 1999, Rubbert et al., 1998, Ostrowski et al., 1998). A recent study has found that gp120 also binds to integrin $\alpha 4 \beta 7$, a transmembrane protein that mediates interaction between adhesion molecules, on CD4⁺ T cells. This attachment mediates the migration of CD4⁺ T cells to the lymphoid tissue in the gastrointestinal tract (GIT) and activates the expression of Lymphocyte Function Associated Antigen-1 (LFA-1), which in turn mediates the formation of virological synapses thereby promoting the dissemination of HIV-1 (Arthos et al., 2008).

The fusion between gp120 and the CD4 receptor on the host cell facilitates the injection of the viral contents into the target cell (Gelderblom et al., 1989). Several studies have also shown that HIV also utilizes lipid rafts from the host membrane to enter the target cell (Graham et al., 2003, Holm et al., 2003, Gulzar

and Copeland, 2004). Viral replication and transcription are initiated by reverse transcriptase, which copies the single stranded viral RNA into its double stranded complementary proviral DNA (Turner and Summers, 1999, Hoffmann et al., 2005). Since the reverse transcriptase of HIV lacks proof reading mechanisms, this step is highly error prone and gives rise to a high number of mutations or quasispecies that enable the virus to escape recognition by the cellular immune system (Malim and Emerman, 2001). A cytoplasmic body called TRIM5 α binds to the HIV Gag capsid protein and accelerated viral uncoating and thus inhibiting HIV replication before the reverse transcription step (Hoffmann et al., 2005, Stremlau et al., 2004, Li et al., 2006).

In resting infected cells, the proviral DNA accumulates within the cell, integrating into the nucleus only once the cell is activated (Siliciano and Siliciano, 2000, Douek et al., 2002). The resting infected cells serve as reservoirs of HIV that cannot be eliminated even by antiretrovirals (ARVs) (Finzi et al., 1997). They pose a threat if antiretroviral therapy is stopped, as they harbour replication competent virus that can mutate into ARV resistant strains (Hoffmann et al., 2005, Finzi et al., 1997).

Upon activation (by antigens, cytokines, mitogens and to a certain extent even gp 120 itself), the complementary DNA is transported into the nucleus where viral integrase incorporates it into the host cell DNA. Transcription of the DNA generates viral mRNA and genomic RNA (Hoffmann et al., 2005, Frankel and

Young, 1998). The production of NF κ B, which promotes the transcription of DNA into mRNA, is upregulated (Griffin et al., 1989, Nabel and Baltimore, 1987). In resting CD4⁺ T cells, NF κ B can be inhibited by a cellular protein Murr1, the activity of which correlates with the inhibition of HIV replication in quiescent CD4⁺ T cells (Ganesh et al., 2003, Hoffmann et al., 2005). The viral mRNA is translated into polyproteins, which are then cleaved by viral and cellular proteases and assembled into new virions (Hoffmann et al., 2005, Turner and Summers, 1999).

2. OVERVIEW OF THE T-CELL MEDIATED IMMUNE RESPONSE

CD4⁺ T lymphocytes (T-helper - Th) are pivotal to the immune system as they coordinate the functioning of the adaptive and cellular immune responses while CD8⁺ T cells (cytotoxic T cells - CTLs) are responsible for the killing of infected cells (Kim et al., 1985, Cantor and Boyse, 1975, Banchereau and Steinman, 1998, Harty et al., 2000). CD4⁺ T cells are activated by professional antigen presenting cells (APCs that comprise B cells, macrophages and dendritic cells - DCs) that bear antigenic peptides presented by Major Histocompatibility Complex (MHC, also called Human Leukocyte Antigen - HLA) Class II molecules. CD8⁺ T cells are activated by cells expressing MHC Class I molecules (present on all nucleated cells in the body) (Janeway et al., 2005, Abbas and Lichtman, 2003, Bretscher and Cohn, 1970, Lafferty and Gill, 1993, Lenschow et al., 1996).

Immature dendritic cells (DCs) take up HIV-1 virions via Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) receptors within tissue surrounding the skin and mucosal sites. They migrate to the lymphoid organs where they develop a mature phenotype and present the virus to CD4⁺ T cells. The interaction between the DC and CD4⁺ T cell is strengthened by the interaction between Inter-Cellular Adhesion Molecule 1 (ICAM-1) on the APC and Lymphocyte Function Associated Antigen-1 (LFA-1) on the CD4⁺ T cell (Geijtenbeek and van Kooyk, 2003, Hoffmann et al., 2005).

T cells bind to the antigen/MHC complex via their T cell receptor (TCR) complex and either the CD4 or the CD8 co-receptor proteins (Lenschow et al., 1996). The α/β chains that form the TCR are generated by the splicing of gene segments and chromosomal rearrangements during T cell development. This generates a vast repertoire of TCRs capable of responding to a large number of invading pathogens (Sompayrac, 2003, Abbas and Lichtman, 2003, Nemazee, 2000).

Upon binding of the MHC-TCR complex, the cytoplasmic end of T cell receptors make contact with cholesterol-rich lipid rafts, located inside the plasma membrane. These lipid rafts “carry” a large number of downstream signaling molecules that ultimately activate the genetic material of the cell via transcription (Xavier et al., 1998, Janes et al., 1999).

It has been known for some time that the TCR-MHC interaction on its own is insufficient to optimally activate T cells. In recent years there has been a surge in studies analyzing costimulatory and inhibitory receptors involved in T cell function (Sompayrac, 2003, Lenschow et al., 1996, Crawford et al., 2009).

Effective stimulation and differentiation of naïve T cells is dependent on coordinated TCR-MHC interaction (antigens presented by MHC class I bind to CD8 TCR and those presented by MHC Class II bind to CD4 TCR), costimulatory signaling and inflammatory molecule signaling. Failure to receive a secondary signal by costimulatory molecules results in the activated cell becoming anergic

in vivo (non-responsive to future antigenic contacts even in the presence of costimulatory signals) , or tolerant in vitro (Lenschow et al., 1996, Schwartz et al., 2003).

Costimulatory and inhibitory molecules are present on both naïve and memory T cells and are mainly derived from the immunoglobulin (Ig) superfamily, specifically the CD28:B7 and the TNF:TNFR subfamilies. In the first group, CD28 and ICOS are costimulatory receptors binding to B7.1/B7.2 and ICOSL respectively. CTLA4, BTLA4, PD-1, PD-L1 are inhibitory members of the CD28 subfamily, binding B7.1/B7.2, HVEM, PD-L1/PDL-2 and B7-1 respectively (Greenwald et al., 2005).

The TNF:TNFR subfamily consists of the following costimulatory receptors: CD40L, OX40, 4-1BB, CD27 and LIGHT, that bind the following stimulatory molecules: CD40, OX40L, 4ABBL, CD70 and HVEM respectively (Watts et al., 2005). This indicates that the same ligands can exert a stimulatory or inhibitory effect depending on the receptor that it binds onto.

The receptors listed above are currently considered to be the most important costimulatory and inhibitory receptors in modulating T cell function. Recent findings have identified several more receptors, among them: LAG-3; 2B4/CD44; CD30-CD30L; Natural Killer cell receptors; TIM molecules 1-4; CD200R and

Siglecs (Crawford et al., 2009). Further research is warranted to develop this field.

Once activated in the lymph node, naïve CD4⁺ and CD8⁺ T cells undergo clonal expansion and differentiate into effector T cells which migrate to the site of infection. Effector CD8 T cells exert their cytotoxic effect by secreting the cytokines IFN- γ , TNF α , IL-2 and the lytic granules – granzyme and perforin. Naïve CD4 T cells exposed to antigen differentiate into T_H1 or T_H2 CD4 T cells that secrete either IFN- γ or IL-4. CD4 T cell clonal expansion drives CD8 T cell function and B cell activation (Kaeche et al., 2002, Sallusto et al., 2004, Messi et al., 2003, Ansel et al., 2003).

In acute infection, this usually results in viral clearance after a few days of infection. Over the next few weeks, more than ninety percent of the effector T cells die during the contraction phase. The surviving T cells differentiate into either central memory (T_{CM}-residing in lymph nodes) or effector memory (T_{EM}-located at peripheral sites) T cells (Kaeche et al., 2002, Sallusto et al., 2004, Messi et al., 2003, Ansel et al., 2003).

After antigen clearance, Treg cells (expressing CD4⁺CD25⁺FoxP3⁺) downregulate the immune response by inhibiting proliferation of naïve and effector T cells. This is accomplished via IL-10 and transforming growth factor-beta (TGF- β) (Levings et al., 2001, Roncarolo et al., 2001). The majority of

effector T cells undergo a form of apoptosis termed Activation Induced Cell Death (AICD), while a small number differentiate into T_{CM} or T_{EM} cells (Sompayrac, 2003, Janeway et al., 2005, Sallusto et al., 2004, Alderson et al., 1995, Ramsdell et al., 1994).

Both CD4⁺ and CD8⁺ T_{CM} cells are referred to as reactive memory cells, are located in the secondary lymphoid organs and have little or no immediate effector function (Williams and Bevan, 2007). They proliferate and differentiate into effector T cells when stimulated by cognate antigens. T_{EM} cells on the other hand are located at the periphery enabling them to exert immediate effector function (Sallusto et al., 2004).

Memory T cells are phenotypically and functionally different to naïve and effector T cells. They can rapidly differentiate into effector cells; shed and reacquire lymph node homing markers (CD62L); have high proliferative capacity; persist without antigen stimulation via IL-7 and IL-15 induced homeostatic proliferation (Wherry, 2011, Virgin, et al., 2009, Williams, et al., 2007, Jameson, et al., 2009).

The efficient generation of memory CD8 T cells is dependent upon the presence of CD4 T helper cells (Sun et al 2003, Janssen et al, 2003). Recent studies on LCMV infected mice and HIV infected LTNP found that virus-specific IL-21 producing CD4 T cells play a crucial role in maintaining virus-specific CD8 T cell function (Elsaesser et al, 2009, Frohlich et al, 2009, Chevalier et al 2011). It is

important to note that these infections were never cleared but controlled to low antigen level.

During chronic viral infection with high levels of viremia, the scenario is slightly different. The prolonged and repetitive stimulation of T cells with antigen can result in a loss of antigen specific T cell functionality (including proliferation), sustained high level expression of inhibitory receptors. Some T cell clones are deleted but the majority persists in a non-functional state for extended periods. This phenomenon has been termed 'T cell exhaustion' (Kaech et al, 2002, Wherry, 2011).

T cell exhaustion can be driven by pathways extrinsic to the cell (via immunoregulatory cytokines) or intrinsic to the cell (via the expression of inhibitory receptors like PD-1). According to recent molecular studies, T cell exhaustion may represent a distinct state of T cell differentiation (Wherry, 2011).

T cell exhaustion occurs gradually. In the early stages, CD8 T cells incur losses of IL-2 production, proliferative and ex vivo killing capacity, followed by TNF production. Severely exhausted CD8 T cells are unable to produce large amounts of IFN- γ , beta chemokines or degranulate. At the end phase, the exhausted cells are deleted. The level of CD8 T cell exhaustion is positively correlated with viral load; amount of antigenic epitopes presented to the T cells; increased expression of inhibitory T cell receptors and the duration of infection

and inversely correlated with CD4 T helper cell activity (Wherry, 2011, Virgin et al., 2009, Wherry et al., 2004, Wherry et al., 2003).

CD4 T cell exhaustion has not been studied to the same extent as CD8 T cell exhaustion. Although the mechanism of CD4 T cell exhaustion is unknown, virus-specific CD4 T cells have been shown to become dysfunctional during chronic viral infections (Wherry, 2011, Kaufmann et al., 2007, Brooks et al., 2005). Loss of CD4 T cell function leads to decreased IL-21 and increased IL-10 levels, which in turn directly decreases CTL anti-viral activity (Wherry 2011, Yi et al., 2009, Brooks et al., 2006).

3. CD4+ AND CD8+ T CELL IMMUNE RESPONSES TO HIV-1

Trafficking of HIV bearing DCs to activate CD4+ T cells in the lymph nodes allows for the dissemination and establishment of HIV in the host cells (Coleman and Wu, 2009). The resultant infection and extensive loss of the preferentially activated CD4+ T cells is the hallmark of HIV-1 infection (Appay and Sauce, 2008, Douek et al., 2002). HIV-1 infected CD4+ T cells are killed by granzyme/perforin and various cytokines secreted by CTLs (Gulzar and Copeland, 2004, Hudig et al., 1993); CTL mediated apoptosis (Rouvier et al., 1993, Sad et al., 1997) and by the budding of newly synthesized HIV-1 virions from the CD4+ T cell (Leonard et al., 1988). A large number of bystander or uninfected CD4+ T cells are also killed by apoptotic mediated pathways (Finkel et al., 1995).

Recent studies have found that naïve T cell homeostasis is dependent on IL-7 produced by T zone fibroblastic reticular cells (Link et al., 2007).

Murine models show that effective viral specific CD8+ T cell responses are dependent on CD4+ T cell activity (Zajac et al., 1998, Shedlock and Shen, 2003, Matloubian et al., 1994, Janssen et al., 2003, Sun and Bevan, 2003). Several B clade based studies investigating the role of CD4+ and CD8+ T cell activity in HIV-1 infection revealed that virus specific CD4+ and CD8+ T cells retain their ability to secrete IFN- γ , but in persons with high viral loads HIV-specific CD4+ and CD8+ T cells had a reduced proliferative capacity in response to HIV antigens. (Pitcher et al., 1999, Rosenberg et al., 2000, Rosenberg et al., 1997,

Kaufmann et al., 2004, Betts et al., 2001a, Draenert et al., 2004, Addo et al., 2003, Lichterfeld et al., 2004, Day et al., 2007).

Apart from secreting cytokines to boost the CTL response, CD4⁺ Th cells have been shown to directly activate CTLs by CD40L binding; enhance CTL activation via DC activation, and generate functional memory CD8⁺ T cells (Janssen et al., 2003, Sun and Bevan, 2003, Shedlock and Shen, 2003, Ridge et al., 1998, Schoenberger et al., 1998, Bennett et al., 1998).

It has also been well documented that CD4⁺ T cells secreting IFN- γ only represent a skewed cytokine response caused by constant exposure to antigen (Boaz et al., 2002, Day and Walker, 2003, Iyasere et al., 2003, Younes et al., 2003). CD4⁺ T cells producing both IFN- γ and IL-2 were negatively associated with viral load, and low frequencies of IL-2 producing CD4⁺T cells were associated with a reduction in their proliferative capacity. Taken together, it was concluded that HIV-1-specific CD4 T cells in untreated patients with progressive disease have a cytokine secreting profile skewed to producing IFN- γ alone that renders them relatively non-functional.

Apart from inducing apoptosis and directly lysing infected cells by perforin/granzyme molecules, CD8⁺ T cells also release macrophage inflammatory proteins (MIP-1 α , MIP-1 β); RANTES (regulated upon activation, normal T cell expressed and secreted) and cell derived antiviral factor (CAF) (Gulzar and Copeland, 2004). The cell surface expression of CD107a on CD8⁺ T cells is positively associated with the release of perforin/granzyme molecules

(Burkett et al., 2005). Classical early studies showed that CTLs are vital in controlling HIV and SIV replication as CD8-depletion of SIV-infected primates resulted in high plasma viral load (Ogg et al., 1998). However, most subsequent studies employed IFN- γ ELISPOT assays as a measurement of HIV-1 specific CD8+ T cell activity and generated conflicting results concerning CD8+ T cell responses and viral load (Ogg et al., 1998, Koup et al., 1994, Addo et al., 2003, Betts et al., 2001b).

This discrepancy and the failure of the Merck T cell-based STEP vaccine trial has led investigators to reassess T cell immunity, in particular secretion of multiple cytokines and anti-viral factors, as well as memory cell phenotyping (Steinbrook, 2007, Almeida et al., 2007). Although multicolour flow cytometry has made this possible, it is not known whether polyfunctional (positive for three or more functions - IFN- γ , TNF- α , IL-2, MIP-1 β or CD107a) CD8+ T cells are a cause or a consequence of low viral load (Brenchley et al., 2008a, Saez-Cirion et al., 2007). Recent findings suggest that the HIV suppressive activity of polyfunctional CD8+ T cells is related to the antigen load (Almeida et al., 2009).

A key feature of chronic untreated HIV-1 infection is a state of sustained immune activation coupled with ongoing viral replication (Appay and Sauce, 2008). This paradox was supported by data showing that increased CD38 levels on CD8+ T cells correlated with disease progression (Appay and Sauce, 2008, Giorgi et al., 1993, Hazenberg et al., 2003). This chronic state of immune activation accounts for the following features observed in HIV-1 infection:

1. enhanced activation-induced T cell death (Hofer and Speck, 2009)
2. disruption of T cell homeostasis, including decreased thymic output (Moses et al., 1998, Douek et al., 1998)
3. reduced T cell proliferation (Maini et al., 1999)
4. aberrant cytokine secretion profile (Harari et al., 2004 (Fan et al., 1993))
5. skewed memory T cell generation (Harari et al., 2004, Burgers et al., 2009)
6. reduced regenerative capacity of immune cells ((Brenchley et al., 2006a, Brenchley et al., 2006b, Hofer and Speck, 2009, Appay and Sauce, 2008, Harari et al., 2004, Burgers et al., 2009, Maini et al., 1999)).

The dysfunctional immune system leads to a large number of CD4⁺ T cells undergoing apoptosis before they expand into effector T cells. CD8⁺ T cells do not seem to follow the exact same fate with a large number of antigen specific CTLs undergoing expansion and differentiation into pre-terminally differentiated effector cells (CD45RA⁻ CCR7⁻) (Appay and Sauce, 2008, De Boer et al., 2003, Ferreira et al., 2000, Homann et al., 2001, Champagne et al., 2001, Harari et al., 2004) .

Another feature of chronic immune activation is the upregulation of PD-1 on virus specific T cells reducing their functionality (Sauce et al., 2007). PD-1 expression on CD8⁺ T cells correlated positively with plasma viral load and apoptotic activity and inversely with CD4 counts, cytokine production and proliferation (Day et al., 2006, Trautmann et al., 2006, Petrovas et al., 2006).

PD-1 expression on HIV-1 specific CD4⁺ T cells was also associated with disease progression (Day et al., 2006). The inhibitory receptor CTLA-4 is likewise associated with HIV-1 disease progression, and correlates negatively with IL-2 production and positively with viral load and may account for anergic CD4⁺ T cell responses in HIV-1 infection (Kaufmann et al., 2007, Leng et al., 2002, Steiner et al., 1999).

With increased understanding of T cell exhaustion, it is becoming clear that the features of untreated chronic HIV-1 infection (high viral load; declining CD4 counts and constant T cell stimulation), ultimately result in T cell exhaustion. However, studies have shown variations to the typical pattern - even towards the end stages of HIV-1 infection, IFN- γ producing T cells do persist, but with diminished capacity to produce IL-2, TNF α , proliferate and exert their cytotoxic effect (Wherry, 2011, Betts et al., 2006). This phenomenon could explain the lack of correlation between the magnitude of IFN- γ producing T cells and viral load (Addo et al, 2003, Ramduth et al 2009).

The past few years have also seen much coverage of Tregs in HIV-1 infection. Two characteristics of Tregs have emerged:

- (A) Tregs have an inhibitory effect on effector T cells mediating antiviral activity; in particular they inhibit the proliferative response to HIV antigens (Weiss et al., 2004)

(B) Tregs reduce the level of immune activation, which could have beneficial effects for HIV-1 immunity (Sempere et al., 2007)

There is also evidence that Tregs skew the CD4⁺ T cell response away from a Th₁ to a Th₂ type response (Tsunemi et al., 2005). Furthermore, Treg cell numbers were increased in progressors compared to non-progressors and appear immune to apoptosis (Sempere et al., 2007). However, the field is also controversial with the markers identifying Tregs undergoing constant revision. The current consensus to identify Tregs are the presence of CD4⁺, CD25^{high}, FoxP3⁺ and CD127^{low} (Sempere et al., 2007), (Ji and Cloyd, 2009, Kinter et al., 2004, Nixon et al., 2005, Tsunemi et al., 2005, Weiss et al., 2004). This has led to no conclusive evidence for the role that Tregs play in controlling or promoting HIV-1 infection.

In addition, several recent studies have found that controlled HIV-1 infection is associated with serum detectable levels of IL-21 produced by CD4 T helper cells and the presence of the IL-21 receptor on effector CD8 T cells. (Chevalier et al, 2011, Lannello et al, 2008, Yeu et al, 2010). Taken together, these findings show that although HIV-1 specific T cells may be present in large numbers, the quality of these cells in mediating an immune response is compromised.

Adding to the complexity of the insufficient immune control is the ability of HIV-1 to escape recognition by CTLs in response to immune selection pressure exerted by CTLs (Price et al., 1997, McMichael and Rowland-Jones, 2001). Given its high replication rate and poor proof reading capacity, the generation of escape

mutants occurs readily in HIV-1 infection (McMichael and Rowland-Jones, 2001). There is mounting evidence that mutations generated are not random though, but influenced by the HLA type of the infected individual (Goulder and Watkins, 2008, Kiepiela et al., 2004, Leslie et al., 2005, Leslie et al., 2004, Ngumbela et al., 2008, Thobakgale et al., 2009). It is perturbing to note that on a global scale, there is an accumulation of HIV escape mutations in populations with a high HLA selection pressure, indicating the evolutionary adaptation of HIV to its host (Payne et al., 2009, Kawashima et al., 2009).

Recently substantial data has suggested that primary HIV-1 and SIV infection occur in gut associated lymphoid tissue (GALT) where the DCs present the virus to resting central memory (CCR5+) CD4+ T cells. This leads to massive depletion of CCR5+ CD4+ T cells by direct infection, apoptosis or direct killing by CTLs and establishes a reservoir of HIV-1 that cannot be eradicated even with HAART (Brenchley et al., 2006a, Veazey et al., 1998, Brenchley et al., 2004, Sankaran et al., 2008, Guadalupe et al., 2003, Mehndru et al., 2004, Anton et al., 2003).

However, the idea that HIV-1 induces a massive early onslaught on CD4+ memory cell numbers has been challenged by a study on sooty mangabeys showing that the memory CD4+ T cells targeted in the GALT are highly activated short-lived effector memory CD4+ T cells and not the central memory CCR5+CD4+ T cells (Grossman et al., 2006). The gradual loss of CD4+ T cells over time could most likely be caused by an activated immune system, increased

CD4⁺ T cell turnover, chronic exposure to HIV-1 and the generation of dysfunctional memory CD4⁺ T cells CD4⁺ T cell rather than direct killing in the first few weeks of infection (Grossman et al., 2006, Kelleher and Zaunders, 2006).

New data suggests that CD4⁺ Th₁₇ cells could be the target in the GALT as they are found in low frequencies in acute SIV and in HIV infection and correlate negatively with plasma virus level (Cecchinato et al., 2008, Brenchley et al., 2008b). In fact, the compromised immune integrity in the GALT allows for the translocation of microbial products from the gastrointestinal tract, which can account for the chronic state of heightened immune activation observed in HIV-1 infection (Brenchley et al., 2006b).

The obliteration of memory CD4⁺ T cells severely impacts on the ability of the adaptive immune system to ward off pathogens it had previously encountered, thereby permitting the establishment of opportunistic infections that characterize HIV/AIDS infections (Veazey and Lackner, 2005, Mattapallil et al., 2005, Haase, 2005).

Two recent SIV vaccine studies have reported some success in generating protective immunity in rhesus monkeys. Hansen et al constructed a Rhesus Cytomegalovirus vector (RhCMV) that incorporated the SIV Gag, Nef-Tat-Rev and Env proteins. Rhesus macaques were vaccinated either with RhCMV vector alone or with RhCMV and adenovirus 5 (Ad5) vector. Both groups generated and maintained robust SIV-specific effector memory T cell responses at the viral entry

sites, and were able to control viral replication to below detectable levels when challenged with the SIVmaq239 strain (Hansen et al, 2009, Hansen et al, 2011).

The second study by Barouch et al vaccinated rhesus macaques with the Adenovirus/poxvirus and adenovirus/adenovirus-vector-based vaccines containing Gag, Pol and Env from the SIV_{SME543} strain. The vaccine reduced the probability of infection by 80% (Barouch et al, 2012).

Thus given the pivotal role that T cells play in the immune system and that sub-Saharan Africa bears the brunt of HIV-1 infection, it is imperative to characterise the relationship between HIV-1 infection and T cells in this setting (UNAIDS, 2008). The few immunologic studies of clade C HIV-1 infection published thus far have been limited to the characterization of CD8+ T cell responses in adult infection (Kiepiela et al., 2004, Kiepiela et al., 2007, Ngumbela et al., 2008, Goulder et al., 2001b, Thobakgale et al., 2007, Novitsky et al., 2006, Gray et al., 1999, Gray et al., 2009). Thus characterizing HIV-1 specific CD4+ T cell activity in adults and infants infected with clade C virus is important in determining the correlates of immune protection.

4. T CELL RESPONSES IN PAEDIATRIC HIV INFECTION

At the time of this study, 72 000 HIV-1 infected infants were born each year in South Africa, and 40% of deaths in children under 5 years were AIDS related (UNICEF, 2005, DOH RSA 2004). Antiretroviral drugs were provided according to criteria stipulated by the South African Department of Health - only when the CD4 percentage was <20% in infants below 18 months of age and <15% in those older than 18 months ¹³.

Although there have been several investigations into pediatric immune responses at a cellular level, these have been limited in the sensitivity of the assays used (ELISAs measuring secreted IL-2 from cord blood), and confined to CD8+ T cell responses in acutely infected infants, and to CD4+ and CD8+ T cell activity in chronically infected children (Kuhn et al., 2001a, Kuhn et al., 2002, Kuhn et al., 2001b, Kuhn et al., 2001c, Kuhn et al., 2007, Thobakgale et al., 2009, Thobakgale et al., 2007, Persaud et al., 2000, Farquhar et al., 2005, Lohman et al., 2005, Slyker et al., 2005). HIV-1 specific T cell responses were rarely detected in infants younger than 24 months, with the magnitude and breadth of responses increasing with age (Predergast et al, 2011).

Just as in adult infection, certain MHC class I molecules influence disease progression by driving the virus to mutate into less fit strains. Slow progressor infants were found to have at least one protective HLA class I allele (B57, B5801, B8101) or have inherited a less fit virus from a mother possessing a protective allele (Thobakgale et al, 2009).

OUTLINE OF THESIS

The mechanistic interaction between CD4⁺ T cells and HIV-1 has been described in the first part of this thesis. Previous literature has alluded to the possible vital role played by CD4⁺ T cells in controlling HIV-1 infection. **Chapter 2 describes** the simultaneous quantification of CD4⁺ and CD8⁺ IFN- γ T cell responses. These responses were mapped to all HIV-1 proteins in a cohort of chronically infected, therapy-naïve adults and the relationship between HIV-1 specific CD4⁺ and CD8⁺ T cell activity was investigated. In **Chapter 3** the study was extended to the developing immune system by assessing HIV-1 specific CD4⁺ T cell cytokine activity (IFN- γ , IL-2 and TNF- α) and proliferative responses in acutely infected infants. In addition, the effect of early HAART on the HIV-1 specific immune responses was also investigated. **Chapter 4** focused on identifying particular HIV-1 CD4⁺ T cell epitopes and their association with viral load. Dual cytokine expression and cross clade activity was also investigated. **Chapter 5** is a coauthor paper characterizing CD8⁺ T cell responses in infants from birth, while **chapter 6** investigated the relationship between HIV protein specific responses and viral load. The investigation of these aspects of HIV-1 infection in Sub-Saharan Africa provides essential information in the design of an HIV vaccine.

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CHAPTER TWO

DIFFERENTIAL IMMUNOGENICITY OF HIV-1 CLADE C PROTEINS IN ELICITING CD8+ AND CD4+ CELL RESPONSES.

Ramduth D, Chetty P, Mngquandaniso NC, Nene N, Harlow JD, Honeyborne I, Ntumba N, Gappoo S, Henry C, Jeena P, Addo MM, Altfeld M, Brander C, Day C, Coovadia H, Kiepiela P, Goulder P, Walker B.

J Infect Dis. 2005 Nov 1; 192(9):1588-96. Epub 2005 Sep 30.

Doctoral Student's contribution to journal articles to be included in a doctoral "Thesis Through Publication"

Student name: D. Ramduth Student no.: 953034661

Title of the article:

Differential Immunogenicity of HIV-1 Clade C Proteins in Eliciting CD8+ and CD4+ Cell Responses

Authors (sequence as in the article):

Ramduth D, Chetty P, Mngquandaniso Nc, Nene N, Harlow JD, Honeyborne I, Ntumba N, Gappoo S, Henry C, Jeena P, Addo Mm, Altfeld M, Brander C, Day C, Coovadia H, Kiepiela P, Goulder P, Walker B

Journal (with year, volume and pages):

Journal of Infectious Diseases 2005 Nov 1; 192(9):1588-96

Please state your contribution to the article under the following headings:

1. Formulation of the hypothesis (if applicable)

I contributed 50% towards formulating the hypothesis, as this was originally a Masters study that was eventually upgraded to a PhD.

2. Study design

I contributed 80% towards the study design by consulting with statisticians on the number of subjects to recruit, reading the latest literature and consulting with my supervisor to incorporate these concepts into the protocol.

3. Work involved in the study

I performed all the laboratory assays, adapted the protocols when necessary and attended the relevant workshops to acquire the required laboratory skills.

4. Data analysis

I performed the entire data analysis of the raw data, drew up the analysis dataset and performed the statistical data after consultation with a statistician.

5. Write-up

I wrote the entire manuscript with constant review and feedback from my supervisor.

I declare this to be a true reflection of my contributions to this journal article.

Signature:



Date: 04 March 2012

CHAPTER THREE

DETECTION OF HIV TYPE 1 GAG-SPECIFIC CD4 (+) T CELL RESPONSES IN ACUTELY INFECTED INFANTS.

Ramduth D, Thobakgale CF, Mkhwanazi NP, De Pierres C, Reddy S, van der Stok M, Mncube Z, Mphatswe W, Blanckenberg N, Cengimbo A, Prendergast A, Tudor-Williams G, Dong K, Jeena P, Coovadia HM, Day CL, Kiepiela P, Goulder PJ, Walker BD.

AIDS Res Hum Retroviruses. 2008 Feb; 24(2):265-70.

Doctoral Student's contribution to journal articles to be included in
a doctoral "Thesis Through Publication"

Student name: D. Ramduth Student no.: 953034661

Title of the article:

Detection of HIV Type 1 Gag-Specific CD4 (+) T Cell Responses In Acutely Infected Infants.

Authors (sequence as in the article):

Ramduth D, Thobakgale CF, Mkhwanazi NP, De Pierres C, Reddy S, van der Stok M, Mncube Z, Mphatswe W, Blanckenberg N, Cengimbo A, Prendergast A, Tudor-Williams G, Dong K, Jeena P, Coovadia HM, Day CL, Kiepiela P, Goulder PJ, Walker BD

Journal (with year, volume and pages):

AIDS Res Hum Retroviruses. 2008 Feb; 24(2):265-70.

Please state your contribution to the article under the following headings:

1. Formulation of the hypothesis (if applicable)

I contributed 75% towards formulating the hypothesis, with special focus on the role of CD4+ T cell responses in pediatric HIV-1 infection.

2. Study design

I contributed 80% towards the study design by consulting with statisticians on the number of subjects to recruit, reading the latest literature and consulting with my supervisor to incorporate these concepts into the protocol.

3. Work involved in the study

I performed all the laboratory assays, adapted the protocols when necessary and attended the relevant workshops to acquire the required laboratory skills.

4. Data analysis

I performed the entire data analysis of the raw data, drew up the analysis dataset and performed the statistical data after consultation with a statistician.

5. Write-up

I wrote the entire manuscript with constant review and feedback from my supervisor.

I declare this to be a true reflection of my contributions to this journal article.

Signature:



Date: 04 March 2012

CHAPTER FOUR

IMMUNODOMINANT HIV-1 CD4+ T CELL EPITOPES IN CHRONIC UNTREATED CLADE C HIV-1 INFECTION.

Ramduth D, Day CL, Thobakgale CF, Mkhwanazi NP, de Pierres C, Reddy S, van der Stok M, Mncube Z, Nair K, Moodley ES, Kaufmann DE, Streeck H, Coovadia HM, Kiepiela P, Goulder PJ, Walker BD.

PLoS ONE. 2009;4(4):e5013. Epub 2009 Apr 7.

Doctoral Student's contribution to journal articles to be included in
a doctoral "Thesis Through Publication"

Student name: D. Ramduth Student no.: 953034661

Title of the article:

Immunodominant HIV-1 CD4+ T Cell Epitopes In Chronic Untreated Clade C HIV-1 Infection.

Authors (sequence as in the article):

Ramduth D, Day CL, Thobakgale CF, Mkhwanazi NP, de Pierres C, Reddy S, van der Stok M, Mncube Z, Nair K, Moodley ES, Kaufmann DE, Streeck H, Coovadia HM, Kiepiela P, Goulder PJ, Walker BD.

Journal (with year, volume and pages):

PLoS ONE. 2009;4(4):e5013. Epub 2009 Apr 7.

Please state your contribution to the article under the following headings:

1. Formulation of the hypothesis (if applicable)

I contributed 80% towards formulating the hypothesis, with the data from this chapter based on data from chapter 2.

2. Study design

I contributed 80% towards the study design by consulting with statisticians on the number of subjects to recruit, reading the latest literature and consulting with my supervisor to incorporate these concepts into the protocol.

3. Work involved in the study

I assisted Christina Thobakgale in performing all the laboratory assays, adapted the protocols when necessary and attended the relevant workshops to acquire the required laboratory skills.

4. Data analysis

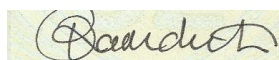
I performed the entire data analysis of the raw data, drew up the analysis dataset and performed the statistical data after consultation with a statistician.

5. Write-up

I provided feedback on the manuscript written by Christina Thobakgale.

I declare this to be a true reflection of my contributions to this journal article.

Signature:



Date: 04 March 2012

CHAPTER FIVE

HUMAN IMMUNODEFICIENCY VIRUS-SPECIFIC CD8+ T-CELL ACTIVITY IS DETECTABLE FROM BIRTH IN THE MAJORITY OF IN UTERO-INFECTED INFANTS

Christina F. Thobakgale, **Dhanwanthie Ramduth**, Sharon Reddy, Nompumelelo Mkhwanazi, Chantal de Pierres, Eshia Moodley, Wendy Mphatswe, Natasha Blanckenberg, Ayanda Cengimbo, Andrew Prendergast, Gareth Tudor-Williams, Krista Dong, Prakash Jeena, Gupreet Kindra, Raziya Bobat, Hoosen Coovadia, Photini Kiepiela, Bruce D. Walker, and Philip J. R. Goulder

Journal of Virology, Dec. 2007, p. 12775–12784

Doctoral Student's contribution to journal articles to be included in
a doctoral "Thesis Through Publication"

Student name: D. Ramduth Student no.: 953034661

Title of the article:

**HUMAN IMMUNODEFICIENCY VIRUS-SPECIFIC CD8+ T-CELL ACTIVITY
IS DETECTABLE FROM BIRTH IN THE MAJORITY OF IN UTERO-INFECTED
INFANTS.**

Authors (sequence as in the article):

Christina F. Thobakgale, **Dhanwanthie Ramduth**, Sharon Reddy, Nompumelelo Mkhwanazi, Chantal de Pierres, Eshia Moodley, Wendy Mphatswe, Natasha Blanckenberg, Ayanda Cengimbo, Andrew Prendergast, Gareth Tudor-Williams, Krista Dong, Prakash Jeena, Gupreet Kindra, Raziya Bobat, Hoosen Coovadia, Photini Kiepiela, Bruce D. Walker, and Philip J. R. Goulder

Journal (with year, volume and pages):

Journal of Virology, Dec. 2007, p. 12775–12784

Please state your contribution to the article under the following headings:

1. Formulation of the hypothesis (if applicable)

I contributed 50% towards formulating the hypothesis, with the data from this chapter complementing the data from chapter 4.

2. Study design

I contributed 40% towards the study design by reading the latest literature and consulting with my supervisor to incorporate these concepts into the protocol.

3. Work involved in the study

I assisted Christina Thobakgale in performing all the laboratory assays, adapted the protocols when necessary and attended the relevant workshops to acquire the required laboratory skills.

4. Data analysis

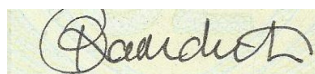
I performed 30% of data analysis of the raw data, and in drawing up the analysis dataset.

5. Write-up

I provided feedback on the manuscript written by Christina Thobakgale.

I declare this to be a true reflection of my contributions to this journal article.

Signature:



Date: 04 March 2012

CHAPTER SIX

CD8+ T-CELL RESPONSES TO DIFFERENT HIV PROTEINS HAVE DISCORDANT ASSOCIATIONS WITH VIRAL LOAD

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CD8+ T-Cell Responses to Different HIV Proteins have Discordant Associations with Viral Load.

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Journal (with year, volume and pages):

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Please state your contribution to the article under the following headings:

1. Formulation of the hypothesis (if applicable)

I contributed 20% towards formulating the hypothesis, with the data from this chapter based on findings in chapter 2.

2. Study design

I did not contribute towards the study design.

3. Work involved in the study

I performed the Elispot assays, intracellular cytokine staining, viral loads and CD4 counts as part of the team in the laboratory.

4. Data analysis

I performed 30% of data analysis of the raw data.

5. Write-up

I provided feedback on the manuscript written by Dr Kiepiela.

I declare this to be a true reflection of my contributions to this journal article.

Signature:



Date: 04 March 2012

CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSION & FUTURE PERSPECTIVES

GENERAL DISCUSSION

An effective cellular mediated immune response against infectious diseases requires CD4⁺ and CD8⁺ T cells functioning in concert against pathogens, leading us to hypothesize that HIV-1 specific CD4⁺ T cell responses are associated with disease control in HIV-1 clade C infection. Our studies found that in untreated adults, CD4⁺ T cell responses correlated with CD8⁺ T cell responses and that the targeting of the Gag region was associated with disease control (chapter 2). In pediatric subjects, we found Gag to be the earliest HIV protein targeted and the level of the immune response was determined by antigen load (chapter 3). Returning to the adult cohort, we were able to identify specific CD4⁺ epitopes (the majority of which were clustered in the Gag region) and found that the breadth of the response was associated with viral control (chapter 4). We discuss these findings in the context of current literature and vaccine development strategies, as well as future directions of CD4⁺ T cell research in HIV immunopathogenesis.

Identification of CD4⁺ T cell Responses in Clade C Infection

At the onset on the study, CD4⁺ T cell responses in HIV-1 clade C infection had been poorly characterized. The studies that had been conducted investigated CD4⁺ T cell responses to a few envelope proteins in cord blood using traditional ELISAs (Kuhn et al., 2001b, Kuhn et al., 2001c) and identified CD8⁺ antigen specific responses by an IFN- γ Elispot assay (Masemola et al., 2004, Goulder et al., 2000, Goulder et al., 2001a). To better characterize HIV-specific CD4⁺ T

cell responses in clade C infection in both adult and pediatric subjects, we utilized a flow cytometry based technique to identify protein specific CD4+ IFN- γ responses and then proceeded to identify individual antigen specific responses by Elispot.

Elispot and ICS have become standard techniques in the assessment of antigen specific cytokine responses (Barouch and Letvin, 2000a, Barouch and Letvin, 2000b, Liu et al., 2009, Barouch et al., 2009, Addo et al., 2003, Goulder et al., 2001, Gray et al., 1999). Each technique has its respective advantages and shortcomings. The Elispot assay is extremely sensitive, capable of detecting 0.01% cytokine producing cells and can be performed with as little as 50 000 cells. However, unlike in ICS, no phenotyping can be conducted during the assay, necessitating prior purification to isolate responses from a particular cellular subset. In our study, this was the best technique to identify individual antigen responses almost immediately ex vivo utilizing a limited number of cells.

The ICS assay on the other hand requires a larger number of cells to begin with (0.5 to 1×10^6) ensuring that a statistically significant number are available for acquisition post several wash steps. At the time of performing the ICS assays, the sensitivity of the assay in our lab (determined by running HIV negative controls) was lower than the Elispot assay, with responses in our control subjects ranging from 0.01-0.03%. Given their complementary characteristics, we

employed both techniques to comprehensively identify and confirm HIV-1 specific CD4⁺ T cell epitopes.

We considered culturing CD4⁺ T cells with protein peptide pools before conducting the Elispot or ICS but this ran the risk of growing out the dominant antigen specific clones, thereby masking the low magnitude responses. We then considered the use of MHC Class II tetramers. Previous studies found that combining tetramer staining with prior cell enrichment techniques; can increase the detection of low frequency antigen specific cells (Scriba et al., 2005, Ceconni et al., 2008) However, in comparison to MHC Class I tetramers, MHC Class II tetramers are not as stable mainly because:

1. the conjugation of Class II tetramers does not always yield stable structures
2. the reorganization of the TCR on the plasma membrane upon T cell activation influences the binding of the tetramers
3. there is variability in the degree of homogenous binding of the CD4 epitope to a monomer arm (Ceconni et al., 2008).

The level of sensitivity of MHC Class I tetramers can be as low as 0.02% and have been successfully utilized in identifying low frequency CD8⁺ T cell clones in chronic clade C infection (Day et al., 2006, Day et al., 2007, Kelleher and Rowland-Jones, 2000). In the context of tetramer staining, an important shortcoming of CD4⁺ T cell epitopes is that they are promiscuous in their binding to multiple MHC Class II alleles which renders the specificity and sensitivity of

MHC Class II tetramers in CD8⁺ T cell depleted cells questionable (Kaufmann et al., 2004). Even if more CD4 epitopes were to be defined, HLA restriction might be difficult, due to the promiscuous nature of CD4⁺ T cell epitopes. Another shortfall is that tetramer staining does not measure any functionality but merely quantifies the number of antigen specific cells (Kelleher and Rowland-Jones, 2000). Apart from these factors, tetramer synthesis is a costly and labour intensive process, making its implementation for comprehensive studies difficult.

Association between CD4⁺ T cell Responses, CD8⁺ T cell Responses and Clinical Parameters in Chronic Clade C HIV-1 Infection

Consistent with previous clade B studies we found that CD4⁺ IFN- γ responses persisted in chronic infection (Pitcher et al., 1999) and correlated positively with CD8⁺ IFN- γ responses. Despite this association, we found no significant relationship between the CD4⁺ T cell IFN- γ responses and viral loads and CD4 counts. This can be explained by several studies which have found that IFN- γ may not be a marker of effective CD4⁺ T cell activity but that antigen specific CD4⁺ T cells producing both IFN- γ and IL-2 concomitantly are correlated with slower disease progression (Boaz et al., 2002, Harari et al., 2004). Chronic exposure to antigen, as in the case of HIV, tends to skew cytokine production to the exhausted non-functional IFN- γ only, pre-terminally differentiated memory phenotype, compromises their capacity to control HIV replication (Harari et al., 2005, Yue et al., 2004, Kaech et al., 2002, Crawford et al., 2009, Wherry, 2011).

A caveat of our study was quantifying CD4⁺ T cell activity by IFN- γ production only. A subsequent study which employed a variation of the intracellular cytokine technique and defined CD4⁺ T cell activity by IFN- γ and IL-2 production in clade C infection also investigated these relationships but arrived at contrasting conclusions (Shalekoff et al., 2008). This study found no relationship between the magnitude of HIV-1 specific CD4⁺ and CD8⁺ T cell responses, possibly due to the analysis of dual cytokine (IFN- γ /IL-2) producing T cells. Similar to our results though, they found HIV-specific CD8⁺ T cell responses to be of a higher magnitude than that of CD4⁺ T cells. This observation may be due to the extensive loss of HIV specific CD4⁺ T cells during infection and to the difference in sizes of the antigenic T cell pools – the number of activated CD4⁺ T cells is much smaller than that of the CD8⁺ T cell pool (Kaeck et al, 2002, Wherry, 2011). Also, IFN- γ producing CD4⁺ T cells are believed to be T_{EM} cells found at peripheral sites (Seder et al 2003). Since T_{CM} cells were not analyzed, we do not have a comprehensive analysis of CD4⁺ T cell function in our cohort.

Taking into account recent findings on the role of inhibitory T cell receptors (such as PD-1 and CTLA-4) and T cell exhaustion, we can hypothesize that the chronically infected, untreated cohort we studied display the classical signs of T cell exhaustion - skewed cytokine profile to IFN- γ only secretion, lack of proliferative responses, uncontrolled viral replication and large number of antigens presented to T cells (Wherry, 2011, Virgin et al., 2009, Wherry et al., 2004, Wherry et al., 2003).

Further definition of HIV-T cell immunology could benefit by employing more sensitive assays like the viral inhibition assay coupled with kinetic studies for both CD4+ and CD8+ T cell activity and in situ tetramer hybridization (ISTH) which combines in situ tetramer (IST) and in situ hybridization (ISH). Recent findings from these techniques found that control of SIV and LCMV infections correlated with the magnitude of effector T cell responses generated very early in the course of infection (Li et al., 2009, (Spentzou et al., 2010). Taken a step further, studies by Hansen et al on RhCMV vectors carrying SIV encoding genes found that the generation of effector memory T cells during early infection had resulted in some level of protection when challenged with SIV (Hansen et al, 2011).

Immunodominance of Gag-Specific T cell Responses in Untreated Chronic Clade C Infection and Detection of Cross Clade Responses

Our initial study showed a dominance of Gag-specific CD8+ T cell responses was associated with lower levels of viremia. This was later extrapolated to a larger cohort and epitope specific analysis, which confirmed our findings (Kiepiela et al., 2007). The study found an inverse relationship between the breadth of Gag-specific responses and viral load, and a positive association between the breadth of envelope responses and viremia (Kiepiela et al., 2007). These conclusions were independent of HLA type and epitope variation and the observation adds weight to previous studies which proposed the inclusion of Gag

as a major immunogen in an HIV vaccine (Leslie et al., 2005, Addo et al., 2003, Kaufmann et al., 2004, Ramduth et al., 2005, McKinney et al., 2004). A following study on clade C infection confirmed Gag to be the dominant protein targeted by both CD4+ and CD8+ T cells and Gag responders to have a lower viral load than non-Gag responders (Shalekoff et al., 2008).

However, we have to be cautious not to interpret these as data as showing that Gag-specific responses driving down the viral load as this is a cross sectional study of chronic infection. Indeed recent SIV vaccine studies by Barouch et al found that the inclusion of the Env protein was important in reducing viral set point and conferred protection to subsequent SIV challenge. Another recent finding is that during chronic HIV infection, IL-6 mediates the differentiation of virus specific CD4+ T helper cells to T follicular (T_{FH}) cells, which enhanced lymph node germinal centre activity, which in turn helped sustain B cell activity in the lymph nodes. It remains to be investigated whether the enhanced B cell activity results in increased neutralization antibody function against HIV Envelope (Harker et al, 2011 Fahey et al, 2011).

Expanding on our first study which identified CD4+ T cell responses to whole peptide pools, we conducted whole genome screening to identify individual epitopes. Here we found a clustering of HIV-1 specific CD4+ T cell epitopes in the Gag region and an inverse correlation between the breadth of the responses and viral load. Unlike the clade B CD4+ T cell epitope mapping study, Nef was

not the dominant target of CD4⁺ T cells. This could be due to differences in the disease and antiretroviral treatment states of the subjects recruited, as the clade B study analyzed acutely infected patients and patients undergoing Structured Treatment Interruption (STI) while our cohort comprised therapy naïve patients (Kaufmann et al., 2004).

A striking observation was that one of the most frequently targeted CD4⁺ epitopes (YVDRFFKTLRAEQATQDV) is a p24 Gag peptide spanning part of the Major Homology Region (MHR). The MHR displays significant homology among multiple genre of retroviruses, with mutations in this region resulting in viral particles that have defects in viral assembly, maturation and infectivity (Freed, 1998). This particular peptide was shown to be frequently targeted in clades B and A/G infections and displayed a high degree of cross reactivity between HIV-1 and HIV-2 infected individuals (Kaufmann et al., 2004, Ondondo et al., 2008). Individuals in our study with a response to this peptide also recognized the clade B sequence with an equal magnitude (Ramduth et al., 2009), probably due to similar biochemical properties between these peptides.

The recognition of CD4⁺ T cell epitopes across multiple clades by the same subject has been investigated by a large number of studies and has yielded positive results (Gupta et al., 2006, Norris et al., 2004, De Groot et al., 2004, Keating et al., 2002, Betts et al., 1997, Boyer et al., 1998, Gotch, 1998). This

feature can be exploited in vaccine design and vaccine testing as it overcomes the obstacle of synthesizing vaccines for each clade (Garber et al., 2004).

HIV-1 specific CD4+ T cell Responses in Acutely Infected Infants

Although there have been several investigations into pediatric immune responses at a cellular level, these have been limited in the sensitivity of the assays used (ELISAs measuring secreted IL-2 from cord blood), and confined to CD8+ T cell responses in acutely infected infants, and to CD4+ and CD8+ T cell activity in chronically infected children (Kuhn et al., 2001a, Kuhn et al., 2002, Kuhn et al., 2001b, Kuhn et al., 2001c, Kuhn et al., 2007, Thobakgale et al., 2009, Thobakgale et al., 2007, Persaud et al., 2000, Farquhar et al., 2005, Lohman et al., 2005, Slyker et al., 2005, Prendergast et al., 2011).

Since there had been no reported data on CD4+ T cell responses in acutely infected infants, we investigated multiple cytokine production and proliferative responses in treated and untreated acutely infected infants (Ramduth et al., 2008).

Cytokine levels in HIV-1 infected untreated infants (IFN- γ , IL-2 and TNF- α) were detected at very low frequencies mainly to the conserved Gag and Pol proteins, and not to the envelope protein. The relative lack of cytokine responses in comparison to adult HIV-1 infection, with 70% of adults having detectable IFN- γ response (Ramduth et al., 2009), could be due to a decreased capacity of the

neonatal immune system to produce cytokines (Holt, 2003, Lewis et al., 1986, Wilson et al., 1986). However in our cohort, this may be restricted to CD4+ T cell responses, as HIV-1 specific CD8+ T cell responses were detected in 70% of infected infants (Thobakgale et al., 2007).

Once again Gag was the dominant target of IFN- γ , IL-2 and TNF- α producing CD4+ T cells and was detected as early as day 6 in an in-utero infected infant, suggesting that even in-utero, Gag is highly immunogenic. However, the envelope protein was targeted more frequently than Gag by CD8+ T cells (Thobakgale et al., 2007), indicating a potential disconnect between the CD4+ and CD8+ immune responses in neonatal HIV infection. It is important that such preferential targeting not be viewed in isolation, but further studies investigating cellular immunity also investigate innate immune responses. The cells constituting the innate immune system also act as professional APCs to CD4+ T cells and their antigen processing may produce different antigenic peptides to those produced by MHC Class I bearing cells.

IFN- γ Gag-specific responses were detected sporadically in infants receiving treatment and increased in magnitude following treatment cessation, suggesting that the level of cytokine response is mediated by antigen load. However, Gag-specific responses were observed intermittently in the untreated infants, indicating a dysfunction in CD4+ T cells chronically exposed to high levels of antigen. Despite the increase in the magnitude of Gag-specific responses after

stopping treatment, the majority of infants had to resume therapy by 24 months of age, implying that the CD4+ Gag-specific IFN- γ responses alone may not exert a long term protective effect but could potentially play a role in modulating the viral set point.

Since HIV has been shown to preferentially infect activated CD4+ T cells, a study investigating the expression of activation markers on CD4+ T cells could yield valuable information. A study which was conducted on adults found that a history of high immune activation (due to the presence of other pathogens) led to a greater loss of CD4+ T cells during the early stages of infection. They also observed that higher levels of CD4counts prior to infection led to a greater loss of CD4+ T cells. Infants may have an immune system that is continuously activated as they adapt to their environment. Associated with a higher absolute number of CD4+ T cells, these are factors which could provide the perfect environment for HIV replication which in turn skews the functioning of the immune system.

An unreported observation is where we compared the HIV-specific IFN- γ CD4 responses in eight infants and nine adults with viral loads >100 000 copies/ul. We found no significant differences in the magnitudes of the responses ($p=0.66$ Student T test). It would be interesting to investigate whether these groups exhibit the same level of T cell exhaustion and compromised memory T cell formation.

Implications for Cell Mediated Vaccine Design and Future Perspectives

Apart from inducing poor cellular immunity, HIV-1 envelope protein also elicits a suboptimal humoral response, as the constantly mutating virus successfully evades neutralizing antibodies (Wyatt et al., 1998, Wyatt and Sodroski, 1998). It was therefore not surprising that the VaxGen trial which aimed to induce neutralizing antibodies failed to protect against HIV infection (Veljkovic et al., 2003, Billich, 2004).

T cell responses though have the potential to be effective therapeutic vaccines as the presence of these responses are associated with a reduced rate of disease progression, and lower viral set points as demonstrated in SIV primate investigations (Barouch and Letvin, 2000a, Barouch and Letvin, 2000b, Liu et al., 2009, Barouch et al., 2009). A low viral set point also reduces transmission, as high viral loads correlate with high transmission rates (Gray et al., 2001, Shiver and Emini, 2004, Coll et al., 1997, Khouri et al., 1995, O'Shea et al., 1998). This would in turn slow the spread of the pandemic (Garber et al., 2004).

Thus identifying HIV-1 epitopes, and in particular Gag epitopes associated with immune control, provides the relevant information for designing a synthetic peptide vaccine (Letvin, 2002, Letvin et al., 2002, Sahni and Nagendra, 2004). A number of studies have focused on defining HIV-1 specific CD8+ T cell epitopes in both clade B and C HIV-1 infections, (Addo et al., 2003, Goulder et al., 2001a,

Gray et al., 1999, Kiepiela et al., 2004, Novitsky et al., 2001, Masemola et al., 2004), while very few have looked at identifying HIV-1 specific CD4+ T cell epitopes, and none defining HIV-specific CD4 T cell epitopes targeted in clade C infection (Boaz et al., 2003, Kaufmann et al., 2004).

Our research has indicated that IFN- γ secreting Gag-specific CD4+ T cell responses are immunodominant in untreated clade C infection, are associated with viral control and that CD4+ T cell responses are directed against multiple epitopes. However, what needs to be determined is whether these responses are the mediators of control or a consequence thereof. A possible approach to this conundrum would be to track HIV-1 specific CD4+ T cell activity from the very early stages of acute infection. In conjunction with multiple phenotypic analyses - host genetic factors (in particular MHC Class II alleles), neutralizing antibody studies, viral fitness assays and host cellular protein analyses will need to be undertaken in a longitudinal study. Determining the level of activation by cell surface markers (eg CD38) and evaluating the number of T cell Receptor Excision Circles (TRECs) would shed light on whether the sub-Saharan population has a higher level of immune activation (due to exposure to more pathogens) which facilitates HIV infection and replication.

In addition, more sensitive techniques are required to detect and characterize this cellular immune response (particularly CD4+ T cell responses) at mucosal sites (Coombs et al., 2003). Present data indicate that Gag-specific CD8+ T cells

in the rectal mucosa are capable of eliciting effector functions (Critchfield et al., 2007). However, increased levels of HIV-1 specific CD8⁺ T cells in the cervix of chronically infected women did not inhibit viral shedding in mucosal tissue (Gumbi et al., 2008). This implies that the field of mucosal immunity requires further investigation since a vaccine capable of eliciting cell mediated immunity and conferring protection at mucosal sites, may reduce the rates of horizontal and vertical HIV transmission (Gupta and Klasse, 2006).

Despite extensive research into the role of T-regulatory (Treg) cells in HIV-1 infection, there is no conclusive evidence that these cells play a pivotal role in either inhibiting or augmenting HIV replication. There is some evidence that natural Tregs (nTregs) can dampen HIV-1 specific immune responses (resulting in high viral loads), as was found in a Ugandan study on therapy naïve individuals (Eggena et al., 2005). Other studies indicate that nTregs reduce cellular activation, which in turn limits the rate of infection (Weiss et al., 2004).

Inducible T-reg (iTregs) are comprised of two subsets: Tr1 and Th3. Both subsets function in the gastrointestinal tract, are activated by cytokines (IL-10, TGF- β , IL-4, IFN- γ , IL-5 and IL-2 and suppress effector T cell function via cytokines, predominantly TGF- β (Sempere et al., 2007, Chatenoud and Bach, 2006).

TGF- β along with (IL-6) is also the cytokine involved in the generation of the newly characterized Th₁₇ CD4⁺ T cells, an additional effector lineage of CD4⁺ T cells (Harrington et al., 2005, Weaver and Murphy, 2007, Acosta-Rodriguez et al., 2007a, Nowak et al., 2009). Studies of HIV-1 infection in the gastrointestinal tract (GIT), found that Th₁₇ CD4⁺ T cells are infected, though not preferentially so; a skewing of Th₁₇ cells towards a Th₁ phenotype with maturation and substantial loss of Th₁₇ cells from the GIT (Cecchinato et al., 2008, Brenchley et al., 2008b, Hofer and Speck, 2009, Locksley, 2009). The findings imply that overall loss of Th₁₇ cells contributes to the compromised integrity of the mucosal membrane, leading to an activated immune system.

In addition, several recent studies have found that controlled HIV-1 infection is associated with serum detectable levels of IL-21 produced by CD4 T helper cells and the presence of the IL-21 receptor on effector CD8 T cells. (Chevalier et al, 2011, Lannello et al, 2008, Yeu et al, 2010). Taken together, these findings show that although HIV-1 specific T cells may be present in large numbers, the quality of these cells in mediating an immune response is compromised.

The immunodominance of Gag is also worthy of more rigorous investigation. A recent finding was that the mutations in one of the Gag cleavage sites resulted in a 50% reduction in viral infectivity (Lee et al., 2009). Thus new avenues in antiretroviral therapy research would be to investigate compounds that inhibit cleavage of Gag at this particular site.

CONCLUSION

The frequency of HIV-1 specific CD4⁺ T cell responses is much lower than HIV-1 specific CD8⁺ T cell responses in untreated pediatric and adult infection. This can be expected given that CD4⁺ T cells are not the primary mediators of the cellular response against viral infections. However, very recent data has revealed that CD4 T cells can exert a cytotoxic effect in addition to their helper activity. SIV studies revealed that Gag and Nef specific CD4 T cells were able to kill virus infected macrophages, which resulted in lower viral set point (Sacha et al, 2009). A very recent longitudinal study conducted on untreated acutely HIV-1 infected subjects found CD4 T cells that killed virus infected cells by secreting granzyme. An early in infection resulted in controlled infection and slower progression to full blown AIDS (Soghoian et al, 2012). Further characterization of CD4⁺ T cell activity in long term non progressor controllers utilizing more sensitive and precise technology has the potential to shed light on correlates of immune protection that can in turn guide the development of protective vaccines.

In addition, characterization of CD4⁺ and CD8⁺ responses in our subjects to other chronic infections such as HCV, EBV, CMV and TB (currently approximately 30% of HIV positive subjects in South Africa are co infected with TB) would be an important comparison to HIV-1 specific immune responses. Characterizing CD4⁺ T cell responses on a per epitope level in models of viral control versus uncontrolled viral replication would provide insight on the immune

status of our cohorts. Given their exposure to a higher number of pathogens compared to subjects in developed countries, our cohorts may have a greater state of immune activation; it is therefore worthwhile studying how this heightened state of activation modulates the immune response to HIV.

A current limitation in the field is the lack of guidelines in establishing cut-off values in the Elispot and ICS assays. The generally accepted approach for a response to be positive in Elispot assay is that the test wells must have at least 50 SFCs/10⁶ PBMC and be twice the background signal (Karlsson A. C., 2003). For the ICS assay, the background response in HIV-1 negative subjects is measured and the standard deviation calculated. A response that was 2SD above the mean background (post correction for non specific response as detected in unstimulated control wells of the test subjects) was considered positive (Karlsson A. C., 2003). Our criteria for a positive response here was 3SD above background of the unstimulated control wells, ensuring that robust responses were evaluated. This discrepancy highlights a need for standardizing the method to define negative responses as these assays have become essential in screening vaccine candidates.

There is accumulating evidence that the effectiveness of the immune response against HIV-1 is influenced by population genetics, and given that clade C infections account for the highest number of HIV-1 infections worldwide (Sub-Saharan Africa, India and Southeast Asia), it is imperative that basic science research be conducted on clade C HIV-1 infection (Kawashima et al., 2009,

Kiepiela et al., 2007, Payne et al., 2009, UNAIDS, 2008). Since the prevalence of HIV-1 is highest in developing countries that are unable to provide ARVs to all infected patients, the development of a vaccine is critical (Letvin, 2006).

Our studies have contributed to HIV vaccine development by identifying immunodominant HIV-1 epitopes and characterized the cytokine secreting profile of HIV-1 specific CD4 T cells. MHC class II tetramers and polychromatic flow cytometry can be used to study low frequency HIV responses in greater detail, which also contributes to our comprehension of the immune system.

From the findings listed in this thesis, our data support the idea that the qualitative (parameters are not yet fully defined) and not quantitative differences in CD4 T cell functions differentiate effective from dysfunctional HIV-1 specific CD4 T cell help.

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