CHARACTERIZATION OF HIV SPECIFIC T CELL RESPONSES DURING ACUTE AND EARLY HIV-1 SUBTYPE C INFECTION

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A thesis submitted to the faculty of Health Sciences, School of Pathology, University of Witwatersrand, Johannesburg, South Africa in fulfilment of the requirements for the Degree of Doctor of Philosophy in Immunology
DECLARATION

I, Mandla Dennis Mlotshwa declare that this thesis is my own, unaided work. It has been submitted for the Degree of Doctor of Philosophy in Immunology at the University of Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at this or any other university.

________________________________________  __________________________
Mandla Mlotshwa                           Date
DEDICATION

I dedicate this work to my late father, Micah Mlotshwa, and late brothers, Nhlanhla Mlotshwa and Bongani Mlotshwa. To my mother and sisters, Bongekile Mlotshwa and Thanduxolo Mlotshwa, thank you for your continuous love and support during the course of my studies. To my lovely daughter, Akhona “Zamaphondla” Mlotshwa, you have been a pillar of my strength and I am eternally grateful for your presence in my life.
ABSTRACT

It is not known what kind of T cell immunity is required to be effective at containing viral replication and abating HIV-1 disease progression. While most studies have examined HIV-1 specific T cell responses in chronic infection, consideration of how these responses evolve during acute and early stages of HIV-1 infection and their influence on viral set point and the course of disease represent important information for understanding potential vaccine induced T cell immunity. To this end, a cohort of 53 individuals identified during acute HIV-1 subtype C infection was followed over the first year of infection to: a) determine whether the magnitude and breadth of HIV-1 specific T cell responses at 3 month post infection were correlated with viral set point at 12 months, b) characterize the hierarchy of HIV-1 specific T cell responses and associated temporal patterns of responses related with viral set point and disease progression, c) study the impact of the polyfunctional nature of CD8+ T cell profiles during acute/early HIV-1 infection that provide for selection of CTL escape mutations, and d) study the dynamics and kinetics of plasma mediators of apoptosis (Fas, TNF-RII and TRAIL) during acute/early HIV-1 infection and associate with viral set point and disease progression. Comprehensive T cell recognition patterns across the complete HIV-1 subtype C proteome were measured using the IFN-\( \gamma \) ELISPOT assay. The polyfunctional nature of CD8+ T cells was measured by simultaneous analysis of IFN-\( \gamma \), TNF-\( \alpha \), MIP-1\( \beta \), IL-2, perforin and CD107 using polychromatic flow cytometry. Plasma levels of apoptosis mediators-Fas, TNF-RII and TRAIL were assessed using ELISA. The magnitude and breadth of IFN-\( \gamma \) ELISPOT responses at 3-months post infection were correlated with
viral set point at 12 months post infection. A strong and diverse pattern of T cell recognition was observed at 3 months post infection, with the recognition of Nef, Gag and Pol being immunodominant as early as 3 weeks post infection. Over 6 months, there was a 23% chance of an increased response to Nef for every week post infection (p=0.0024), followed by non-significant increase to Pol (4.6%) and Gag (3.2%). Responses to Env and regulatory proteins appeared to remain stable. The magnitude of T cell responses fluctuated widely over the first year of infection and three distinguishing temporal patterns of T cell recognition could be observed: persistent, lost and new. Relating these patterns of T cell recognition with disease progression showed that the proportion of persistent T cell responses were significantly higher (p=0.0037) in slow progressing (85%) compared to rapid progressing (20%) individuals. New T cell responses tended to associate with rapid progression (p=0.06) and lost responses, which were associated with autologous sequence escape (88%) had no bearing on disease progression. The median time to autologous viral escape was found to be directly associated with time to loss of IFN-γ ELISPOT responses (r=0.61, p=0.019), where 80-100% of responses that were lost occurred at an average of 14 weeks (95% C.I: 4.4-24 weeks) after epitope escape. In four of these individuals, (I) the total magnitude of epitope-specific CD8+ T cells was associated with the time of viral escape mutant selection and (II) there was no association between the polyfunctional nature of CD8+ T cells, perforin expression or memory maturation with selection of early or late viral escape mutant epitopes. In addition, the magnitude and polyfunctionality of early and late mutating epitope specific CD8+ T cells were significantly reduced (p=0.003) over time following selection of viral escape mutants. A further analysis of twenty-one
individuals for plasma levels of TRAIL, TNF-RII and Fas showed a significantly elevated levels of TNF-RII in acute/early infection and was associated with changes in viraemia and CD4+ and CD8+ T cell activation over the first year of infection. Overall, the findings presented in this thesis provides an insight on the character of T cell immunity in the containment of viraemia in acute HIV-1 infection and also highlights the rapidity of T cell evolution and the likely unpredictable nature of T cell recognition patterns during acute to early infection.
ACKNOWLEDGEMENTS

I owe my deepest thanks to all CAPRISA 002 participants who take part in this study. Without them this study would not have been possible.

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PRESENTATIONS

1. **Poster presentation**- AIDS Vaccine, International Convention Centre, Cape Town, South Africa, October 13-16, 2008-Rapid evolution of HIV-1 specific T cell responses within the first six months of subtype C HIV-1 infection.


3. **Oral presentation**- 3rd Annual CHAVI retreat, Sheraton Imperial Hotel, Durham, North Caroline, 27710, 29 Sep – 03 Oct 2007-HIV-1 specific T cell responses at acute stage of subtype C infection.

4. **Oral presentation**- Uganda AIDS Conference, Sheraton Imperial Hotel, Kampala, 7-8 Dec 2006-Detection of HIV-1 specific T cell responses within the first 3-8 weeks of subtype C infection.

5. **Poster presentation**- XVI International AIDS Conference, Toronto, Canada, 13-18 Aug 2006-Magnitude and breadth of HIV-1 specific T cell responses at 3-months post infection is inversely associated with viral load.
PUBLICATIONS

Direct publications


Associated publications


<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ACD</td>
<td>Acid Citrate Dextrose (ACD)</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
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<tr>
<td>APOBEC</td>
<td>Apolipoprotein B mRNA-editing enzyme catalytic polypeptide like 3G (APOBEC)</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell antigen binding receptor</td>
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<tr>
<td>Bcl-2</td>
<td>B cell lymphoma 2</td>
</tr>
<tr>
<td>BID</td>
<td>BH-3 interacting death domain</td>
</tr>
<tr>
<td>CA</td>
<td>Capsid protein</td>
</tr>
<tr>
<td>CAPT</td>
<td>Canadian Africa Prevention Trial Network</td>
</tr>
<tr>
<td>CAPRISA</td>
<td>Centre for AIDS Programme Research in South Africa</td>
</tr>
<tr>
<td>CCR5</td>
<td>Chemokine (C-C motif) receptor 5</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CEF</td>
<td>Cytomegalovirus, Epstein-Barr virus and Influenza virus</td>
</tr>
<tr>
<td>CRF</td>
<td>Circulating recombinant form</td>
</tr>
<tr>
<td>CTL</td>
<td>CD8 cytotoxic lymphocyte</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytotoxic T-lymphocyte antigen 4</td>
</tr>
<tr>
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<td>CXC chemokine receptor 4</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
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<td>ELISPOT</td>
<td>Enzyme immunosorbent spot</td>
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<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<td>FADD</td>
<td>Fas-associated death domain</td>
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<tr>
<td>Gag</td>
<td>Group specific antigen</td>
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<tr>
<td>Acronym</td>
<td>Abbreviation</td>
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</tr>
<tr>
<td>GATA</td>
<td>Globin transcription factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Growth macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>GEE</td>
<td>Generalized estimating equation</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>Gp</td>
<td>Glycoprotein</td>
</tr>
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<td>Human T lymphotrophic retroviruses</td>
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<td>Human leukocyte antigen</td>
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<tr>
<td>HCP5</td>
<td>HLA complex 5</td>
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<td>Interleukin</td>
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<td>Immunoglobulin</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine based activation motif</td>
</tr>
<tr>
<td>ICS</td>
<td>Intracellular cytokine staining</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
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<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IN</td>
<td>Integrase</td>
</tr>
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<td>Linkage disequilibrium</td>
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<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>LTNP</td>
<td>Long term nonprogressors</td>
</tr>
<tr>
<td>MA</td>
<td>Matrix protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>NC</td>
<td>Nucleocapsid protein (NC)</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative factor</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>Acronym/Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>----------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
</tr>
<tr>
<td>PIC</td>
<td>Pre-integration complex</td>
</tr>
<tr>
<td>PR</td>
<td>Protease</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rAd5</td>
<td>Recombinant adenovirus serotype 5</td>
</tr>
<tr>
<td>Rev</td>
<td>Regulatory of virion</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal cell derived factor 1</td>
</tr>
<tr>
<td>SFU</td>
<td>Spot forming unit</td>
</tr>
<tr>
<td>SGA</td>
<td>Single genome amplification</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian Immunodeficiency virus</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TAP</td>
<td>Transport associated with antigen</td>
</tr>
<tr>
<td>Tat</td>
<td>Trans-activator of transcription</td>
</tr>
<tr>
<td>T-bet</td>
<td>T-box family of transcription factor</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Tumour growth factor beta</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumour necrosis factor receptor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumour necrosis factor-related apoptosis inducing ligand</td>
</tr>
<tr>
<td>TRADD</td>
<td>Tumour necrosis factor receptor associated death domain</td>
</tr>
<tr>
<td>URF</td>
<td>Unique recombinant form</td>
</tr>
<tr>
<td>UNAIDS</td>
<td>United Nations Programme on HIV/AIDS</td>
</tr>
<tr>
<td>Vpu</td>
<td>Viral protein U</td>
</tr>
<tr>
<td>Vpr</td>
<td>Viral protein R</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral infectivity factor</td>
</tr>
<tr>
<td>VVTRV</td>
<td>Vif, Vpr, Tat, Rev, Vpu</td>
</tr>
<tr>
<td>ZNDR1</td>
<td>Zinc ribbon domain-containing protein</td>
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CHAPTER 1
REVIEW OF RELEVANT LITERATURE

1.0 Introduction

Human Immunodeficiency virus (HIV), a member of the lentivirus genus of *Retroviridae* family, is the causative agent of AIDS and in 2010, there were an estimated 34 million people living with HIV, 2.7 million new infections and 1.8 million AIDS-related deaths (http://www.unaids.org/globalreport/global_report.htm). HIV/AIDS was first reported among homosexual men in the United State in 1981. This syndrome, AIDS, was characterized by a marked reduction of CD4+ T cells leading to an increased susceptibility to a wide variety of opportunistic infections (*Mycobacterium tuberculosis*, human herpes virus 8, Candidiasis and *Pneumocystis carinii*) and cancers such as non-Hodgkin’s lymphoma and Kaposi’s sarcoma (http://www.unaids.org/globalreport/global_report.htm). The causal agent of AIDS was initially thought to belong to the family of human T lymphotropic retroviruses (HTLV) but differed biologically and morphologically from previous isolates, HTLV-I and HLTV-II (Barre-Sinoussi et al., 1983; Gallo et al., 1983; Levy et al., 1984). This discovery, along with the subsequent confirmatory reports in 1986 which revealed HIV as the causative agent of AIDS (Coffin et al., 1986). Despite more than two decades of intensive research in exploring the biology of HIV, AIDS continues to wreak havoc on a global scale, with a brunt of the pandemic being borne by sub-Saharan Africa. According to the Joint United Nations Programme on HIV/AIDS (UNAIDS) epidemic update in 2010 and 2011, sub-Saharan Africa account for more 68% of all new HIV infections globally (http://www.unaids.org/globalreport/global_report.htm). Although important
data from prevention studies suggest that the use of combination antiretroviral therapy (ART) as pre-exposure prophylaxis can be effective in the prevention of HIV infection (Baeten et al., 2012; Van Damme et al., 2012), the development of a safe and effective HIV-1 vaccine would be the best global solution to curb the AIDS pandemic. However, the extraordinary diversity of HIV-1, the capacity of the virus to evade immune responses, along with the lack of knowledge of what constitutes durable and effective protective immune responses against the virus, represent unprecedented challenges for vaccine development. Understanding the various aspects of HIV transmission, viral entry, host genetics, gene expression and the mechanisms by which HIV proteins manipulate host cells to facilitate virus replication and immune evasion is important to grasp so that the design of an effective HIV vaccine can be realized. The purpose of this chapter is to provide an overview of some key concepts of the immune system in the biology of HIV, host genetic factors and immune evasion strategies employed by HIV.

1.1 The human immune system

The immune system is a network of cells, tissues and organs that work together to defend the body against foreign pathogens such as bacteria, parasite and viruses. The main functions of the immune system are the recognition and subsequent elimination of foreign antigens, formation of immunologic memory, and development of tolerance to self-antigens. The immune system involves an interplay between the innate and adaptive immunity (Figure 1.1), which collectively determines the outcome to pathogenic organisms (Murphy et al., 2008).
Fig 1. Schematic representation showing different stages in the induction and regulation of the immune systems against pathogens. Pathogens can be taken up by antigen presenting cells such as Macrophages (Mφ), dendritic cells (DCs) or B cells. Antigens are presented to naïve T cells by activated DCs through MHC-TCR signalling leading to activation and expansion of antigen-specific T cells. Cytokine production and innate immune response activation involving pathogen-associated molecular patterns (PAMPs) with Toll like receptors (TLR), cytokine milieu (IL-4, IL-12, IL-18), transcription factors (T-bet, STAT-1, STAT-4, STAT-6 and GATA) lead to activation and differentiation of naïve T cells into either Th1 or Th2 cell lineage. The cellular and humoral immunity are mediated through Th1 and Th2 cell lineage respectively. Th1 cells activate macrophages and CD8+ cytotoxic T cells (CTL) through secretion of cytokines enable them to destroy pathogens or induced apoptosis in infected cells. Th2 cells activate B-cells through production of cytokine (IL-4, IL-5, IL-10, IL-13 and TGF-β) and induce B-cell differentiation, antibody class switching and affinity maturation. Th1 effector functions are characterized by killing of the ingested microbes through maximized killing efficacy of macrophages by producing inducible nitric oxide synthase (iNOS) and reactive oxygen species intermediate (ROI) and lysis of infected cells by CTL through cytokine secretions, perforin and granzymes. Th2 effector functions represent increased neutralizing antibody production, opsonization and phagocytosis. Adapted from (Thakur et al., 2012).
The immune system is anatomically organised throughout a complex of organs and tissues such as tonsil, lymph nodes, lymphatic vessels, thymus, spleens, peyer’s patches and bone marrow (Murphy et al., 2008). The primary lymphoid organs, the bone marrow and thymus are sites of haematopoiesis and clonal selection of B and T cells. The immune response begins in the secondary lymphoid organs: spleen, lymph nodes, and organized lymphoid tissues associated with mucosal surfaces including peyer’s patches, tonsils, bronchial, nasal and gut-associated lymphoid tissues.

1.1.1 Innate immunity

Innate immunity is the first line of defence against invading pathogens. Upon infections, key elements of innate immunity, most important of which are phagocytic cells and natural killer cells, may successfully limit and non-specifically clear invading pathogens by inducing acute inflammatory responses, including production of IFN-γ (Murphy et al., 2008). Cells of the innate immune system include those of the mononuclear phagocytic and polymorphonuclear cells such neutrophils, macrophages, dendritic cells, eosinophils, basophils and natural killer (NK) cells. These also include barrier mechanisms, such as epithelial cell layers that express tight cell-cell contacts and the secreted mucus layer that overlays the epithelium in the respiratory, gastrointestinal and genitourinary tracts, and the epithelial cilia. The innate immune response includes soluble proteins (cytokines, chemokines, and lipid mediators) and variety of receptors such as Toll like receptors (TLRs) that recognize a diverse array of molecular patterns foreign to mammalian organism but commonly found on pathogens (Murphy et al., 2008).
1.1.2 Adaptive immunity

Adaptive immunity comprises of a dual system consisting of humoral and cellular mediated immunity. B and T lymphocytes along with those of professional antigen presenting cells such as dendritic cells and macrophage are amongst the central elements of adaptive immunity. Adaptive immunity recognizes specific molecular structures and depends on the generation of large numbers of antigen specific receptors expressed on the surfaces of T and B cells (Murphy et al., 2008). The first step in adaptive immunity following viral infection is the priming and activation of naïve T cells by antigen presenting cells (APCs) in the lymphoid organs (Parkin and Cohen, 2001).

T cells originate from pluripotent haematopoietic stem cells in the bone marrow before maturing in the thymus (Figure 1.2). In the thymus, the thymocytes undergo a complex process involving both positive and negative selection. In particular, binding of MHC-peptide to T cell receptor (TCR) will either induce positive selection in the thymocytes, results in maturation of T cells capable of recognizing of peptides in the context of MHCs or negative selective resulting in apoptosis of autoreactive thymocytes. The cells surviving these selection processes mature into naïve T cells, which can respond to viral antigens presented as exogenous peptides in the context of self-MHC molecules on the surface of antigen presenting cells. B cells, like T cells are derived from pluripotential haematopoietic stem cells in the bone marrow, where they differentiate into pro-B cells, pre-B cells and immature B cells (Parkin and Cohen, 2001). Immature B cells are characterized by the appearance of surface IgM in the absence of IgD. Immature B cells that are not self-reactive then leave the bone marrow as transitional B cells and migrate in
the peripheral circulation, where they mature into naïve B cells. On the other hand, self-reactive immature B cells will undergo apoptosis and/or generate new B cell receptor by receptor editing or become unresponsive to antigen. The differentiation of naïve B cells into memory B cells occurs within germinal centres in secondary lymphoid organs, where activated naïve B cells undergo vigorous proliferation, somatic hypermutation of immunoglobulin variable (V) region genes, isotype switching, interaction with antigens, antigen-driven selection, and differentiation into memory B cells and plasma cells (Hokibara et al., 2000; Kelsoe, 1995).

![Diagram of B and T cell immune responses to antigen](image)

**Fig1. 2 The development of B and T cell immune responses to antigen.** First, antigen is presented to recognized by the antigen specific T or B cell leading to cell priming, activation and differentiation, which usually occurs within the specialised environment of lymphoid tissues. The cells that emerge from the thymus and bone marrow having undergone gene rearrangement are naïve T and B cells. Once receptor rearrangement has occurred, these cells able to respond to antigen and induce an immune response. Adapted from (Parkin and Cohen, 2001).
1.1.2.1 B cell-mediated (humoral) immunity

Humoral immunity involves the production of antibody molecules in response to antigen and is mediated by B cells (Murphy et al., 2008). B cells express an antigen specific membrane associated B-cell antigen-binding receptors (BCR) that is secreted upon antigen binding and activation of the B cell. B-cell antigen-binding receptors (BCR) are dimers of immunoglobulin heavy and light chains. The secreted forms of BCR are known as antibodies or immunoglobulins. There are five different types of immunoglobulins (Ig), IgM, IgD, IgE, IgA and IgG. These serve to neutralize toxins, prevent organisms adhering to mucosal surfaces, activate complement, opsonize bacteria for phagocytosis and sensitize tumour and infected cells for antibody-dependent cytotoxic attack by killer cells (Parkin and Cohen, 2001). Different classes of antibody predominate at different compartment of the body: a) immunoglobulin G (IgG), is the main antibody in the blood and tissues, b) IgM is found in the intravascular, c) IgA is found in the serum and in various body secretions, d) IgD is very small amount in the bloodstream and e) IgE is typically seen in people who are having an allergic reaction.

1.1.2.2 T cell-mediated (cellular) immunity

T cell-mediated immunity is an adaptive process of developing antigen-specific T lymphocytes to eliminate viral, bacterial and parasitic infection. Antigen specificity of T lymphocytes is based on recognition through the T cell receptor of unique antigenic peptides presented by MHC molecules on antigen presenting cells. The major class of T cells are defined by their surface expression of the αβ T cell receptor (TCR). The function of this receptor is to recognize peptide antigens presented in a complex with
MHC class I or class II molecules. T cells differentiate into several subsets, CD8+ T lymphocytes (Cytotoxic T lymphocytes) and CD4+ T lymphocytes, which act primarily to kill cells infected with intracellular microbes and CD4+ T lymphocytes (T Helper cells), which are involved in the regulation of CD8+ T cells and B cells. CD8+ T cells are highly specific for a limited number of peptide epitopes that are found on the surface of antigen presenting cells. These peptides are typically 8-11 amino acids in length and are presented to CD8+ T cells by MHC class I molecules. CD4+ T cells are critical for preservation and maintenance of functional CD8+ T cell functions (Battegay et al., 1994; Janssen et al., 2003; Matloubian et al., 1994; Shedlock and Shen, 2003; Sun and Bevan, 2003); and the formation of germinal centre reactions and affinity maturation of B cells, which are critical for the generation of neutralizing antibodies and formation of memory B cells (Crotty et al., 2003; McHeyzer-Williams and Ahmed, 1999). CD4+ T helper cells can be further subdivided into T\( _{H1} \), T\( _{H2} \), T\( _{H9} \), T\( _{H17} \), Treg and T\( _{FH} \) (Follicular T cells) subsets based on production of signature cytokines. T\( _{H1} \) cells are characterized by the production of IFN-\( \gamma \), TNF-\( \alpha \), IL-2, granulocyte macrophage colony stimulating factor (GM-CSF), and express transcriptional factor T bet, whereas T\( _{H2} \) cells predominantly secrete IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, tumour growth factor (TGF)-\( \beta \) and express transcriptional factor GATA3. For examples, T\( _{H1} \) cells are mainly involved in activating macrophages infected with intracellular pathogens whereas TH\( _2 \) cells predominantly help B cells in the induction of antibodies and can be essential in the downregulation of inflammatory responses by secretion of IL-10 (Murphy et al., 2008). T\( _{H17} \) cells predominantly produce IL-17 and IL-22 and are characterized by transcriptional factors ROR-\( \gamma t \), ROR\( \alpha \) and STAT3. Treg T helper cells can be divided into different subsets
based on the expression of FoxP3 and the production of IL-10, TGF-β and IL-35. Follicular T helper cells (T_{FH}) are specialized T helper cell subset that regulate the evolution of effector and memory B cell responses, and play a critical role in the development of autoimmune diseases (Murphy et al., 2008). T_{H}9 cells were initially characterized as a subset of Th2 cells but have recently been classified as IL-9 secreting Th9 cells.

### 1.1.2.3 Antigen presenting cells

Antigen presenting cells (APCs) are haematopoietic cells that have a specialized role in the processing and presenting of antigens on the surface of infected cells. They include dendritic cells, macrophages, B cells, veiled cells in the blood and Langerhan’s cells in the skin. The most potent types of APCs are dendritic cells (DCs), bone marrow derived cells, which are present in most tissues and are concentrated in the secondary lymphoid tissues (Murphy et al., 2008). Dendritic cells initiate an immune response by presenting the captured antigen, which is in the form of peptide-major histocompatibility complex (MHC) molecules, to naïve T cells in lymphoid tissues. Distinct T cell responses are generated depending on whether antigen is captured by DCs in peripheral tissues or directly in lymph nodes (Murphy et al., 2008). Macrophages are resident at most sites, but are actively recruited to sites of inflammation, where they engulf pathogenic microbes and use intracellular vacuoles to focus toxic effector molecules (nitric oxide, superoxide and degradative enzymes) in an effort to destroy the organisms (Murphy et al., 2008).
1.1.2.4 Antigen presentation and MHC molecules

Antigen presenting cells express high levels of major histocompatibility complex (MHC), that are required to permit recognition of processed antigen by T cell receptor (TCR) on T cells, and co-stimulatory molecules on their cell surface, that are essential for the activation of T cells (Figure 1.3). T cell receptors on the surface of cells are associated with the CD3 complex of molecules that transmit signals into the cell when antigen is bound to the T cell receptor. Interaction of the TCR/CD3 complex with antigenic peptide presented by MHC molecules provides only a partial signal for cell activation. For optimal activation, this requires additional participation of a co-stimulation such as CD80 (B7-1), CD86 (B7-2), and CD40, which bind to CD28, CTLA-4 and CD40 ligand on T cell respectively (Murphy et al., 2008). There are two important different types of MHC molecules that are involved in T cell immunity, MHC class I and class II. The main biological functions of MHC class I and class II molecules are the presentation of endogenously and exogenously processed peptides (Figure 1.4). In particular, HLA class I molecules, HLA-A, B and C, are present in most nucleated cells and synthesized in the endoplasmic reticulum, where they bind peptides in their peptide cleft. These peptides are derived from the proteins that have endogenously synthesized in the cytosol and are degraded by the action of proteasomes and presented on the surface of CD8+ T cells.

The MHC class II molecules, HLA-DR, DP and DQ, are almost exclusively expressed on the surface of antigen presenting cells and present antigenic peptides recognized by helper CD4+ T cells. In particular, MHC class II molecules are transported from the Golgi to the endosomes as complex bound to non-polymorphic invariant chain instead of
a peptide. This invariant chain is then degraded and replaced with peptides generated by vesicular acid proteases at acid pH in the endosomal compartments. MHC class II/peptide complexes also found on the surface of activated T cells and antigen presenting cells including macrophages, B cells and dendritic cells. While it has been known that endogenous and exogenous peptides were only presented by MHC class I and Class II respectively, there is some evidence to suggest that MHC class I molecules are also capable of presenting exogenous peptides through cross presentation, whereby APC present an antigen transferred from other cells.

Fig 1.3 Schematic representation showing interaction between antigen presenting cells and TCR-MHC complex. The complete TCR complex includes rearranged TCR α and β chains and the CD3γ, CD3δ, CD3ε and CD3ζ chains. The CD3 chains contain ITAMs in their cytoplasmic domains that can be phosphorylated to activate the intracellular signal cascade for T cell activation. TCR engagement by MHC-peptide in the absence and presence of co-stimulatory results in T cell anergy (A) and T cell activation (B). Polyclonal activation of T cells can be induced by superantigens, which interact outside the peptide-binding groove with the β1 chain of the class II and with all Vβ chains. Adapted from (Chaplin, 2003).
Fig 1.4  **Antigen presentation pathways by MHC class I and II molecules.** Following an infection, a complex multi-step involving the processing of endogenous and exogenous proteins leads to generation of antigenic peptides. MHC class I molecules bind antigenic peptides, which originate in the cytosol of APC as result of a multimolecular complex of proteases (proteasomes) and are transported to the endoplasmic reticulum by TAP-1 and TAP-2 (transporter associated with Antigen processing-1 and 2). MHC class II molecules bind peptides derived from intracellular proteins internalized by endocytosis. Another mechanism of presenting antigenic peptides is through cross-presentation, a process in which APCs may present an antigen transferred from other cells. This enables extracellular antigen to be presented by MHC class I and to activate CTL. Adapted from (Villadangos and Schnorrer, 2007).
1.1.2.5 Memory T cell development

During a typical immune response to an acute viral antigen, antigen specific T cells are activated in lymphoid tissues and initiate a cascade of proliferation and differentiation into effector T cells, which then migrate to the peripheral sites and coordinate viral clearance (Jameson and Masopust, 2009; Wherry and Ahmed, 2004). Most of these effector cells die after virus is cleared, leaving behind a surviving fraction that persist as long-lived memory cells. There are three stages that T cells pass through as they develop into memory cells: a period of initial activation and expansion, a contraction or death phase; the establishment and maintenance of memory (Kaech et al., 2002; Verhoeven et al., 2008). The first stage, known as the expansion phase, is initiated in the lymphoid tissues, where encounter with antigen induces naïve T cells to clonally expand and differentiate into effector T cells. Over the weeks that follow pathogen clearance, the majority of effector T cells die and this second phase is often referred to as death phase. The surviving T cells enter the third phase referred to as the memory phase, in which the number of memory T cells stabilize, and these cells are maintained for long periods of time (Kaech et al., 2002). Several models for the lineage of memory T-cell development have proposed and this illustrated in Figure 1.5 (Kaech et al., 2002). In transgenic mice, virus specific effector T cells marked with CRE/LOXP systems showed that these cells were maintained in the memory pool, which indicated that these cells were direct descendants of effector T cells (Figure 1.5A). A second approach using adoptive transfer of effector T cells showed that memory T cells can arise directly from this population (Figure 1.5B).
Fig 1.5  Models of memory T-cell differentiation. (a) In this model, naïve T cells can bypass an effector cell stage and develop directly into memory T cells. (b) Model 2 represents a linear-differentiation pathway, whereby memory T cells are directed against descendants of effector cells inferring that memory T cell development does not occur until antigen (Ag) is removed or greatly reduced in concentration. (c) Model 3 is a variation of model 2, whereby a short and longer duration of antigenic stimulation favours the development of central and effector memory T cells respectively. (d) Model 4 represents the decreasing-potential hypothesis, which suggests that effector T-cell functions steadily decrease as a consequence of persistent antigen as seen in chronic infection. Adapted from Kaech (Kaech et al., 2002).
In addition, studies using bromodeoxyuridine and CFSE-labelling techniques to determine proliferation of effector T cells showed that the memory T cell population is not generated from a subset of effector cells that proliferate during the contraction phase, but rather, is formed directly from the effector T cells themselves (Figure 1.5C) (Kaech et al., 2002). It has also been proposed that the primary factor that distinguishes effector T cells that die from those that survive and differentiate into memory T cells is directly related to the duration and the level of antigenic stimulation to which the T cells are exposed and is known as the “decreased potential hypothesis” (Figure 1.5D) (Kaech et al., 2002). Several surface markers have been used to delineate the lineage of memory T cells and these have been largely based on the expression of CD45RA, CD45RO, CD27, CD28, CCR7 and CD62L (Appay et al., 2002; Champagne et al., 2001; Sallusto et al., 1999). For example, based on the expression patterns of CD45RA, CD27 and CCR7, the following lineage model for CD8+ T cell memory differentiation have been proposed: 

\[
\text{CD45RA}^+\text{CD27}^+\text{CCR7}^+ \Rightarrow \text{CD45RA}^-\text{CD27}^+\text{CCR7}^+ \Rightarrow \text{CD45RA}^-\text{CD27}^-\text{CCR7}^- \Rightarrow \text{CD45RA}^+\text{CD27}^-\text{CCR7}^- \Rightarrow \text{CD45RA}^-\text{CD27}^+\text{CCR7}^- 
\]

(CD4+ memory T cell lineage also appears to be heterogenic and at least five subsets have been identified in human, naïve, central memory, transitional memory, effector memory and effector T cells (Appay et al., 2008; Burgers et al., 2009; Champagne et al., 2001; Sallusto et al., 1999). Distinctions in effector functions of CD4+ and CD8+ T cells are reflected by the expression of different intracellular molecules. For instance, CCR5 and CXCR3 discriminates CD4+ T cells with a TH1 cytokine profile,
while CCR3, CCR4 and CRTh2 expression identifies CD4+ T cells with a T\(_h2\) cytokine profile (Sallusto et al., 1999; Wherry and Ahmed, 2004). In addition, expression of CCR6 and CCR4 identifies a homogenous subset of CD4+ T cells that produce IL-17 but not IFN-\(\gamma\) in humans referred to as T\(_h17\) whereas expression of CCR6 and CCR3 are composed of T\(_h1\) CD4+ T cells subset that produce both IFN-\(\gamma\) and IL-17 (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007). Effector and memory CD8+ T cells have the abilities to produce and secrete various cytokines (IFN-\(\gamma\), TNF-\(\alpha\) and IL-2) and chemokines (MIP-1\(\beta\) and MIP-1\(\alpha\)) that can be critical to the control of viral infections (Appay et al., 2002; Sallusto et al., 1999; Wherry and Ahmed, 2004). Apart from producing and secreting various cytokines and chemokines, CD8+ T cells also possess the ability to directly kill infected target cells via apoptosis or perforin (Figure 1.6).

**Fig 1.6** Mechanisms of CD8+ T cell mediated cytotoxicity via perforin/granzyme B and Fas mediated apoptosis pathways. Perforin creates pores in the membrane of target cell to enable granzyme B to enter into the cell. Granzyme activate caspases that induce apoptosis. TCR-mediated activation induces CD95L expression on the CTL. Binding of CD95 on the target cells induce sequential caspase activation leading to apoptosis. Adapted from Nijkamp 2011(Nijkamp et al., 2011)
The predominant killing pathways comprise of preformed granules containing various apoptosis inducing proteins such as perforin and granzyme A and B that are secreted via exocytosis following recognition of target cells (Nijkamp et al., 2011). Granzymes are effector molecules capable of inducing apoptosis in target cells via caspase-dependent and independent mechanisms. Granzymes enter into the target cell directly via plasma membrane pores formed by perforin or via receptor-mediated endocytosis. The other mechanisms involves the engagement of death receptors of tumour necrosis factor such Fas, TRAIL and TNF-RI/II on target cells that induces caspase dependent apoptosis (Nijkamp et al., 2011).

The next section will focus on the biology of HIV infection in relation to HIV structure, transmission, pathogenesis, disease progression, viral protein expression and anti-HIV immunity which is the core of the data that will be presented in this thesis.

1.2 The biology of HIV infection

1.2.1 HIV classification

Human immunodeficiency virus (HIV) is classified into a subgroup of retroviruses called the lentiviridae based on morphological, genetic and biological characteristics. Retroviruses are ribonucleic acid (RNA) viruses that contain reverse transcriptase, an enzyme that transcribes deoxyribonucleic acid (DNA) from RNA. HIV isolates are currently grouped into two types; HIV type 1 (HIV-1) and HIV type 2 (HIV-2), both of which derive from cross-species infections. HIV-1 is more predominant and HIV-2 is restricted to some regions of Western and Central Africa (Barre-Sinoussi et al., 1983;
Gallo et al., 1983; Sarngadharan et al., 1984). HIV-1 can be subdivided into four major groups based on the genetic sequences of gag and env genes, group M (main), group O (outlier), group N (non-M/non-O) and the recently identified group P (Ayouba et al., 2000; Gurtler et al., 1994; Plantier et al., 2009; Simon et al., 1998; Vallari et al., 2011). This diversity is largely driven by several factors including the high error rates of reverse transcriptase (Op de Coul et al., 2001; Roberts et al., 1988), rapid turnover of HIV-1 in vivo (Ho et al., 1995), host selective immune mediated pressure (Goulder and Watkins, 2004) and recombination events during replication (Temin, 1993). Group M is the most prevalent among the four groups, responsible for most of the HIV pandemic worldwide and can be further subdivided into 9 phylogenetic subtypes (A-D, F-H, J, K) (Gao et al., 2001a; Robertson et al., 2000). In contrast, HIV-1 group O and N appear to be rare and are found almost exclusively in restricted areas in the Central-West African countries, Equatorial Guinea and Cameroon.

1.2.2 HIV-1 structure and genomic organization

The structure of HIV-1 follows the typical pattern of retrovirus family, comprising a single stranded RNA genome of 9.7 kilobases (Leis et al., 1988). The HIV-1 RNA genome encodes the essential retrovirus genes gag, pol and env, as well as the regulatory genes; tat and rev; and accessory genes, vif, vpr, vpu and nef flanked by two identical long terminal repeat (LTR) regions, that have control elements required for transcription of the viral genome (Figure 1.7). The gag (group specific antigen) gene encodes for the p17 matrix protein (MA), the p24 capsid protein (CA), the p7 nucleocapsid protein (NC) and the p6 link protein. These group specific antigen proteins function together to
coordinate membrane binding and Gag-Gag lattice interaction in immature virions (Ganser-Pornillos et al., 2008). The matrix protein, p17, contains a nuclear localization domain that facilitates the nuclear transport of the viral genome and is involved in the early stages of viral replication cycle (Bukrinsky et al., 1993). The p24 capsid protein forms the conical core of viral particles and is important for proper folding of the HIV virion particles and also following entry into a new target cells (Gottlinger, 2001). The nucleocapsid protein p7 binds directly viral genome whereas the p6 link protein links the viral membrane to the viral capsid (Gottlinger, 2001). The pol gene encodes the viral enzymes, reverse transcriptase (RT), integrase (IN) and protease (PR), and other viral and cellular gene products, which are essential for viral integration into the host genome (Leis et al., 1988). The env gene encodes for the viral glycoprotein gp160, which is cleaved inside the lumen of the Golgi apparatus into the noncovalently associated proteins, surface glycoprotein gp120 and transmembrane glycoprotein, gp41 by the cellular furin-protease (Leis et al., 1988). The gp120 subunit binds to CD4 and a core-receptor either CCR5 or CXCR4 on target cells, whereas gp41 functions in viral-host membrane fusion interactions (Moore et al., 1997; Wyatt and Sodroski, 1998). Vpu (viral protein U) promotes degradation of CD4 in the endoplasmic reticulum transport of HIV-1 Env to the cell surface (Chen et al., 1993; Willey et al., 1992); and Vpr (viral protein R) participates in the nuclear transport of the HIV-1 pre-integration complex (PIC), facilitation of RT, induction of apoptosis and disruption of cell-cycle control (Heinzinger et al., 1994). Vif (viral infectivity factor) is essential for viral maturation as well as infectivity and binds apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC) proteins and excludes them from incorporation into virions (Mangeat et al., 2003). Nef
(negative factor) induces downregulation of the CD4 receptor and MHC class I molecules (Aiken et al., 1994; Miller et al., 1994). Tat regulates transcription of HIV-1 genes and is also known to downregulate the expression of Bcl-2 and MIP-1α (Klotman et al., 1991). Rev is an RNA binding protein and is involved in the export of unspliced and single spliced mRNA from the nucleus to the cytoplasm (Hope and Pomerantz, 1995).

Figure 1.7 Genomic organization of the HIV-1 proviral genome. The principle features are depicted in diagrammatic form includes the 5’ and 3’ long terminal repeats (LTR), the structural and enzymatic proteins encoded by the gag, pol and env genes. Regulatory gene products are encoded by the tat and rev genes and the major regulatory proteins are encoded by the vif, vpu, vpr and nef.

1.2.3 The life cycle of HIV-1

The life cycle of HIV-1 comprises the series of events that is initiated by sequential receptor binding of gp120, first to CD4 and then to a specific chemokine core-receptors (CCR5 or CXCR4) on the host cell surface (Alkhatib, 2009). The basic steps of HIV-1 life cycle are outlined in Figure 1.8. Upon interaction between gp120 and the host chemokine receptors, this induces Env-mediated fusion of the viral and plasma membranes, allowing the viral core to enter the host cell. The core is then uncoated in
the cytoplasm to allow for reverse transcription of the genomic RNA that yields a double stranded complementary DNA, which is then integrated to the host chromosomal DNA, thereby forming the provirus which is expressed by the host transcriptional machinery. The integrated viral genome, called proviral DNA, is then transcribed by the cellular RNA polymerase II into messenger RNA (mRNA). The integrated viral genome is flanked on both ends by long terminal repeats (LTR) made of U3, R and U5 illustrated in Figure 1.7. The LTR contains cis active elements that are vital for the viral DNA integration into the host genome and the transcription of retroviral genome (Klotman et al., 1991). The transcribed mRNA may either be spliced in preparation for translation of viral proteins or exported from the nucleus in an unspliced form for packaging into newly produced virions (Furtado et al., 1999). In the cytoplasm, mRNA is translated into HIV specific structural proteins that are integrated with the viral core particles. This is a process where double spliced RNA species are translated into the viral proteins Tat, Rev and Nef; and single spliced long length RNA is translated into Gag, Pol and Env (Goto et al., 1998). The completion of processing of polyproteins after release from the host cell induces a structural reorganization of the virion, changing from immature assembly structure to a mature and fully infectious particle. The released mature virus can then infect another host cell, starting the replication cycle over again.
1.2.4 Kinetics of viral protein expression

Several lines of evidence suggest that expression of viral gene products during the viral life cycle following primary HIV-1 occurs with different kinetics and in a distinct hierarchical order (Lichterfeld et al., 2005; Loffredo et al., 2005a; van Baalen et al., 2002;
Van Baalen et al., 1998; Yang et al., 2003b). It has been hypothesized that CD8+ T cells which recognized epitopes derived from early expression of Nef, Rev and Tat encoded by multiply spliced mRNA during the viral replication cycle might be more effective than those directed against epitopes located in the late structural proteins Gag, Pol and Env (van Baalen et al., 2002; Van Baalen et al., 1998; Yang et al., 2003b). This is supported by studies that have shown that translocation of reverse transcriptase (RT) or Gag CD8+ T cell epitopes into the early expressed Nef protein can enhance the ability of RT or Gag specific CD8+ T cell clones to suppress viral replication following *in vitro* infection of isolated human CD4+ T cells (Ali et al., 2004; van Baalen et al., 2002). Additional reports for the central role of early expressed proteins comes from studies of SIV infection, where early expression Nef and Tat did not only elicit a strong and robust total magnitude of SIV-specific CD8+ T cells but T cell epitopes directed against these proteins were the first ones to exhibit CD8+ T cell escape mutations (Allen et al., 2000; O’Connor et al., 2003). In contrast, a study by Barouch and colleague (Barouch et al., 2002) has shown that although Gag derived T cell epitopes were targeted during acute SIV infection, escape mutations in Gag were only observed in chronic infection. It has also been shown that the majority of HIV-1 infected patients develop CD8+ T cell responses against these early expressed viral proteins during acute HIV-1 infection (Cao et al., 2003). In addition, studies on CD8+ T cell responses in acute/early HIV-1 infection have demonstrated a clear hierarchical targeting of HIV-1 proteins by CTL in Nef, Gag and Pol (Gray et al., 2009; Lichterfeld et al., 2004; Masemola et al., 2004a; Streeck et al., 2007). This hierarchical targeting by CD8+ T cells are frequently associated to low set point of viral replication (Streeck et al., 2009).
1.2.5 Transmission of HIV

Transmission of HIV occurs mainly through mucosal surfaces in the genital or rectal tract, vertically by mother-to-child transmission, by blood transfusion and injecting of drug use. The most common route of transmission is through sexual intercourse accounting for more 75% of cases worldwide (Galvin and Cohen, 2004; Haaland et al., 2009; Kozlowski and Neutra, 2003). Data on in vivo transmission in SIV and HIV infection suggest that mucosal transmission can occur without epithelial cell damage of the rectal and genital mucosae (Kozlowski and Neutra, 2003; Milman and Sharma, 1994). It has also been shown in 80% of cases that mucosal transmission of HIV-1 is caused by a single variant from amongst a complex of viral quasispecies (Abrahams et al., 2009; Boeras et al., 2011; Cichutek et al., 1992; Haaland et al., 2009; Keele et al., 2008; Keele et al., 2009). The first effective immune responses by the HIV-1 infected host are directed against this transmitted founder virus and appear to occur in a relatively ordered profile (McMichael et al., 2009). After an eclipse phase, lasting a period of approximately 10 days, the progeny of the transmitted/founder virus give rise to a productive systemic infection resulting in the dissemination and seeding of the virus in all peripheral organs as well as mucosa-associated lymphoid tissues (McMichael et al., 2009). The intestinal mucosal tissue is an earliest target organ of HIV/SIV infection and studies in acute infection suggest that the vast majority of T cells that are infected and depleted are activated memory CD4+ T cells expressing CCR5 in gut-associated lymphoid tissue (GALT) (Allen et al., 2005; Guadalupe et al., 2003; Veazey et al., 1998). This observation is consistent with the notion that the majority of newly transmitted HIV strains, as well as the commonly used SIV of macaques are CCR5-tropic (Zhang et al.,...
The mucosal surface of the gastrointestinal tract (GI) is a unique anatomical and physiological site and functions as a structural and immunological barrier against microorganisms. As the GI tract is also responsible for absorption of food and nutrients during digestion, the intestinal immune homeostasis is maintained by an immunological barrier imparted by the gut-associated lymphoid tissue (GALT), which harbours the vast majority of lymphoid tissue in the body. This vital lymphoid compartment has also been shown to be a persistent viral reservoir as well as an earliest target for the HIV-1/SIV during acute infection (Guadalupe et al., 2003). The first description of the early depletion of intestinal mucosal CD4+ T cells was illustrated in SIV by Veazey et al (Veazey et al., 1998), who demonstrated that 70-95% of CD4+ T cells were lost in the jejunum, ileum and colon by 21 days post SIV-Mac239 infection. Data on studies elucidating the mechanisms underlying early SIV-associated mucosal CD4+ T cell depletion suggest that up to 60% of CD4+ T cells that are lost harbour SIV DNA by day 10 post infection, suggesting that depletion of CD4 cells is through a direct virus-mediated killing of infected CD4+ T cells (Mattapallil et al., 2005). In addition, gene expression profiles of GI tract biopsies reveal that genes associated with cell cycle regulation, lipid metabolism, and epithelial cell barrier and digestive functions are downregulated in HIV-infected individuals (Sankaran et al., 2005).

1.2.6 Mechanisms of pathogenesis in HIV-1 infection

The mechanisms for depletion of CD4+ T cells that results from HIV-1 infection is largely unresolved despite considerable progress made in unravelling the pathogenesis of AIDS. Studies that investigate the mechanism by which HIV-1 causes depletion of
CD4+ T cell counts have brought forward two major hypotheses: a) direct killing of infected target cells by HIV proteins leading to cytopathicity; and b) indirect immune mediated destruction of bystander CD4+ T cells and apoptosis due to the release of pro-apoptotic virus proteins on infected T cells (Badley et al., 2000; Gougeon, 2003; Petrovas et al., 2005).

Increasing evidence indicates that pathogenesis of HIV infection is linked to the ability of the virus to induce chronic immune activation, fuelled in part by ongoing viral replication and resulting in CD4+ T cells depletion, immune dysfunction and disease progression (Giorgi et al., 1999; Grossman et al., 2002; Hazenberg et al., 2000). This chronic immune activation is characterized by polyclonal B cell activation (Lane et al., 1983), increased T cell proliferation (Hellerstein et al., 1999), apoptosis (Gougeon et al., 1996), T cell functional impairment (Papagno et al., 2004), upregulation of chemokines and proinflammatory cytokines (Brenchley et al., 2006; Valdez and Lederman, 1997). The status of immune activation is measured by increased expression of CD38, HLA-DR and Ki67, and has been shown to be as good as a predictor of disease progression as viral load (Deeks et al., 2004; Giorgi et al., 1999; Hazenberg et al., 2003; Sousa et al., 2002, Hunt et al., 2008). Immune activation is a major determinant that distinguishes pathogenic from non-pathogenic SIV infection in different animal models (Paiardini et al., 2009; Silvestri et al., 2007; Sousa et al., 2002). In SIV-infected sooty Mangabeys and African green monkeys, the natural hosts of SIV, which do not progress to AIDS, exhibit minimal levels of systemic immune activation despite evidence of persistent high viral loads (Estes et al., 2008; Silvestri et al., 2007). In humans infected with the less pathogenic
HIV-2, experience a mild or slow disease progression and usually display significantly less immune activation than HIV-1 infected individuals (Leligdowicz et al., 2007; Sousa et al., 2002). It has been shown in HIV-1 controllers that progressive loss of CD4+ T cell in the absence of detectable viral load correlates with high levels of immune activation (Hunt et al., 2008). Emerging data also suggest that there is a direct link between immune activation in chronic HIV infection and depletion of CCR5+ CD4+ memory T cells that occurs at mucosal surfaces of the GI tract during acute HIV infection (Brenchley et al., 2004; Li et al., 2005; Mattapallil et al., 2005; Mehandru et al., 2004).

Increased plasma LPS levels, an indicator of microbial translocation, has been demonstrated in HIV-1 infection and was found to be directly associated with markers of immune activation (Brenchley et al., 2006; Hunt et al., 2008). More recently, the preferential loss of Th17, a subset of T cells (CD161+ CD4+ T cells) that are defined by the secretion of IL-17 in GI tract was reported in SIV and HIV infection (Brenchley et al., 2008; Cecchinato et al., 2008). Th17 cells are thought to be critical in defence against bacteria and fungi, especially at mucosal surfaces and also contribute to the homeostasis of enterocytes (Brenchley et al., 2008). These cells are potential targets for HIV-1 as they upregulate the CCR5 chemokine core-receptor and secrete low levels of CCR5 ligands, MIP-1α and MIP-1β (El Hed et al., 2010). Together, these data have brought some insight into the potential mechanisms that implicate microbial translocation as a cause of systemic immune activation in chronic HIV infection and thus provide a direct link between the damage to the GI tract and progression to AIDS.
1.2.7 Categories of HIV-1 Disease progression

The disease course in the majority of HIV-1 infected patients can be categorized into three main stages, acute, chronic asymptomatic and AIDS (Figure 1.9) (Fauci et al., 1996). During the acute phase of HIV-1 infection, patients experience flu-like symptoms as viral load peaks and CD4+ T cell counts decline. The peak in viraemia coincides with an expansion of immune responses, release of soluble proteins, apoptotic microparticles, multiple viral escape variants (Figure 1.10) (McMichael et al., 2009). Within a few weeks, viraemia ultimately levels to a steady-state known as the “viral set point” that typically lasts for several years in the chronic phase of infection during which patients are generally asymptomatic (Figure 1.9). During this phase, HIV-1 associated pathogenic effects persist and induce a slow but progressive loss of CD4+ T cells and impairment of the adaptive immune system. Progression of disease is characterized by the destruction of the lymphoid tissue architecture, which has been proposed to be a direct consequence of virus replication and chronic immune activation (Giorgi et al., 1999; Grossman et al., 2002; Hazenberg et al., 2000). As discussed above, there is an extensive viral replication in the gut lamina propria and submucosa and in draining lymph nodes with depletion of gut associated lymphoid tissue, where activated CCR5+ CD4+ memory T cells are present in high numbers and directly infected by virus (Brenchley et al., 2008; Brenchley et al., 2004).
Of note, it has been shown during this period of infection that the degree of viral load set point is inversely associated with the rate of disease progression (Mellors et al., 1995). This rate at which HIV-1 infected patients progress to AIDS can be categorized into (i) fast progressors who cannot control vireamia and develop AIDS from as early 6 months to three years of infection (Anzala et al., 1995; O'Brien et al., 2001), (ii) viraemic controllers, defined as having viral load between 50 and 2000 RNA copies/ml (Pereyra et al., 2008), (iii) long-term non-progressors (LNTP) who can maintain stable CD4+ T cell counts and low virus load (>or <500 RNA copies/ml) for ten or more years (Mikhail et al., 2003), (iv) elite controllers (EC), represent just 1% of HIV-1 infected persons who control vireamia below the limit of detection (<50 RNA copies/ml) without antiviral
therapy (Baker et al., 2009; Blankson et al., 2007; Deeks et al., 2004), and (v) intermediate progressors, represent the vast majority of antiretroviral untreated HIV-1 infected persons, who progress to AIDS within 10 to 15 years of infection. The progression of HIV-1 disease depends on the capacity of the host to contain virus replication and also to reconstitute the pool of memory CD4+ T cells within the gut associated lymphoid tissue (GALT) and lymph nodes.

Fig 1.10 Early events in acute HIV-1 infection following transmission of the founder virus. Adapted McMichael et al (McMichael et al., 2009).

In absence of virus containment, the destruction of GALT and lymphoid tissues persists and CD4+ T cell counts continues to drop to levels below 200 cells/µl in blood which signal the onset of AIDS and increased susceptibility to opportunistic infections. It has
been shown that this is followed by an increase in viral load as well as a decrease in anti-HIV specific CTL responses and neutralizing antibodies (Badley et al., 2000; Fauci et al., 1996; Boulder and Watkins, 2004). In the absence of antiretroviral treatment (ART), the mean time from infection to the onset AIDS-related death ranges from 4-11 years and death usually occurs within 2 years of AIDS (http://www.unaids.org/globalreport/global_report.htm).

1.2.8 Adaptive immune responses to HIV-1 infection

Adaptive immunity comprises of a dual system consisting of cell-mediated and humoral antibody responses. Over the last decade, considerable progress has been made in exploring the role of adaptive immune responses in HIV-1 infection. This section of the chapter will give an overview into current insights on the role of cellular mediated (i.e. CD8+ and CD4+ T cells) and humoral (i.e. B cells) responses, and major histocompatibility complex molecules in HIV-1 infection.

1.2.8.1 Role of CD8+ T cell responses in HIV infection

Several studies have demonstrated the important role of virus specific CD8+ cytotoxic T lymphocytes (CTL) responses in the control of HIV-1 and SIV during primary and chronic infection (Borrow et al., 1994; Geldmacher et al., 2007a; Goonetilleke et al., 2009; Kiepiela et al., 2007; Koup et al., 1994; Masemola et al., 2004a; Schmitz et al., 1999; Streeck et al., 2009). In particular, the first emerging virus specific CD8+ T cell responses during primary HIV-1 infection coincides with decline in peak vireamia and
the establishment of viral set point (Borrow et al., 1994; Koup et al., 1994). Depletion of CD8+ T cells in SIV-infected macaques results in increased vireamia (Jin et al., 1999; Matano et al., 1998; Schmitz et al., 1999). Polymorphisms in HLA class I restricting CTL responses are associated with differential HIV-1 disease outcomes (Carrington and O'Brien, 2003; Kaslow et al., 1996; Kiepiela et al., 2007; Migueles et al., 2000; O'Brien et al., 2001). Moreover, in studies of acute and chronic SIV or HIV-1 infection there is strong evidence of selection for immune escape variants within the targeted HLA class I restricted CTL epitopes (Borrow et al., 1997; Goulder and Watkins, 2004; O'Connor et al., 2002; Price et al., 1997). In most cases, the initial virus specific CD8+ T cell responses in acute HIV-1 infection are of low magnitude and narrowly directed against a limited number of epitopes, which are typically structured in a clear hierarchical order (Altfeld et al., 2006; Altfeld et al., 2001b; Gray et al., 2009; Lichterfeld et al., 2004; Yu et al., 2002). This hierarchy of virus specific CD8+ T cell responses has been proposed to be crucial for the effectiveness of immune responses identified in acute HIV-1 infection linked to a low ensuing viral set points. (Altfeld et al., 2006; Streeck et al., 2009). Analysis of the magnitude, breadth and function of virus specific CD8+ T cell responses in chronic HIV-1 infection have also provided some clues as to the specificity of CD8+ T cell responses associated with effective control of HIV replication (Betts et al., 2006; Geldmacher et al., 2007a; Kiepiela et al., 2007; Masemola et al., 2004a).

For example, preferential targeting of class I restricted CTL epitopes in Gag are strongly associated with low vireamia in early and chronic HIV-1 infection whereas Env or Nef class I restricted CTL epitopes are associated with high vireamia (Geldmacher et al.,
Depending on the relative contribution of virus specific CD8+ T cell responses to the overall magnitude within a given individuals, these responses can be classified as dominant, co-dominant or subdominant (Lichterfeld et al., 2005). It has been shown that restriction of dominant and subdominant CTL epitopes by specific HLA alleles are associated with better viral control (Frahm et al., 2006; Friedrich et al., 2007; Geldmacher et al., 2007b; Kiepiela et al., 2004; Liu et al., 2009). However, despite strong evidence for CTL responses in controlling HIV-1, most ARV-untreated HIV-1 infected individuals experience ongoing viral replication and eventually progress to AIDS. This finding has led to a hypothesis that the qualitative nature of virus specific CTL responses could be critical in achieving successful immune control of HIV. Polyfunctionality has been described as the ability of T cells to secrete effector cytokines (i.e. CD107a, IFN-γ, IL-2, TNF-α and perforin) and chemokines (MIP-1β and MIP-1α), and has been shown to be associated with CD8+ T cell mediated virus control (Almeida et al., 2009; Betts et al., 2006). Other studies have also shown that polyfunctional and/or fully differentiation, and suppressive activity of virus specific CD8+ T cell responses could constitute a correlate of protection (Addo et al., 2007; Almeida et al., 2007; Almeida et al., 2009; Barbour et al., 2009; Betts et al., 2006; Julg et al., 2010; Payne et al., 2010; Saez-Cirion et al., 2007). The observation that immunization with vaccinia virus induced a polyfunctional and phenotypically distinct CD8+ T cells responses associated with lifelong protection against smallpox further reinforces this hypothesis (Precopio et al., 2007). Moreover, a strong and robust inverse relationship between proliferative capacity of virus specific class I restricted CTL epitopes and low vireamia
was demonstrated in chronic HIV-1 infection suggesting that proliferation of T cells might play an important role in the control of viral replication (Day et al., 2007). This is further supported by a recent study of Card and colleagues (Card et al., 2012) who have shown that the proliferation of CD4+ and CD8+ T-cell directed against Gag epitopes were more frequent among HIV controllers than noncontrollers. Increasing evidence also suggest that “elite controllers” exhibit a significant proportion of virus specific CD4+ and CD8+ T cells endowed with capacity to produce IL-2 and IFN-γ than HIV-1 progressors (Owen et al., 2010; Pereyra et al., 2008). In addition, a recent study by Hersperger et al (Hersperger et al., 2010) examined CD8+ T cell responses in elite controllers and demonstrated an association between control of virus replication and increased perforin expression of terminal effector CD8+ T cell populations. Additional studies on HLA-mediated viral evolution have also shown the impact of CD8+ T cell responses on viral fitness and replication capacity (Brockman et al., 2010; Crawford et al., 2009; Miura et al., 2010; Prado et al., 2009). Brockman and colleagues recently (Brockman et al., 2010) demonstrated that CTL escape have a detrimental effect on virus fitness and that replication defects were pronounced in CTL escape epitopes restricted by B*13, B*57 and B*58 during acute HIV infection. Data on the kinetics of epitope specific CD8+ T cells suggest that antiviral activities of CD8+ T cells are attributed to the different kinetics of epitope presentation (Payne et al., 2010). For example, rapid presentation of HLA-B*2705 Gag KK10 and Pol KY9 restricted epitopes were shown to elicit earlier antiviral activity of CD8+ T cells than HLA-B*2705 Vpr VL-9 specific CD8+ T cell epitopes (Payne et al., 2010). Another study by Julg et al (Julg et al., 2010) demonstrated that antiviral activity of CD8+ T cells are enhanced in individuals targeting diverse
epitopes in Gag compared those with minimal or no Gag specific CD8+ T cell responses. Together, these data suggest that the presentation of T cell epitopes early in the viral replication cycle may be important in the containment of viraemia.

1.2.8.2 Role of CD4+ T cell responses in HIV infection

CD4+ T cells are the main target, as well as the major determinant of coordinated effective adaptive immune responses during HIV-1 infection (Chomont et al., 2009; Chun et al., 1997; Douek et al., 2002; Finzi et al., 1999; Schacker et al., 2001). The important role of virus specific CD4+ T cells have been extensively demonstrated in HIV-1, HCV and CMV infections (Gerlach et al., 1999; Kalams and Walker, 1998; Lechner et al., 2000; Rosenberg et al., 2000; Rosenberg et al., 1997). The preservation of virus specific CD4+ T cells endowed with proliferative capacity has been shown to be associated with better control of HIV-1 replication (Rosenberg et al., 2000; Rosenberg et al., 1997). Immunodominant HLA class-II restricted epitopes have been described in chronic HIV-1 infection and the highest density of CD4+ T cell epitopes were identified in Gag and Nef proteins (Kaufmann et al., 2004). More recently, the frequency of Gag specific IFN-γ CD4+ T cell responses was associated with low vireamia and high CD4+ T cell count in chronic HIV-1 subtype C infection (Ramduth et al., 2009). In addition, higher frequencies of virus specific CD4+ T cells with the abilities to produce multiple cytokines were observed in individuals with non-progressive HIV-1 infection (Kannanganat et al., 2007; Pereyra et al., 2008; Potter et al., 2007). Moreover, the preservation of central memory and activated effector memory CD4+ T cells were reported to be higher among HIV-1 controllers than viraemic and HAART treated
individuals (Potter et al., 2007). Taken together, these data support the role of CD4+ T cell responses in mediating viral control in HIV infection.

1.2.8.3 Role of Humoral responses in HIV-1 infection

While much effort has focused on exploring the mechanisms and specificity of virus specific T cell immune responses, less is known about virus specific neutralizing antibody responses. Advances in the understanding of Env structure and function have begun to shed some insight why the in vivo generation of broadly reactive neutralizing antibodies against HIV-1 have been so difficult (Kwong et al., 1998; Montefiori et al., 2007; Wyatt et al., 1998). The HIV-1 Env glycoprotein is a trimer on the virion surface with extensive N-linked glycosylation that effectively shields many conserved potential neutralization epitopes from eliciting or binding to antibodies (Kwong et al., 2012). Among the large number of antibodies generated during natural infection by HIV-1, includes those directed to a V1/V2 conformational epitope, the CD4 binding site and to outer domain glycans of gp120 surface unit and those that target the membrane proximal external region (MPER) of gp41 (Kwong et al., 2012). The recent discovery of monoclonal antibodies PG9 and PG16, binding mainly to a quaternary epitope on the V2 loop in the viral Env trimer and VRC01, directed at the CD4 binding site was shown to display a greatly enhanced breadth of neutralization and potency against different HIV-1 virus strains (Walker et al., 2009; Zhou et al., 2010). Subsequent studies by Walker and colleagues in 2011 (Walker et al., 2011) also identified several antibodies that directly target glycan (PGT121-PGT123, PGT125-PGT128, PGT130, PGT131 and PGT135-PGT137) which broadly neutralize all HIV-1 subtypes with an exception of PGT128
which neutralize 70% of viruses. The identification of these broadly neutralizing antibodies raised hopes that engaging protective humoral responses to design an efficacious vaccine against HIV is durable. Emerging data from RV 144 vaccine trial in Thailand, which showed a modest level of efficacy (31%) in reducing HIV-1 infection rates has also fostered fresh hopes that design of a protective vaccine against HIV may be achievable (Rerks-Ngarm et al., 2009).

1.2.8.4 Role of MHC molecules in HIV-1 infection

There is increasing evidence that human leukocyte antigens (HLA) molecules influence the rate of disease progression in HIV-1 infection (Altfeld et al., 2003a; Carrington and O'Brien, 2003; Goulder and Watkins, 2008; Hunt and Carrington, 2008; Stephens, 2005). HLA are a group of genes belonging to the major histocompatibility complex (MHC), which contains over 200 genes in a 4 Mb region of chromosome 6p2.1.3. HLA class I and II loci are the most polymorphic genes known in the human genome encoding cell-surface heterodimers that play an important role in antigen presentation, tolerance, self and non-self recognition (An and Winkler, 2010; Kaur and Mehra, 2009; Martin and Carrington, 2005). HLA Class I loci include HLA-A, -B and –C are involved in CD8+ cytotoxic T cell-mediated immunity, which is the primary immune responses against virus infected cells. HLA Class II loci include HLA-DR, -DP and –DQ are involved in CD4+ helper cell-mediated immunity, which activates immune responses against extracellular infection. The HLA class I alleles have been consistently identified to have a significant impact on the rate of HIV-1 disease progression (Carrington and O'Brien, 2003; Goulder and Watkins, 2008). Of the three HLA class I loci, HLA-B is the most
polymorphic, with 2605 different HLA-B molecules described, compared to 2013 and 1551 distinct HLA-A and HLA-C respectively (http://www.ebi.ac.uk/imgt/hla/stats.html). HLA allele variation differs between populations, with some exhibiting a greater diversity compared to others. The section on this part of the thesis will focus on reviewing HLA-B allele as this has been shown to have a dominant influence in mediating viral replication in HIV-1 infection. The distribution of some selected HLA-B class I alleles worldwide are shown in Figure 1.11. One of the strongest clues for the role of HLA class I alleles in mediating immune control of HIV/SIV comes from the studies that have shown a consistent association of viral control and slow disease progression with HLA*B57, B*5801, B*27, B*13 and B*51 in humans; and Mamu A*01, B*17 and B*08 in rhesus macaques (Carrington and O'Brien, 2003; Goulder and Watkins, 2008; Honeyborne et al., 2007; Kiepiela et al., 2004; Leslie et al., 2010; Prado et al., 2009). In contrast, HLA-B*5802, B*35 and B*53 class I alleles are often associated with relatively lower CD4+ T cell counts and higher viral load (Carrington and O'Brien, 2003; Gao et al., 2001b; Kiepiela et al., 2007; Ngumbela et al., 2008). Data from Elite controllers also suggest that there is an enrichment of HLA*57, B*5801, B*13 and B*81 in these individuals compared to viremic non-controllers further providing strong evidence for the role of CD8+ T cell responses restricted by these alleles in determining the rate of disease progression (Emu et al., 2008; Pereyra et al., 2008). Moreover, the observation that mutations in HLA*B57 and B*27 Gag restricted epitopes select CTL escape that impose a fitness cost on the virus and that these escape mutants are likely to revert to wild type following transmission to HLA-mismatched recipient suggests a critical role of CD8+ T cells for the initial selection of escape variants in the
transmitting individuals (Chopera et al., 2008; Feeney et al., 2004; Goepfert et al., 2008; Goulder et al., 2001b; Leslie et al., 2004). Emerging data have also demonstrated a link between the selection of escape mutations in Gag that reduce in vitro viral fitness and association of the selecting HLA class I alleles with long-term control of HIV (Matthews et al., 2008; Miura et al., 2009; Miura et al., 2010; Rousseau et al., 2008). A previous study by Altfeld and colleagues (Altfeld et al., 2006) suggest that HLA alleles associated with slow disease progression contributes strongly to the initial CD8+ T cell responses against HIV during primary infection. It was demonstrated that Elite controllers select for rare B*57/5801-Gag restricted variant epitopes associated with impaired viral replication capacity and strong CTL responses (Miura et al., 2009). In addition to HLA class I alleles that have been relatively associated with faster disease progression, there is some evidence to suggest that homozygosity at one or more HLA loci might influence progression to AIDS (Carrington and O'Brien, 2003). This stems from the hypothesis that heterozygosity at each HLA class I allele allows for presentation of a diverse range of epitopes, producing a greater breadth of T cell responses and enabling effective control of HIV replication (Carrington and O'Brien, 2003). In addition, studies on whole genome wide analysis have begun to shed some light on factors not previously reported in influencing viral control and disease progression (Dalmasso et al., 2008; Fellay et al., 2007; Le Clerc et al., 2009; Limou et al., 2009).
A study by Fellay et al. (Fellay et al., 2007) identified three single nucleotide polymorphisms (SNPs) including HLA complex 5 (HCP5), zinc ribbon domain-containing protein 1 (ZNDR1) and HLA-C within the MHC locus on chromosome 6 that were consistently associated with lower HIV-1 viral load set points in chronic infection. HCP5 is in near absolute linkage disequilibrium (LD) with HLA-B*57, which has the strong influence on HIV-1 disease progression and has been associated with low viral load (Fellay et al., 2007). The role of these SNPs are yet to be defined and warrant further investigation that would provide new insights into HIV pathogenesis.
1.2.9 Vaccine and immune responses

The challenge of developing an effective HIV vaccine globally is hindered by multifaceted factors including the high genetic diversity of HIV-1, the capacity of the virus to evade adaptive immune responses, the inability to induce broadly neutralizing antibodies, the early establishment of latent reservoirs and the lack of clear immune correlates of protection (Barouch, 2008; Barouch and Korber, 2010). Recent advances in understanding the biology of HIV-1 have greatly facilitated the current strategies for design of an HIV-1 vaccine. Most vaccine strategies are designed to either evoke potent cellular or humoral immunity to HIV-1 infection (Barouch and Korber, 2010; McMichael et al., 2009). Neutralizing antibodies are known to protect against many viruses and initial efforts to test this strategy involves purified monomeric Env gp120 immunogens in two phase III efficacy trials (Vax 003 and Vax 004) sponsored by VaxGene Biotechnology company, revealed that these vaccine candidates afforded no detectable protective efficacy, suggesting that antibodies elicited by this vaccine were insufficient to protect against HIV-1 infection in human (Flynn et al., 2005; Pitisuttithum et al., 2006).

The challenges associated with antibody-based vaccine candidates led to intense efforts in the development of novel T cell-based vaccine based on the premise that this vaccine will induce cell-mediated responses that can potentially lower virus load set point and thereby slow disease progression and reduce rates of transmission. However, early-phase 2B efficacy trials (STEP trial) utilizing replication-incompetent recombinant adenovirus serotype 5 (rAd5) vectors expressing HIV-1 Gag, Pol and Env, were unexpectedly terminated when the first planned interim analysis showed that this T cell-based vaccine appeared to be futile and failed to protect against HIV acquisition and reducing virus load.
after infection (Barouch and Korber, 2010). Latterly, encouraging data from the RV144 phase III efficacy trials in Thailand offered the first evidence of modest vaccine-induced partial protection (31%) against HIV-1 infection (Rerks-Ngarm et al., 2009). Subsequent analysis of immune correlates of risk of infection in this clinical trial by Haynes and colleague (Haynes et al., 2012) demonstrated that IgG antibodies targeting the vaccine Env gp120 V1/V2 region negatively correlated with infection risk, while IgA Env binding antibodies to Env positively correlated with infection risk. In another study, it was demonstrated that the vaccine regimen elicited antibodies that mediate antibody dependent cellular cytotoxicity (ADCC) in the majority of vaccinees and that gp120 C1 region-specific A32 like antibodies significantly contributed to the overall ADCC responses (Bonsignori et al., 2012). The identification of A32-like monoclonal antibodies (mAbs) in vaccine recipients suggests that the gp120 epitope recognized by the A32 mAb could be an immunodominant region in response to natural infection as well as upon vaccination.

One of the main challenges to vaccine development is the genetic diversity and mutability of HIV-1, and its ability to evade immune response.

1.2.10 How HIV-1 evades immune responses

Like many other viruses, HIV-1 has evolved different strategies to circumvent antiviral of innate and adaptive immune responses. A better understanding of how HIV evades the immune system can help with understanding disease mechanisms and the challenges for developing prophylactic and therapeutic vaccines. The following is an overview of three
features that HIV-1 uses to evade the immune systems: a) viral escape, b) Apoptosis and c) down-regulation of MHC molecules.

1.2.10.1 Virus escape and HIV-1 infection

One of the major factors limiting the effectiveness of virus specific CD8+ T cell responses is the propensity for HIV to evade immune recognition through sequence evolution or viral escape mutations (Allen et al., 2005; Barouch et al., 2002; Bernardin et al., 2005; Borrow et al., 1997). Most notably, selection of CTL escape variants in SIV and HIV-1 infections have been documented during both acute and chronic infections (Allen et al., 2005; Bernardin et al., 2005; Goulder and Watkins, 2004; Li et al., 2007; O'Connor et al., 2002). Such CTL escape variants have been shown to interfere with intracellular epitope processing, abrogate peptide-HLA binding or disrupt recognition of peptide/HLA complex by the T cell receptor (Allen et al., 2004; Draenert et al., 2004; Goulder and Watkins, 2004; Kelleher et al., 2001). Viruses with mutations that abrogate or interfere with CTL recognition have been shown to be preferentially selected for, suggesting that viral quasispecies continuously evolve to evade CTL recognition (Allen et al., 2005; Goulder and Watkins, 2004; O'Connor et al., 2004). For example, in some cases particular CTL escape mutations can result in loss of control of viraemia and disease progression (Borrow et al., 1997; Feeney et al., 2004; Goulder et al., 1997; Kelleher et al., 2001). On the other hand, certain CTL escape mutations can impose a significant fitness cost on the ability of the virus to replicate, thereby facilitating subsequent control of viraemia (Chopera et al., 2008; Fernandez et al., 2005; Goepfert et al., 2008; Liu et al., 2007; Martinez-Picado et al., 2006; Miura et al., 2009; Peyerl et al.,
2004; Prado et al., 2009). This phenomenon is best illustrated in long-term non-progressors and elite controllers, where both mutations within the flanking region of HLA-B*57/58:01 TW10 restricted epitope is associated with greater reduction in viral replication capacity (Miura et al., 2009; Navis et al., 2007). In addition, it has been shown that some of TW10 escape variants may be targeted by specific CTL responses (Feeney et al., 2005; Miura et al., 2009). These data suggest a dual mechanism involving both a strong CTL responses and CTL induced escape that incurs a fitness cost to the virus. There is now clear evidence supporting HIV-1 adaptation to HLA class I-mediated CTL selection pressure; and that escape and reversion represents a major driving force of HIV evolution and diversity across the genome at both the individual and population levels (Bansal et al., 2010; Berger et al., 2010; Brumme et al., 2007; Brumme et al., 2009b; Duda et al., 2009; Kawashima et al., 2009; Rousseau et al., 2008; Treurnicht et al., 2010; Wang et al., 2009). These HLA-associated CTL escape mutations have been demonstrated in both early and chronic HIV-1 infection; and across HIV subtypes (Brumme et al., 2008a; Brumme et al., 2009b; Kawashima et al., 2009; Rousseau et al., 2008; Treurnicht et al., 2010). Gag, Pol and Nef proteins were found to have the highest proportion of HLA-associated mutation sites compared to Env and other regulatory proteins (Brumme et al., 2007; Brumme et al., 2009b; Rousseau et al., 2008; Wang et al., 2009). Some of these HLA associated mutation sites have been shown to have a significant impact on viral set point and the fitness cost (Brumme et al., 2009a; Brumme et al., 2008b; Matthews et al., 2008; Rousseau et al., 2008). In particular, Rousseau et al. 2008 (Rousseau et al., 2008) identified seven HLA associated polymorphism sites that were significantly associated with low vireamia and further suggested that there was a
greater fitness cost for escape associated with these sites. Most notably, the highest fitness cost ranked in order of hierarchy was on Vpr> Gag> Rev> Pol> Nef> Vif> Tat> Env> Vpu (Rousseau et al., 2008).

1.2.10.2 Apoptosis and HIV-1 infection

Another mechanism of immune evasion is through apoptosis, which is a regulated form of programmed cell death that plays an essential role in embryogenesis, organ development, inflammation, tissue homeostasis and proliferation, and the safe and efficient clearance of deleterious cells from multi-cellular organisms. At the cellular level, this process is characterized by the redistribution of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, cellular morphological patterns such as chromatin condensation, nuclear fragmentation, cytoplasmic shrinkage, membrane blebbing, the formation of vesicles, and consequently phagocytosis by immune cells (Gougeon, 2003; Saelens et al., 2004; Strasser et al., 2000).

1.2.10.2.1 Apoptosis pathways

Apoptosis occurs through two principal pathways (Figure 1.12): the extrinsic pathway, which is mediated by death receptors of the tumour necrosis factor (TNF) receptors superfamily and the intrinsic pathway, which is mediated by Bcl-2 related proteins (Saelens et al., 2004). The extrinsic pathway is initiated by the binding of tumour necrosis factor-family death receptor ligands to their cognate death receptors such as Fas ligand (FasL), TNF-related apoptosis inducing ligand (TRAIL), TNF-receptor 1 (TNFR1)
and TNF-receptor 2 (TNFR2). Binding of tumour necrosis factor-family death receptor ligands to their respective death receptors leads to recruitment of an adapter proteins, TNFR-associated death domain (TRADD) and FADD (Fas-associated death domain, which function as molecular bridge to caspase 8. This facilitates the binding and activation of caspase 8, which in turns stimulate apoptosis via two parallel cascades: the first directly cleaves and activates caspase 3; and the second cleaves pro-apoptotic protein BID (BH3-interacting death domain). Truncated BID (tBID) then translocates to mitochondria, inducing cytochrome C release, which sequentially activates caspase 9 and 3. The intrinsic pathway is initiated by internal sensors, such as p53, which activates BH3-containing proteins and mediate the assembly of pro-apoptotic members of the Bcl2 family, including BAX and BAK, into hetero-oligomeric pores in the mitochondrial membrane. BAX and BAK facilitate the release of pro-apoptotic proteins such as cytochrome C, Smac/DIABLO and Omi/HtrA2 into the cytoplasm, whereas anti-apoptotic Bcl-2/Bcl-xL inhibits the release of these pro-apoptotic regulator proteins. BH3 containing proteins (Bad, Bim, Bid, Noxa and Puma) selectively interact with either pro-apoptotic or anti-apoptotic proteins to promote apoptosis. The release of cytochrome C into the cytosol triggers activation of caspase 3 through formation of the apoptosome, which includes APAF1 (apoptotic protease activating factor 1) and pro-caspase-9, whereas Smac and Omi/HtrA2 promote caspase activation through neutralizing the inhibitors of apoptosis proteins (Gougeon, 2003; Saelens et al., 2004; Strasser et al., 2000). There is also some evidence to suggest that the intrinsic and extrinsic pathways are linked and that molecules in each pathway can influence the other (Shedlock et al., 2008). There is an additional pathway that involves T-cell mediated cytotoxicity and
perforin-granzyme-dependent killing of target cells which induce apoptosis via either granzyme A or B (Trapani and Smyth, 2002). This effect is mediated through amplification of death signal by specific cleavage of BID, induction cytochrome C release and direct activation of caspase-3 (Barry and Bleackley, 2002; Russell and Ley, 2002; Shedlock et al., 2008).

Fig 1.12 Intrinsic and extrinsic apoptotic pathways triggered by HIV-1 proteins. Adapted from Fevrier et al (Fevrier et al., 2011).
1.2.10.2.2 Apoptosis in HIV-1 infection

While apoptosis in HIV infection may result from the effects of continuous immune activation, possibly driven by viral replication, there is considerable evidence to indicate that there are additional distinct mechanisms by which HIV induces apoptosis (Badley et al., 2000; Buenz and Badley, 2004; Cummins and Badley, 2010; Gougeon, 2003). From the host perspective, HIV-1 has evolved several mechanisms to evade the host immune system and one of the strategies is to activate apoptotic programmes that destroy immune effectors function (Andrews and Koup, 1996; Gougeon, 2003; Petrovas et al., 2005). These strategies include direct killing of infected target cells by HIV protein and cytopathicity, death of bystander cells by pro-apoptotic virus proteins that are released by infected cells, killing of HIV-specific effector cells following their recruitment to infected lymphoid tissues, and altered expression of cellular apoptosis regulatory molecules by lymphocytes and antigen presenting cells as a consequence of HIV mediated immune activation (Buenz and Badley, 2004; Gougeon, 2003). Early in vitro studies from HIV infection suggest that CD4+ and CD8+ T cells are sensitive to spontaneous apoptosis, activation induced cell death, TRAIL, FAS, TNF-R1 and TNFR-2 mediated apoptosis (de Oliveira Pinto et al., 2002; Gougeon et al., 1996; Herbeuval et al., 2005a; Petrovas et al., 2006). Data from in vivo studies suggest that the vast of majority of T cells undergoing apoptosis in SIV and HIV-1 infection are non-infected bystander cells (Finkel et al., 1995; Muro-Cacho et al., 1995). In addition, enhanced in vitro T cell apoptosis associated with in vivo pathogenicity of lentiviral infection was demonstrated in SIV-infected macaques supporting the critical role of apoptosis in disease progression (Estaquier et al., 1994; Gougeon, 2003). Other studies have also reported a direct link
between the magnitude of apoptosis and different stage of HIV disease progression and changes in apoptosis during antiretroviral therapy (Liegler et al., 1998; Prati et al., 1997). Moreover, several HIV-1 proteins (Figure 1.12), including Env, Nef, Vpr, Vif, Vpu, Tat and Rev have been shown to trigger apoptosis in both HIV-infected and uninfected cells (Arnoult et al., 2004; Buenz and Badley, 2004; Gougeon, 2003).

1.2.10.3 Downregulation of MHC molecules

Central to the function of adaptive immune responses is the presentation of antigen as epitopes of endogenous and exogenous origin by class I and II MHC molecules. Downregulation of MHC class I molecules from the surface of HIV-1 infected cells by Nef is thought to be an important factor in the ability of HIV to evade virus specific CD8+ T cell responses (Le Gall et al., 1998; Schwartz et al., 1996). Nef has been shown to interfere with the expression of MHC class I molecules on the cell surface, and infected primary cells expressing high levels of Nef are resistant to destruction by MHC class I restricted CD8+ T cell mediated lysis (Collins and Baltimore, 1999; Collins et al., 1998; Schwartz et al., 1996). Aside from downregulation of MHC class I, Nef stimulates the upregulation of FasL on infected CD4+ T cells and macrophages which triggers bystander apoptosis of CD4+ and CD8+ T cells (Badley et al., 2000; Buenz and Badley, 2004). There is also some evidence to suggest Tat might be involved in the downregulation of MHC molecules. Tat is believed to indirectly affect MHC class I presentation by inhibiting dendritic cell phagocytosis of apoptosed cells (Poggi et al., 2002).
1.3 Study Hypothesis

This PhD will examine aspects of T cell immunity that are related with immune control as well as two potential mechanisms of immune evasion. The identity of T cell immunity during early infection that would serve as a predictor of viral set point, and hence disease progression, would be important not only for understanding pathogenesis, but also providing insight into immunological markers that could be used in vaccine trials. It is not known what kind of T cell immunity is required to be effective at containing viral replication or abating disease progression. Whether it is immunodominant T cell responses to HIV or the quality and specificity of virus epitope specific CD8+ T cells is open to investigation. The rationale of these investigations is based on the need to identify CD8+ T cell responses that associate with effective immunity against HIV. This PhD thesis will address three central hypotheses:

1.3.1 That the magnitude and breadth of HIV-1 specific CD8+ T cell responses during primary infection would correlate with viral set point (Chapters 3 and 4).

1.3.2 That the multifunctional nature of CD8+ T cells during primary infection would correlate with selection of CTL escape mutants (Chapter 5).

1.3.3 That the level of apoptosis and features associated with apoptosis during primary HIV-1 infection would correlate with viral set point (Chapter 6).
1.4 Objectives

To test these hypothesis, this PhD has the following objectives:

1.4.1 To determine whether the magnitude and breadth of HIV-1 specific T cell responses at 3 months post HIV-1 infection correlate viral set point at 12 month post infection (Chapter 3).

1.4.2 To associate the dynamics and kinetics of HIV-1 specific T cell responses during the first year of infection with viral set point and disease progression (Chapter 4).

1.4.3 To study the impact of the multifunctional nature of CD8+ T cell profiles during acute/early HIV-1 infection that provide for the selection of CTL escape mutation (Chapter 5).

1.4.4 To study the dynamics and kinetics of plasma cell death inducing ligands (soluble TRAIL, TNF-RII and FAS) and associate with viral set point and disease progression (Chapter 6).

1.4.5 To delineate the sensitivity of memory and activated CD4+ and CD8+ T cells to TNF-RII and FAS induced apoptosis in HIV infected and uninfected individuals (Chapter 6).
CHAPTER 2

MATERIALS AND METHODS

2.1 Study cohorts

2.1.1 CAPRISA 002 Acute HIV infection study cohort

The data generated from this cohort are presented in chapters 3, 4, 5 and 6. Participants enrolled in this study were part of the Centre for AIDS Programme Research in South Africa (CAPRISA) 002 cohort investigating the role of viral and immunological factors in HIV disease progression during acute/early HIV-1 subtype C infection (Gray et al., 2009; van Loggerenberg et al., 2008). A cohort of 245 HIV-negative high-risk sex workers was followed over time to identify acute HIV-1 infection. The timing of infection was estimated either to be at the estimated midpoint between the last antibody negative test and first antibody positive enzyme-linked immunosorbent assay test or to be 14 days during which participants were RNA PCR positive and antibody negative as illustrated in Figure 2.1 (Karim et al., 2007). These participants were monitored clinically and virologically every week for the first month and at intervals of 1-3 months thereafter. Freshly isolated PBMC’s were used immediately in the IFN-γ ELISPOT assay at each time point and the rest of PBMC’s were cryopreserved in liquid nitrogen and plasma samples stored at -80°C until needed for further analysis. The Human Research Ethics Committees of University of KwaZulu-Natal, University of Witwatersrand and University of Cape Town institutional review boards approved this study, and all the
subjects provided written informed consent for participation in this study. Ethical clearance certificate: M121001 and M040202 (Appendix 1A and 1B, pages 199-200)

Fig. 2.1 HIV testing algorithm used to define acute HIV infection in the CAPRISA 002 cohort. Figure is Adapted from Karim et al (Karim et al., 2007).

2.1.2 CAPT 001 cohort

A cohort of HIV serodiscordant couples were recruited from the Perinatal HIV Research Unit (PHRU), Soweto, South Africa and were enrolled as a part of Canada-Africa Prevention Trial Network (CAPT) study. As part of the recruitment process, each partner of a couple was provided with independent informed consent for screening and eligibility. Heterosexual couples were defined as sexual partners of the opposite gender who are married, have been living together, or otherwise consider each other a primary
partner. One partner in the couple was infected with HIV at enrollment. For the purpose of this PhD thesis, PBMC and plasma samples isolated from twenty HIV-infected participants and ten HIV-uninfected controls were used in the apoptosis studies and the quantification of plasma markers of apoptosis in Chapter 6. The Human Research Ethics Committee of the University of Witwatersrand approved this study, and all the subjects provided written informed consent for participation in this study. Ethical clearance certificate: M121001 and M070249 (Appendix 1A and 1C, pages 199 and 201).

2.2 Methods

2.2.1 Quantification of plasma viral load, CD4+ T cell counts and HLA typing

CD4+ T cell counts were assessed using a FACScalibur flow cytometry and plasma viral load were measured using a COBAS AMPLICOR™ HIV-1 monitor test version 1.5 (Roche Diagnostic). For HLA typing, DNA was extracted from either PBMC’s or granulocytes using the Pel-Freez DNA isolation kits (Dynal, Invitrogen Corporation). Dr Debbie De Asis Rosa at the National Institute for Communicable Diseases performed high-resolution HLA class I typing and the results were provided for the analysis in this thesis. High-resolution HLA class I typing was performed by sequencing of exons 2, 3 and 4 using Atria Allele SEQR kits (Abbott Diagnostics) and sequencing-based typing (assign SBT 3.5) kits (Applied Biosystems, Foster City, CA) as previously described (Gray et al., 2009; Masemola et al., 2004b). The sequencing products were analysed using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Analysis of the resulting sequences and subsequent allele were performed using
MatchMarker Allele Identification Software (Applied Biosystems, Foster City, CA). Any ambiguities resulting from either polymorphisms outside the sequenced exons or identical heterozygote combinations were resolved using sequence-specific primers. Briefly, two systems were adopted to resolve ambiguities, the first was the design of sequencing primers to bind and sequence one of the alleles, where amplicon was available for sequencing and the second was the use of high resolution allele-specific Pel-Freez SSP-PCR-based kits (Dynal, Invitrogen Corporation).

2.2.2 Cell preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood collected in tubes containing Acid Citrate Dextrose (ACD) and layered onto Ficoll-Hypaque using density gradient centrifugation method (Amershan Pharmacia, Uppsala, Sweden). Isolated PBMCs were then counted using a Guava PCA® system (Guava Technologies, USA) and either used immediately in the ELISPOT assay or cryopreserved at 10x10^6 cells/ml in 90% heat-inactivated fetal bovine serum (Invitrogen, Paisley, United Kingdom) plus 10% dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO) and stored in liquid nitrogen until needed for further analysis. Frozen PBMC’s were thawed at 37°C in a water-bath and spun twice at 250 x g for 10 minutes, counted using a Guava PCA® system and rested in RPMI 1640 (Invitrogen, Paisley, United Kingdom) containing 10% heat in-activated fetal bovine serum and 50 U gentamicin (Invitrogen, Paisley, United Kingdom) at 37°C, 5% CO₂ incubator for 12-18 hour prior to use in ELISPOT and intracellular cytokine staining assays.
2.3 Synthetic subtype C HIV-1 peptides

Four hundred and thirty-two (432) peptide sets spanning the entire HIV-1 clade C proteome (Table 2.1 and in Appendix 1D for design of matrix pools, page 202) corresponding to gene products from the HIV-1 consensus C (Gag, Vif, Vpr and Vpu); isolate Du151 (Pol, Nef, Tat and Rev) and isolate Du179 (gp160 Env) were synthesized using 9-fluorenylmethoxy carbonyl chemistry and standard-based solid-phase techniques (Natural and Medical Sciences Institute, University of Tubingen, Tubigen, Germany). The peptide sets based on Du151 were sequences matching clade C vaccine candidates in clinical trials (Williamson et al., 2003). The estimated purities of the peptides were >80% as measured by high-performance liquid chromatography and mass spectrometry. Lyophilized individual peptides were dissolved in dimethyl sulfoxide at a concentration of 10mg/ml, further diluted in phosphate buffer saline (Invitrogen, Paisley, United Kingdom) as a working stock of 40µg/ml and stored at -80°C as previously described (Masemola et al., 2004a).

Table 2.1 The pooling of a series of peptides in a contiguous fashion that makes up the nine HIV-1 gene regions.

<table>
<thead>
<tr>
<th>Gene region</th>
<th>No. of pools</th>
<th>No. of matrix</th>
<th>No. of peptide/pool</th>
<th>Peptide length (aa)</th>
<th>Overlap (aa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gag</td>
<td>5</td>
<td>14</td>
<td>14</td>
<td>15-18</td>
<td>10</td>
</tr>
<tr>
<td>Pol</td>
<td>5*</td>
<td>24§</td>
<td>24</td>
<td>15-18</td>
<td>10</td>
</tr>
<tr>
<td>Vif</td>
<td>2</td>
<td>24§</td>
<td>12</td>
<td>15-18</td>
<td>10</td>
</tr>
<tr>
<td>Vpr</td>
<td>1</td>
<td>24§</td>
<td>11</td>
<td>15-18</td>
<td>10</td>
</tr>
<tr>
<td>Tat</td>
<td>1</td>
<td>24§</td>
<td>12</td>
<td>15-18</td>
<td>10</td>
</tr>
<tr>
<td>Rev</td>
<td>1</td>
<td>24§</td>
<td>14</td>
<td>15-18</td>
<td>10</td>
</tr>
<tr>
<td>Vpu</td>
<td>1</td>
<td>24§</td>
<td>9</td>
<td>15-18</td>
<td>10</td>
</tr>
<tr>
<td>Env</td>
<td>5</td>
<td>24§</td>
<td>24</td>
<td>15-18</td>
<td>10</td>
</tr>
<tr>
<td>Nef</td>
<td>5</td>
<td>10</td>
<td>10</td>
<td>15</td>
<td>11</td>
</tr>
</tbody>
</table>

*Pool 5 of Pol contains integrase, which is subdivided into six pools and matrixes with 6 peptides. § Pools from Pol 1-4, Vif, Vpr, Tat, Rev, Vpu and Env were arranged in a 24 matrixes with 12 peptides.
2.4 IFN-γ ELISPOT assay

HIV-1 specific T cell responses were quantified using the gamma interferon (IFN-γ) ELISPOT assay and the set of 432 overlapping peptides spanning the entire HIV-1 C proteome arranged in a pool-matrix format as described previously (Gray et al., 2009; Masemola et al., 2004a). Using this approach, regions that were rich in T cell epitopes across the entire HIV-1 proteome were identified after deconvoluting the pools/matrix reactive peptides in the initial screening and mapped to the single peptides in follow-up IFN-γ ELISPOT assays. Briefly, PBMC’s were plated at 50,000 - 100,000 cells/ml with HIV-1 peptide pools (2µg/ml) and CEF peptide pools (1µg/ml) in 96-well polyvinylidene difluoride plates (MAIP S45; Millipore, Johannesburg, South Africa) that had been coated with 5µg/ml anti-IFN-γ monoclonal antibody 1-DIK (Mabtech, Stockholm, Sweden) overnight at 4°C. Phytohemagglutinin (Calbiochem, San Diego, CA), at 4µg/ml and no peptide stimulation (medium alone) were used as a positive and negative control respectively. For quality assurance, thawed PBMC that had been tested previously for responses to a pool of optimal peptides corresponding to Cytomegalo-virus, Epstein-Barr virus and Influenza viruses (CEF) were included on the same plate as a positive quality control sample to monitor assay consistency. The plates were incubated overnight at 37°C, 5% CO₂, and developed using Nova Red substrate (Vector laboratories, CA) as previously described (Masemola et al., 2004a). Individual spots were counted with an automated CTL ImmunoSpot plate reader (Cellular Technology Ltd., Cleveland, OH) and expressed as spot forming units (sfu) per million PBMC. Responses were initially evaluated by reacting PBMC with peptides arranged in a pool-matrix format and followed with a second round ELISPOT assay on selected participants to confirm
positive responses at the single-peptide level in triplicate. The following criteria were used to define positive responses: (i) reactivities to peptide pools of $> 67$ SFU/10$^6$ PBMC after background subtraction and at least three times greater than mean background activity and (ii) a matching peptide in the matrix pool array. The data events generated using this assays are presented in Chapter 3 and 4.

2.5 Shannon entropy scores estimation

As a measure of peptide diversity in Chapter 4, the average entropy scores for all 432 peptide sets spanning the entire HIV-1 clade C were estimated as previously described (Treurnicht et al., 2010). Shannon entropy ($\sum p_i \ln p_i$, where $p$ is the probability of the $i^{th}$ form of the epitope$^i$), is an information theory measure of the uncertainty in a variable (Shannon, 1948; Yusim et al., 2002). Briefly, HIV-1 clade C sequences derived from the alignment of 20 full-length HIV-1 clade C genome from primary infection in the HIV sequence database (http://hiv.lanl.gov/components/sequence/HIV/) were used to determine the database frequency of amino acids at alignment for gp41, gp120, Gag, Nef, Rev, Vif, Vpr and Pol as described previously (Treurnicht et al., 2010).

2.6 Autologous virus sequencing

Dr Denis Chopera and Mellisa-Rose Abrahams at the University of Cape Town performed the autologous virus sequencing used in this PhD thesis. RNA was isolated from plasma samples using the Magna-Pure Compact Nucleic Extractor (Roche Diagnostics Corporation, Indianapolis, USA) and reverse transcribed using the Invitrogen
Thermoscript Reverse Transcription Kit (Invitrogen, Paisley, United Kingdom) as previously described (Abrahams et al., 2009; Choper et al., 2008). The sequencing details can be found in appendix 1E (page 203) and data events generated using these assays are presented in Chapter 4 and 5.

2.7 Plasma soluble markers of apoptosis detection by ELISA kits

Enzyme-linked immunosorbent assays (ELISA) for human soluble TRAIL and Fas (Diaclone, Besancon Ceder, France) and for TNF-RII (Hycult Biotech, Uden, The Netherlands) levels were performed in plasma using commercial ELISA kits according to manufacturer instructions (TRAIL and FAS, Diaclone, Besancon Ceder, France). Briefly, plasma samples and diluted reference standards provided on the kits were directly added in duplicates to a Microwell plate strips precoated with antibody to TRAIL, FAS and TNF-RII followed by addition of biotinylated anti-TRAIL, FAS and TNF-RII antibodies; and streptavidin-HRP solution. After washing, a ready-to-use TMB substrate solution was added to each microwell plate strips and the enzyme-substrate reaction was stopped by the addition of sulphuric or oxalic acid. Absorbance from each well was read on a spectrophotometer using 450 nm as the primary wavelength and 620 nm as the reference wavelength. Plasma samples were assayed undiluted for TRAIL or diluted 1:10 for FAS and TNF-RII. The reference standards for TRAIL, Fas and TNF-RII were diluted from 93.75 to 3000 pg/ml, 93.75 to 3000 pg/ml and 16 to 1000 pg/ml respectively. The concentrations of TRAIL, Fas and TNF-RII were interpolated using the
standard curves run in duplicate with each test (Appendix 1F, page 204). The data events for ELISA analysis are presented in Chapter 6.

2.8 Induction of apoptosis in CD4+ and CD8+ T cells

As a measure of apoptosis in Chapter 6, PBMC (1 -1.5 x 10^6) were cultured in 24- well plates in the absence or presence of plate-bound 5µg/ml anti-CD95/Fas (IgM, CH11, Beckman Coulter, South Africa), 25µg/ml anti-TNR-RII (Sigma-Aldrich, South Africa) and 50µg/ml Etoposide (Sigma-Aldrich, South Africa) for 14 to 24 hours at 37οC, 5% CO2 as previously described (Petrovas et al., 2004, de Oliveira Pinto, 2002 #95). Briefly, cells were harvested, washed and surface stained with Live/Dead® Fixable violet fluorescent reactive dye and various combination of surface antibodies in Table 2.2 (Apoptosis Panel) for 30 minutes. After fixation and permeabilization (Cytofix/Cytoperm buffer kit, BD Pharmingen), cells were intracellular stained with antibodies against active caspase-3 for 1 hour on ice. Data were collected on an LSRII flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo v8.8.6 (Tree Star, Inc, San Carlos, CA).

2.9 T cell Phenotyping and intracellular cytokine staining assay using Multicolour flow cytometry

Intracellular cytokine and surface staining of CD4+ and CD8+ T cells were performed as described previously with some modification (Lamoreaux et al., 2006). Briefly, a total of 1 – 2 x10^6 PBMC were stimulated with either HIV-1 peptides (1 µg/ml), staphylococcal
enterotoxin B (1 µg/ml), or RPMI 1640 supplemented with 10% heat in-activated fetal bovine serum and 50 U gentamicin in the presence of co-stimulatory antibodies (αCD28 and αCD49d, 1µg/ml) and brefeldin A (5 µg/ml, Sigma-Aldrich, St Louis, MO) for 6 hour at 37°C, 5% CO₂. After a 6 hour incubation, cells were washed once with phosphate buffer saline (PBS) and stained with Live/Dead® Fixable Red, Aqua or Violet stain fluorescence (Molecular Probes®, Eugene, OR) for 10 minutes at room temperature. Cells were then washed with PBS supplemented with 1% heat inactivated fetal bovine serum (1% PBS) and surface stained with a panel of fluorescently labelled antibodies 

(Table 2.2, Multifunctional Panel) for 30 minutes at room temperature. Following surface staining, cells were washed in 1% PBS, fixed with Cytofix/Cytoperm buffer kit (BD Pharmingen) for 20 minutes at room temperature and permeabilized with 1 x Perm Wash (BD Pharmingen). Cells were then incubated for 1 hour at 4°C with a cocktail of intracellular antibodies as shown in Table 2.2 (Multifunctional Panel). Electronic compensation was conducted with antibody capture beads (BD Bioscience, San Diego, CA) stained separately with individual monoclonal antibodies used in a test samples. The T cell phenotype and ICS data are presented in Chapter 5.
<table>
<thead>
<tr>
<th>Flourophores</th>
<th>Antibodies</th>
<th>Volume/µl</th>
<th>Flourophores</th>
<th>Antibodies</th>
<th>Volume/µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqua blue&lt;sup&gt;a&lt;/sup&gt;</td>
<td>V-amine</td>
<td>4µl (1:60)</td>
<td>Aqua blue&lt;sup&gt;a&lt;/sup&gt;</td>
<td>V-amine</td>
<td>4µl (1:60)</td>
</tr>
<tr>
<td>ECD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CD14</td>
<td>3µl</td>
<td>Cy7 APC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>CD14</td>
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</tr>
<tr>
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<td>3µl</td>
<td>Cy7 APC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>CD19</td>
<td>1µl</td>
</tr>
<tr>
<td>Pacific blue&lt;sup&gt;c&lt;/sup&gt;</td>
<td>CD3</td>
<td>2µl (1:10)</td>
<td>Qdot 655&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CD3</td>
<td>1µl</td>
</tr>
<tr>
<td>Cy5.5 PE&lt;sup&gt;d&lt;/sup&gt;</td>
<td>CD4</td>
<td>0.42µl</td>
<td>Cy5.5 PE&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CD4</td>
<td>0.42µl</td>
</tr>
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<td>Qdot 705&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CD8</td>
<td>1.5µl</td>
<td>Qdot 705&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CD8</td>
<td>1µl</td>
</tr>
<tr>
<td>Qdot 605&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>1.5µl</td>
<td>ECD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CD45RO</td>
<td>4µl</td>
</tr>
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<td>4µl</td>
<td>Cy5 PE&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CD27</td>
<td>5µl</td>
</tr>
<tr>
<td>Cy7 PE&lt;sup&gt;e&lt;/sup&gt;</td>
<td>CCR7</td>
<td>4µl</td>
<td>Qdot 565&lt;sup&gt;f&lt;/sup&gt;</td>
<td>CD57</td>
<td>3µl</td>
</tr>
<tr>
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<td>CD38</td>
<td>10µl</td>
<td>FITC&lt;sup&gt;e&lt;/sup&gt;</td>
<td>CD107a</td>
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<td>HLA-DR</td>
<td>7µl</td>
<td>Alexa 700&lt;sup&gt;e&lt;/sup&gt;</td>
<td>IFN-γ</td>
<td>0.25µl</td>
</tr>
<tr>
<td>FITC&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Caspase 3</td>
<td>5µl</td>
<td>APC&lt;sup&gt;e&lt;/sup&gt;</td>
<td>IL-2</td>
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<td>-</td>
<td>-</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>Cy7 PE&lt;sup&gt;e&lt;/sup&gt;</td>
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</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>PE&lt;sup&gt;e&lt;/sup&gt;</td>
<td>MIP-1β</td>
<td>0.6µl</td>
</tr>
</tbody>
</table>

<sup>a</sup>Invitrogen, Paisley, UK; <sup>b</sup>Beckman Coulter Inc., Brea, CA; <sup>c</sup>eBioscience Inc., San Diego, CA; <sup>d</sup>Caltag laboratories, Burlingame, CA; <sup>e</sup>BD Pharmigen, San Diego, CA; <sup>f</sup>BD Custom conjugation at University of Pennsylvania, Dr Michael R. Betts.
2.10 Data analysis

2.10.1 Generalized Estimation Equation (GEE)

The Generalized Estimating Equation (GEE) models for longitudinal data was performed using SAS version 9.1.3 (SAS, Institute, Inc., Cary, NC). The generalized estimating equations introduced by Liang and Zeger (Liang, 1986) and Zeger and Liang (Zeger, 1986) were used to model the frequency of HIV protein recognition using a binomial distribution and logit link, effectively modeling a logistic regression model and adjusting for repeated measures. Due to the overdispersion in the data, a Poisson regression model was not appropriate for this analysis and hence a GEE approach using a negative binomial model was used to model the rate of change of the magnitude of responses over weeks post infection in Chapter 4 (Diggle, 2002; McCullagh, 1989). A GEE model, assuming a normal distribution with an identity link and unstructured covariance for repeated measures was also fitted to log viral load and CD4+ T cell counts in order to model the association with plasma sTNF-RII, sFas and sTRAIL. A GEE approach is often used to analyse longitudinal and other correlated response data, particularly if responses are binary (Liang, 1986; Zeger, 1986) and the model fitted to the data was given: $U(\beta) = \sum_{i=1}^{N} \left( \frac{\partial \mu_i}{\partial \beta} \right)^T V_i^{-1} (y_i - \mu_i) = 0$ where $Y_i = (Y_{i1}, \ldots, Y_{in})^T$ represent the vector of $n_i$ measurements on the $i$th cluster and $X_i = (x_{i1}, \ldots, x_{ip})^T$ the $p$ vectors of independent variables (the $p$-explanatory variables) on the $i$th cluster. The mean vector of $Y_i$ is assumed to $\mu_i = h^{-1}(X_i \beta)$ where $h$ is a link function. $V_i$ is a working covariance matrix for $Y_i$, given by $V_i = \phi A_i^{1/2} R_i A_i^{-1/2}$, where $\phi$ is a scale parameter following the quasi-
likelihood approach (where the variance of $Y_{ij}$ is expressed as a known function of the expectation $\mu_{ij}$, that is, $\text{var}(Y_{ij}) = \phi g(\mu_{ij})$, where $\phi$ is treated as a nuisance parameter and the focus of quasi-likelihood is on methods for inference about $\beta$); $A_i$ is an $n_i \times n_i$ diagonal matrix with $g(\mu_{ij})$ as the $j$th diagonal element; $R_i$ is a $n_i \times n_i$ “working” correlation matrix for $Y_i$.

2.10.2 Flow cytometry acquisition and analysis

A minimum of 500,000 events were acquired per sample on a custom designed LSRII flow cytometer (BD Bioscience, San Jose, CA) using FACSDiva software. The LSRII analyser has a four-laser optical platform, blue (488nm), green (532nm), red (635nm) and violet (405nm) and is equipped with 18 fluorescence detectors, along with forward and side scatter detectors for a total capacity to measure 20 parameters (Appendix 1G, page 205). Data analysis was performed using FlowJo (Version 8.8.6, Tree Star, Inc, San Carlos, CA), Pestle (Version 1.6.2) for background subtraction and SPICE (Version 5.2) for graph formatting and frequency analysis of CD8+ T cell polyfunctional profiles. Pestle and SPICE were kindly provided by Dr M. Roederer (Vaccine Research Center, NIH, Bethesda, MD). A threshold of $> 0.05\%$ positive cytokine responses after background subtraction (unstimulated PBMC) and at least twice background were considered for analysis.
2.10.3 Statistical analysis

Statistical analysis and graphical representation were performed using Graphpad Prism version 5.0 software and InStat version 3.0. Data was expressed as a median with interquartile range or (mean ± standard deviation) and analyzed by the use of nonparametric statistics. Statistical analysis of significance was performed with either Mann-Whitney or Kruskal-Wallis analysis of variance using Dunn’s test for multiple comparisons and Fisher’s exact test for the analysis of proportion of recognition among HIV-1 proteins or between group differences. Heat maps for the proportion of HIV-1 proteins data were generated in Microsoft Excel 2008. All tests are two tailed and a p value of <0.05 was considered as statistically significant. The relationships between virus specific T cell responses, memory/functional profiles of CD4+ and CD8+ T cells, soluble apoptotic plasma markers, CD4+ T cell counts and viral load was analyzed using Spearman rank correlation test and linear regression analysis was used to determine the slope.
CHAPTER 3
CHARACTERIZATION OF HIV-SPECIFIC IFN-γ T CELL RESPONSES IN ACUTE/EARLY SUBTYPE C HIV-1 INFECTION: ASSOCIATIONS WITH VIRAL SET POINT AND DISEASE PROGRESSION.

3.1 Introduction

For over two decades, there has been a wealth of evidence to show that HIV-specific cytotoxic T cell (CTL) responses play a role during HIV-1 and SIV infection. Most notably, the following observations have been made which implicate these cells as probable effectors for imparting protective responses: (i) the resolution of peak vireamia during primary HIV-1 infection coincides with the emergence of CTL responses (Borrow et al., 1994; Goonetilleke et al., 2009; Koup et al., 1994), (ii) depletion of CD8+ T cells in SIV-infection results in elevated vireamia (Schmitz et al., 1999), (iii) polymorphisms in HLA class I restricted CTL responses are associated with differential HIV-1 disease outcomes (Goulder and Watkins, 2008) and (iv) the emergence of viral escape with CTL epitopes during acute and chronic HIV-1 or SIV infection demonstrates the effectiveness of CD8+ T cell responses to exert viral selection pressure (Goulder and Watkins, 2004). These observations are, however, confounded by the finding that, high frequencies of virus-specific CD8+ T cells are detectable both in individuals who progress rapidly to AIDS and those who spontaneously control vireamia without treatment (Frahm et al., 2004; Kiepiela et al., 2007; Masemola et al., 2004a). Defining the specificity of virus specific-CD8+ T cell responses that associates with control of viral replication during acute/early infection is important to understand from the viewpoint of understanding
pathogenesis and determining the specific nature of T cell immunity that may facilitate vaccine design and aid in immunogenicity measurements. Multiple studies have defined class I restricted CTL epitopes in most expressed gene products in HIV, but the majority of these responses have been defined either in early or chronic HIV infection (Frahm et al., 2004; Geldmacher et al., 2007a; Kiepiela et al., 2007; Masemola et al., 2004a; Zuniga et al., 2006). As discussed in chapter 1, preferential targeting of class I restricted CTL epitopes in Gag are associated with lower vireamia, whereas Env and Nef specific CD8+ T cell responses are associated with high vireamia (Geldmacher et al., 2007a; Kiepiela et al., 2007; Masemola et al., 2004a). However, there have been very few studies designed to characterized the magnitude, breadth and hierarchy of epitope CTL responses during acute/early HIV-1 infection and the association with disease progression. The data presented in this chapter describe some of the earliest T cell responses that occurs within the first 3 months of HIV-1 subtype C infection and their association with viral set point at 12 months post infection. It is hypothesized that the magnitude and breadth of HIV-specific T cell responses at 3 months postinfection would correlate with the viral set point at 12 months.

3.2 Results

3.2.1 Study subjects

Fifty-three subtype C HIV-1 infected participants enrolled into the CAPRISA 002 acute infection cohort were recruited within a median of 6 weeks post-infection (range: 2-15). Table 3.1 shows the median log_{10} RNA copies and CD4+ T cell counts at three time points during the first year of infection, showing an overall decline in vireamia of 0.376
log_{10} RNA copies/ml (p=0.045) across the cohort, with a non-significant parallel reduction of 62 CD4+ T cells/µl (p=0.07) at 40-56 weeks postinfection. The median viral load and CD4+ T cell counts at enrolment were 4.74 log_{10} RNA copies/ml (range: 2.62 – 6.74) and 494 cells/µl (range: 197 - 989) respectively. Comprehensive T cell recognition patterns of IFN-γ ELISPOT assay responses were undertaken at a median of 12 weeks postinfection (range: 10 – 17) using a panel of 432 overlapping peptide spanning the entire expressed subtype C HIV-1 genome (see section 2.3 material and methods).

Table 3.1  Clinical characteristics of the cohort at enrolment, 10 to 17, and 40 to 56 weeks post infection

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Enrolment</th>
<th>10-17 weeks post infection</th>
<th>40 to 56 weeks post infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weeks PI</td>
<td>RNA copies/ml</td>
<td>CD4 T cells/µl</td>
</tr>
<tr>
<td>Median</td>
<td>6.0</td>
<td>4.74*</td>
<td>494</td>
</tr>
<tr>
<td>95% CI limits</td>
<td>4.43-4.936</td>
<td>477-589</td>
<td>4.31-4.77</td>
</tr>
<tr>
<td>Range</td>
<td>2-15</td>
<td>2.62-6.74</td>
<td>197-989</td>
</tr>
</tbody>
</table>

*p<0.05, CI: confidence interval limits, PI: Post infection

3.2.2  The hierarchy of regions recognised at 3-months post-infection.

Figure 3.1A shows the cumulative magnitude of IFN-γ ELISPOT assay responses at 12 weeks post-infection, where Nef specific IFN-γ T cell responses were found to be highly dominant. Statistical analysis showed that the magnitude of Nef specific IFN-γ T cell responses were significantly higher than that of Gag, Env, Vif, Vpr, Tat, Rev and Vpu (p<0.01). The hierarchy of the total sum of responses was as follows: Nef (65,780
sfu/10^6 PBMC) > Pol (32, 475 sfu/10^6 PBMC) > Gag (29198 sfu/10^6 PBMC) > Env (19,585 sfu/10^6 PBMC) > Rev (16,428 sfu/10^6 PBMC) > Vif (8,825 sfu/10^6 PBMC) > Vpr (6,368 sfu/10^6 PBMC) > Vpu (2038 sfu/10^6 PBMC) > Tat (430 sfu/10^6 PBMC).

When corrected for responses per amino acid to account for density of responses per expressed gene, and on a per participant basis, the order of recognized proteins became Nef > Gag > Pol > Rev > Vif > Env > Vpr > Vpu > Tat (Figure 3.1B), with Nef being highly immunodominant.

Fig.3.1 Magnitude, frequency and hierarchy of IFN-γ ELISPOT assay responses at 3-months post-infection. (A) Cumulative magnitude of IFN-γ ELISPOT assay responses across the expressed subtype C HIV-1 genome showing statistical significance between Gag, Pol, Vif, Vpr, Tat, Rev, Vpu, Env and Nef. The magnitude is shown as the total responses of all pools for each gene region, and each point represents a response per participant measured as sfu/10^6 PBMC. Data is represented as median ± interquartile ranges of 53 participants. The number below each plot indicates the proportion of responders to each gene regions. (B) Order of corrected magnitudes of IFN-γ ELISPOT assay across the genome, calculated by dividing the total magnitude (sfu/10^6 PBMC) of the response per protein by the amino acid length for each protein and expressing that number by the proportion of responders recognizing that region per peptide.
Forty-eight of the fifth-three participants targeted epitopic regions within Nef (91%), followed by Pol (79%), Gag (68%), Env (43%), Vif (36%), Vpr (26%), Rev (17%), Vpu (11%) and Tat (6%) (Figure 3.1A). Together, these data show that HIV-1 specific IFN-γ T cell responses were directed against all viral proteins, with the majority of responses dominated and preferentially targeting Nef at 3-months post-infection.

3.2.3 Immunodominant regions recognized at 3-months post infection.

To identify the exact immunodominant epitopic regions targeted at 3-months post-infection, responses were broken down into peptide pools spanning subregions within Gag (p17, p24 and p15), Pol (Protease, Reverse transcriptase and Integrase), Env (gp120 and gp41) and Nef (Figure 3.2A and B). Within Gag, p17 (aa 1-218) and p24 (aa 219-422) subregions were frequently targeted and contributed to more than 90% of the total Gag magnitude (Figure 3.2B). Gag p17 and p24 were targeted at higher magnitude than that of p15 (aa423-495) region (Figure 3.2A) (p<0.05). Integrase (aa690-980) and reverse transcriptase (aa355-689) were the most dominant targeted subregions in Pol. Protease (aa1-175) was the least targeted subregion within Pol and contributed to less than 5% of the total magnitude in Pol (Figure 3.2B). Statistical analysis of sub-regions within Pol showed that the magnitude of responses to Integrase was significantly higher than those of peptide pools spanning Reverse transcriptase and Protease (p<0.01) and contributed to 40% of the total magnitude in Pol. Within Env, there appeared a more uniform spread of responses within gp41 (aa524-826) and gp120 (aa1-523) sub-regions, with gp120 contributing to more than 75% of the total magnitude towards Env. Within Nef, the distribution of responses was focused between amino acids 1 and 171, and less
recognition in the more hypervariable N-terminus (amino acids 172-206). Responses directed against the C-terminus (aa1-51) and Central region (aa52-171) of Nef made up more 90% of the total cumulative magnitude in Nef. This region of Nef is considered to be the most immunogenic component of HIV-1 and has been shown to be rich in T cell epitopes (Currier et al., 2006; Frahm et al., 2004; Mashishi et al., 2001).

Fig.3.2 Magnitude and frequency of IFN-γ ELISPOT assay responses in Gag, Pol, Env and Nef subregions at 3-months postinfection. (A) Cumulative magnitude of IFN-γ ELISPOT responses showing immunodominant regions in Gag (p17, p24 and p15), Pol (Protease, RT and Integrase), Env (gp41 and gp120) and Nef. (B) Pie charts depict the relative contribution of each pool to the cumulative magnitude in Gag, Pol, Env and Nef. are shown in the insert on top right in each plots.
3.2.4 Narrow and focused recognition of Nef at 3-months postinfection.

By using a peptide pool-matrix, as described in appendix 1D (page 202) and by (Masemola et al., 2004a), it was possible to identify regions within the Nef protein that were rich in T cell epitopes. Table 3.2 shows the six peptides frequently targeted in Nef, where over 70% of responses were accounted for by three peptides in the central region of Nef (aa52-171): 77RPMTYKAADFSLFFLKEKG$^{95}$, 101IHSKRRQDILDLVYYHTQG$^{119}$, and 113PGPGVRYPLTFGWCFKLV$^{147}$. The peptide corresponding to the most dominant responses (80%, 113PGPGVRYPLTFGWCFKLV$^{147}$) possessed up to six previously described CTL epitopes found in the HIV Immunology database (http://www.hiv.lanl.gov/content/sequenceHIV/mainpage.html). Based on the participants HLA background, A*2301-RYPLTFGW, B*0702-YPLTFGWCF and B*1801-YPLTFGWCF restricted epitopes were predicted to be the optimal epitopes within 113PGPGVRYPLTFGWCFKLV$^{147}$ stretch. Similarly, A*0301-ILDLWVYHT, B*1801-RRQDILDLVY, B*4403-RRQDILDLVY, Cw*0701-RRQDILDLVY and B*1302-RQDILDLVW restricted epitopes were predicted to be the optimal epitopes within 101IHSKRRQDILDLVYYHTQG$^{119}$. Within the 7RPMTYKAADFSLFFLKEKG$^{95}$ peptide stretch, B*5801-KAAFDLSFF, A*0201-AAFDLSFFL, A*6802- AAFDLSFFL and Cw*06/-08-AAFDLSFFL restricted epitopes were predicted to be the optimal epitopes. Together, these data show that narrowly focused responses to selected epitopes in Nef during acute HIV-1 subtype C infection account for the majority of the dominant IFN-γ ELISPOT responses.
Table 3.2  Highly reactive regions in Nef (aa1-171) and known epitopes within sequences.

<table>
<thead>
<tr>
<th>Reactive peptide sequence</th>
<th>Responders</th>
<th>Mean Responses ± SD, sfu/10^6 PBMC</th>
<th>Range (sfu/10^6 PBMC)</th>
<th>Described CTL epitopes in the Los Alamos HIV database (<a href="http://www.Hiv.lanl.gov/content/immunology/ctl_search">http://www.Hiv.lanl.gov/content/immunology/ctl_search</a>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EVGFPVRPQVPLRPMTYKA</td>
<td>16% (3/9)</td>
<td>554 ± 214</td>
<td>390-960</td>
<td>FPVRPQVPL (B7; B3501); RPQQVPLRPM (B4201; B8101); PQVPLRPMTY (B35); QVPLRPMTYK (A3; A11); VPLRPMTYB35; B42</td>
</tr>
<tr>
<td>RPMTYKAAFDLSFLKEKG</td>
<td>36% (9/25)</td>
<td>382 ± 320</td>
<td>110-1255</td>
<td>KAAFDLSFF (B5701; B5801); AAPDLSFL (B5703, A6802, A0201, Cw0602, Cw0802); DLSFLKEK (A3; A11)</td>
</tr>
<tr>
<td>IHSKRRQDILDLWVYYHTQG</td>
<td>24% (6/25)</td>
<td>1240 ± 1113</td>
<td>150-3915</td>
<td>RQDILDLWV (B1302); RQDILDLWVY (Cw07; B7); ILDLWYHT (A2)</td>
</tr>
<tr>
<td>WYHTQGYFPDWQNYTPGP</td>
<td>12% (3/25)</td>
<td>1567 ± 1578</td>
<td>145-3710</td>
<td>YHTQGYFPDW (B17); HTQGYFPDW (B57; B5801); TQGYFPDWQNY (B15); GYFPDWQNY (A24); YFPDWQNY (A29; B57; B5801); YFPDWQNYT (A01)</td>
</tr>
<tr>
<td>PGPGVRYPLTFGWCFKLVP</td>
<td>80% (8/10)</td>
<td>2005 ±1503</td>
<td>170-4800</td>
<td>TPGPGVRYPLF (B7; GPGVRYPLTF (B35); VRYPLTFGW (B27); RYPLTFGW (A2301; A24); YPLTFGWCF (B18; B35; B5301); LTGFWCFLK (A2)</td>
</tr>
<tr>
<td>NNCLLHPMSQHGMEDADRE</td>
<td>14% (1/7)</td>
<td>570 ± 169</td>
<td></td>
<td>No previously defined epitopes</td>
</tr>
</tbody>
</table>

Chapter 3: Association of IFN-γ T cell responses with viral set point and disease progression 73
3.2.5 HIV-1 specific IFN-γ T cell responses at 3-months postinfection are not associated with the course of vireamia in the first 12 months.

To assess whether the qualitative nature of HIV-1 specific T cell responses mounted during the first few weeks of subtype C HIV-1 infection were likely to be critical in associating with subsequent immune control of vireamia over the first 12 months of infection, an association between IFN-γ ELISPOT assay responses and changes in the viral load between 3 and 12 months were investigated. This was based on the premise that the magnitude of early HIV-1 specific T cell responses at 3 months would determine the course of vireamia. Teasing out individual viral load changes within the cohort (Table 3.1, page 68) revealed positive and negative differences in viraemia between 3 and 12 months. Figure 3.3A shows the change in log_{10} viraemia, where some individuals showed a >1-log_{10} RNA copies/ml increase in viraemia and some displayed <1-log_{10} RNA copies/ml lower viraemia. These differences were used to track the course of viraemia for each participant and relate these changes with the IFN-γ ELISPOT assay response profile for each participant. Figure 3.3B shows the total proportions of IFN-γ ELISPOT assay response across the expressed genome for each participant shown as a heat map, where no clustering of responses was evident in relation to viral load differences (Figure 3.3A). Most notably, focused responses to Nef were omnipresent regardless of the negative or positive changes in the viral load at 12 months. Figure 3.3C quantifies this lack of association with viraemia over the first year. This lack of association between viral load and responses to Gag, Env and Nef appears to be in contrast with previous findings (Geldmacher et al., 2007a; Kiepiela et al., 2007; Masemola et al., 2004a), which demonstrated an association between Gag, Env or Nef
with low and high concurrent viral load respectively. The data presented in this chapter focuses on the predictive power of the IFN-γ ELISPOT responses at 3 months with the course of viraemia and set point at 12 months.

Fig.3. 3 Association of viral load differences between 3 and 12 months with the magnitudes of ELISPOT assay responses at 3-months post-infection. (A) Tracking of log_{10} RNA copies/ml between 3 and 12 months for each of the participants arranged from negative to positive differences between the two time points. The dotted line demarcates the calculated 95% confidence limits for the mean of three log10 viral load measurements between 48 and 56 weeks postinfection (0.58 log_{10} RNA copies/ml) (Table 3.1). (B) Heat map reflecting the proportions of Gag; Pol; combined Vif, Vpr, Tat, Rev, and Vpu (VVTRV) and Env responses out of the total ELISPOT assay response across the complete proteome for each participant at 3 months. Statistical differences in the proportions of responses are shown (*, p<0.05, and **, p<0.001). (C) Spearman rank correlations between the percent responses for Gag, Pol, Env and Nef and the difference in log_{10} RNA copies/ml between 3 and 12 months.
3.2.6 Lack of correlation between HIV-1 specific IFN-γ T cell responses at 3 months with viral load set point at 12 months post-infection.

The central hypothesis of this thesis was that the magnitude and breadth of HIV-1 specific IFN-γ T cell responses measured at 3 months post-infection were correlated with viral set point at 12-months. To account for viral spikes, the mean log10 plasma RNA copies/ml at three time points around 52 weeks, ranging from 48 to 56 weeks were calculated (see appendix 2A, page 206) as viral set point at 12-months. Figure 3.4 shows plots of Spearman coefficients (r) for the total magnitude and breadth of HIV-1 specific IFN-γ T cell responses for each region at 3-months post-infection, depicting the lower and upper 95% confidence intervals. Correlating the magnitude (r, range: -0.270 to 0.1758) of HIV-1 specific IFN-γ T cell responses with the viral set point at 12 months revealed no significant association for each protein region (Figure 3.4A). Similar results were observed when the breadth (r, range: 0.0200 to 0.2734) of HIV-1 specific IFN-γ T cell responses at 3-months post-infection were correlated with the viral set point at 12-months (Figure 3.4B). These findings suggest that the magnitude and breadth of IFN-γ ELISPOT responses detected during acute/early HIV-1 subtype C infection had no impact on plasma viral set point. There were also no associations with plasma viral set point when subregions within Gag (p17, p24 and p15), Pol (Protease, reverse transcriptase and integrase), and Env (gp120 and gp41) were analysed (Figure 3.4C). Taken together, these data suggest that neither the magnitude nor breadth of HIV-1 specific T cell responses, measured at 3 months post-infection, associate with the viral set point at 12 months and that the IFN-γ ELISPOT responses at 3 months have no predictive power for viral set point.
Fig. 3.4 Correlation of the magnitude and breadth of IFN-γ ELISPOT assay responses with viral set point. Spearman correlation coefficients with 95% confidence interval for the magnitudes (A) and breadths (B) of Gag-, Pol-, Env-, and Nef-specific T cell responses with viral set point. (C) Spearman correlation coefficients with 95% confidence interval for the magnitudes of p17, p24, p15, protease, reverse transcriptase (RT), integrase, gp120 and gp41 HIV-1 encoded subregions. The number above in C plots depict the values for spearman correlation coefficient.
3.2.7 Definition of early disease profiles during the first year of HIV-1 infection.

During 15 months of follow-up, it was possible to define disease profiles in this cohort by additionally factoring in the course of absolute CD4+ T cell count and viral load changes. Figure 3.5 shows representative examples of the time course of viral load and CD4 counts in six participants showing rapid, slow and intermediate disease progression (see appendix 3.1). Rapid progression was defined as study participants who had CD4 counts below 350 cells/ml and viral loads above 100,000 RNA copies/ml on two consecutive measurements between 10-15 months post-infection (Figure 3.5A); slow progression was defined as participants having CD4 counts above 350 cells/ml and viral loads below 2000 RNA copies/ml on two consecutive measurements between 10-15 months post-infection (Figure 3.5B) and intermediate progression as those who fitted neither the rapid nor slow progression category (Figure 3.5C). By utilizing a mean of 15 ± 1.6 CD4 time points over 52 weeks post-infection to calculate CD4 slopes, it was possible to validate the usefulness of using CD4 T cell counts to define disease profiles. Figure 3.6A shows participants who were progressing slowly and spontaneous controlling viraemia below 2000 RNA copies/ml having a median positive CD4 slope compared to a median negative slope found in rapid (p<0.0071) and intermediate (p<0.05) progressors. Relating the changes in CD4 slope with viral set point at 12 months post infection showed that there was a strong relationship between loss of CD4 T cells and a high viral set point and vice versa for low viral set points (Figure 3.6B).
Rapid progressors

A

Slow progressors

B

Intermediate progressors

C

Fig. 3. Representative examples from six participants showing the kinetics of viral load and CD4 T cell counts over the first year of HIV infection in rapid (A), slow (B) and intermediate (C) disease progression. Horizontal dash lines demarcate CD4 T cell count at 350 cells/µl (blue), viral load at 2000 (Orange) and 100000 (Red) RNA copies/ml.
Fig. 3.6 Correlation of CD4 slopes with log_{10} RNA copies/ml viral set point. (A) CD4 slope differences between rapid (n=12), slow (n=8) and int (n=32) progressors. CD4 slopes were calculated using linear regression analysis in Prism and utilized a mean of 15 ± 1.6 CD4 measurements over 52 weeks. Scatter plots show median ± interquartile range limits for CD4 slopes. (B) Correlation plot of log_{10} RNA copies/ml set point with CD4 slopes.
3.2.8 Lack of association between HIV-1 specific IFN-γ T cell responses at 3 months post-infection with disease progression.

To further evaluate whether the breadth and magnitude of IFN-γ ELISPOT assay responses associated with categories of disease progression, HIV-1 specific IFN-γ T cell responses at 3-months post infection were compared between rapid, intermediate and slow progressors (see section 3.2.7 for definition, page 78). Figure 3.7A and B shows the magnitude and frequency of IFN-γ ELISPOT responses respectively, where no discernible difference in profiles of T cell responses was evident between disease categories. There was no statistically significant difference between the magnitude of Gag, Pol, VVTRV, Env and Nef specific T cell responses in rapid, intermediate and slow progressors (Figure 3.7A). This was also the case when the proportion of Gag, Pol, VVTRV, Env and Nef specific T cell responses was compared among the disease categories (Figure 3.7B). Table 3.3 A and B shows the magnitude and breadth of confirmed peptide responses found in participants who are rapidly or slowly progressing. Although there was no difference in the mean magnitude of response between rapid and slow progressors, there was a non-significant trend of increased breadth of response (rapid, 3-11 vs slow, 0-8, mean of peptide pools). Interestingly, there was no response to any peptide protein at 3 months in individual CAP269, despite virus control (Table 3.3 B). By teasing out single peptide responses in the subset of eight controllers and twelve rapid progressors, it was evident that rapid progressors possessed a diverse epitope recognition pattern, with Nef and Vif being dominant (16 and 6 peptides, respectively, out of a total 39 detected). Among the rapid progressors, only two peptides in Gag could
be identified (in individuals CAP8 and CAP210), as opposed to three of the eight in slow progressors (CAP222, CAP228 and CAP262).

Fig. 3.7 Magnitude and frequency of the IFN-γ ELISPOT assay responses in rapid, intermediate and slow progressors at 3-months post-infection. (A) The magnitude is shown as the total response of all pools in Gag, Pol, VVTRV (Vif, Vpr, Tat, Rev and Vpu), Env and Nef, and each point represents a response per participant measured as sfu/10⁶ PBMC. (B) Pie charts depicting the relative contribution of Gag, Pol, VVTRV, Env and Nef to the total magnitude of HIV-1 specific T cell responses in rapid, intermediate and slow progressors at 3-months post-infection.
It was not possible to draw any conclusions or interpret differences between slow and rapid progressors as a complete dissection of the single peptide responses was limited due to the availability of samples. Additionally, the use of consensus peptides might underestimate T cell responses, especially the variable regions within HIV genome. What was notable, however, was that 98% (52/53) of peptide responses identified in both slow and rapid progressors matched previously described CTL epitopes when the HLA allele background of each individuals were taken into account (Table 3.3 A and B). Although fine mapping of epitopes and HLA restrictions was not performed, these data imply that globally common CTL epitope responses are elicited during acute and early subtype C infection which are also found later during chronic infection.
Table 3.3A  Selected confirmed peptide responses identified in the first 12 weeks post-infection in rapid progressors

<table>
<thead>
<tr>
<th>(Disease category)</th>
<th>Total sfu/10^6 PBMC</th>
<th>Breadth</th>
<th>HLA background</th>
<th>Confirmed peptides with known previously described HLA restricted epitopes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP0063 (RP)</td>
<td>2850</td>
<td>6</td>
<td>A<em>0201/2301, B</em>4501/4501, Cw*0401/1601</td>
<td>ND</td>
</tr>
<tr>
<td>CAP0206 (RP)</td>
<td>5950</td>
<td>11</td>
<td>A<em>0301/3201, B</em>0701/4403, Cw*0201/7002</td>
<td>gp120 Env p54: &quot;STITPCRIKQINNN&quot;, Nef p32: &quot;QNYTPGKRVPYPLTF&quot;, Nef p33-34: &quot;PGFGVRYPLTGCFKLV&quot;</td>
</tr>
<tr>
<td>CAP0260 (RP)</td>
<td>1780</td>
<td>6</td>
<td>A<em>2902/4301, B</em>1503/4403, Cw*118</td>
<td>Nef p26-27: &quot;INSKQRQDILDLWYHTQG&quot;</td>
</tr>
<tr>
<td>CAP0270 (RP)</td>
<td>3115</td>
<td>5</td>
<td>A<em>0301/3002, B</em>0801/5801, Cw*0701/0701</td>
<td>Nef p20-21: &quot;RVMTYKAAFDLFLKEG&quot;</td>
</tr>
</tbody>
</table>

Median 4125 6

Range 1831-12440 3-11
Table 3.3B. Selected confirmed peptide responses identified in the first 12 weeks post-infection in slow progressors

<table>
<thead>
<tr>
<th>(Disease category)</th>
<th>Total sfu/10^6 PBMC</th>
<th>Breadth</th>
<th>HLA background</th>
<th>Confirmed peptides with known previously described HLA restricted epitopes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP0045 (SP)</td>
<td>11400</td>
<td>8</td>
<td>A<em>2301/2902, B</em>1510/4501, Cw*0602/1601</td>
<td>Vif p10: 17LQTGERWLHGLGVSI, Rev p7-8: 44WRARQGRIHISERILSTCLGR, Nef p33-34: 12PGPGVRPLTFGWCFKLVP</td>
</tr>
<tr>
<td>CAP0061 (SP)</td>
<td>345</td>
<td>8</td>
<td>A<em>6602/6802, B</em>1401/4201, Cw*0802/1701</td>
<td>ND</td>
</tr>
<tr>
<td>CAP0220 (SP)</td>
<td>3480</td>
<td>5</td>
<td>A<em>3004/7401, B</em>4201/4201, Cw*1701/1701</td>
<td>ND</td>
</tr>
<tr>
<td>CAP0228 (SP)</td>
<td>6800</td>
<td>3</td>
<td>A<em>2301/2636, B</em>4403/5101, Cw*0303/0701</td>
<td>Gag p23: 17AFSPVEIPMTAL, Vif p8: 59HLGEARLVIKTYWGL, Nef p33-34: 17PGPGVRPLTFGWCFKLVP</td>
</tr>
<tr>
<td>CAP0269 (SP)</td>
<td>0</td>
<td>0</td>
<td>A<em>0205/6802, B</em>0702/5802, Cw*06/07</td>
<td>ND</td>
</tr>
<tr>
<td>Median</td>
<td>2415</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0-11400</td>
<td>0-8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Chapter 3: Association of IFN-γ T cell responses with viral set point and disease progression
3.2.9 HLA class I allele distribution among rapid, intermediate and slow disease progression

Prior studies have shown that certain HLA class I alleles, particularly those encoded by HLA-B, are associated with slow HIV disease progression, supporting the important role HLA class I-restricted CTL responses play in controlling viral replication (Altfeld et al., 2003a; Emu et al., 2008; Kiepiela et al., 2004; Leslie et al., 2010; O'Brien et al., 2001; Pereyra et al., 2008). Among individuals with slow disease progression, an over-representation of certain HLA class I alleles has been reported, in particular, HLA-B*57, B*58:01, B*27, B*13, B*51 and B*8101 whereas HLA-B*58:02, B*35 and B*18 are more commonly associated with rapid disease progression (Emu et al., 2008; Goulder and Watkins, 2008; Kiepiela et al., 2007; Pereyra et al., 2008). To determine the potential role of certain HLA class I alleles in mediating viral control and influence the outcome of disease progression in this cohort, the distribution of HLA class I alleles were analysed in rapid (n=12), slow (n=8) and intermediate (n=32) progressors (Figure 3.8). Among the individuals with slow disease progression, HLA-B*07 supertype [B*07:02, B*39:10, B*42:01 and B*42:01] were the most common alleles (56% in slow versus 17% in rapid progressors, Fisher exact test, p=0.015) whilst HLA B*58:02 (17% in rapids versus 6% in slow progressors) and B*15:03/15:10 (29% in rapids versus 6% in slow progressors) were more frequent in individuals with rapid disease progression (Figure 3.8). One striking observation is that HLA B*57 and B*58:01 alleles, which have been consistently associated with slow disease progression and low viral load in several studies (Emu et al., 2008; Kiepiela et al., 2007; Pereyra et al., 2008) were less common in individuals with slow disease progression and measurable CD8+ T cell responses. Conversely, one of
seven participants (CAP0270) who was HLA B*58:01 in this cohort was a rapid progressor during the follow-up and the rest of individuals met the criteria for intermediate disease progression. Although the HLA*58:01 class I allele has been reported to be consistently associated with favourable clinical disease outcome (Kiepiela et al., 2007; Pereyra et al., 2008), the data presented in this chapter suggest that the presence of this allele was under represented in individuals with slow disease progression. The B*15:03 and B*15:10 alleles, which were more frequent in individuals with rapid disease progression are in agreement with previously associations of high viral load in subtype C chronic HIV-1 infection (Kiepiela et al., 2004; Leslie et al., 2010). Individuals with rapid disease progression also expressed HLA-B*58:02 allele more frequently than slow progressors.

Fig.3. Frequency of HLA class I alleles and of the IFN-γ ELISPOT assay responses among rapid (n=12), intermediate (n=32) and slow (n=8) progressors. HLA class I allele distributions are shown for B*15:03/15:10, B*07:02/39:10/42:01/81:01/39:10, B*57/58:01 and B*58:02 in rapid, intermediate and slow progressors. Fisher’s exact was used for statistical comparisons between the proportion of HLA class I allele among the groups.

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This allele is in linkage disequilibrium with Cw*06:02 and is known to be associated with rapid disease progression and high viral load in chronic subtype C HIV-1 infection (Kiepiela et al., 2004; Leslie et al., 2010; Ngumbela et al., 2008). It is also worthwhile to mention that HLA-B*07:02 contributed 6.25% to the frequency of B*07 supertype (38% for B*42:01 and B*81:01 for 12.5%) in individuals with slow disease progression and low viral load. Both B*42:01 and B*81:01 have been shown to be associated with a modest protective effect in subtype C chronic infection (Kiepiela et al., 2004; Leslie et al., 2010) and the findings in this chapter are agreement with the association between these alleles and low viral load and slow disease progression.
3.3 Discussion

The character of immunity that associates with viral control in HIV infection is unknown, despite a wealth of evidence supporting the role of virus specific CD8+ T cell responses in the control of viral replication (Borrow et al., 1994; Geldmacher et al., 2007a; Kiepiela et al., 2007; Koup et al., 1994; Masemola et al., 2004a; Zuniga et al., 2006). As the global HIV epidemic continues unabated in Sub Saharan Africa, and South Africa in particular, the need to implement a preventative vaccine in the public health sector remains a critical goal. To inform on a rational design of prophylactic vaccines and therapeutic intervention strategies to curb infection, it is important to characterize immune responses to HIV, which can provide further evidence for T cell immunity and control of viral replication. The data presented in this chapter has explored the relationship between the magnitude and breadth of HIV-1 specific T cell responses at 3-months post infection and viral load set point at 12-months. The initial hypothesis was that T cell responses during early HIV infection at 3-months would determine the course of disease. Thus, the first level of analysis consisted of comprehensive screening of IFN-γ ELISPOT responses across the expressed HIV-1 subtype C genome at 3-months post infection. The data in this chapter revealed a distinct hierarchy of targeting in Nef, Pol and Gag. The highest density of targeted epitopes in Nef were located within the highly conserved region of this protein (a.a 52-171), with responses contributing to almost one-third of the total HIV-specific T cell responses at this early time post infection. This specific region of Nef is considered to be the most immunogenic domain of HIV-1 and is rich in T cell epitopes (Currier et al., 2006; Lichterfeld et al., 2004; Masemola et al.,
Within Pol, a high density of epitopes were found in the highly conserved regions, reverse transcriptase (RT) and the C-terminal portion of integrase. The targeting of integrase contributed to 40% of the total magnitude in Pol and is in agreement with multiple ethnicity studies by Frahm et al. (Frahm et al., 2004) demonstrating that integrase was the most frequent targeted protein along with Gag and Nef. Within Gag, p17 and p24 subregions were the most targeted proteins and the conserved regions have been shown to be rich in CTL epitopes restricted by multiple HLA class I molecules (Masemola et al., 2004b). Among the structural proteins, Env was the least recognized protein, targeted at a frequency of 43%. One of the caveats to this finding was the use in this study of peptide sets based on a mixture of consensus and subtype C vaccine-matched strains (Williamson et al., 2003). This may lead to a proportion of T cell responses, especially in the more variable regions, being missed due to a lack of matching between epitopes potentially recognized and the peptide reagents used (Altfeld et al., 2003b; Barouch et al., 2010; Goonetilleke et al., 2009; Malhotra et al., 2007; Santra et al., 2010). Among the accessory and regulatory proteins, Vif and Vpr were the most frequently targeted proteins and is consistent with previous findings of Altfeld et al. (Altfeld et al., 2001a) and Addo et al. (Addo et al., 2002) demonstrating that Vif and Vpr proteins are frequently targeted by CTL in HIV infection. Taken together, the data presented in this chapter suggest that all HIV-1 proteins are targeted by the initial wave of HIV-1 specific CD8+ T cell responses to emerge upon HIV infection and that Nef is the most immunogenic HIV-1 protein. Identification of immunodominant regions in early HIV-1 subtype C infection may be important in the design and testing of candidate HIV-1 vaccines. The extent to which these early immunodominant regions
might impact on subsequent viral set point and disease progression forms the second level of analysis. The relationship between the magnitude and breadth of HIV-1 specific T cell responses in primary infection and the viral set point at 12-months post infection was further investigated. The viral set point has been defined as stable viraemia over time, and from subtype B infections, viraemia has been shown to reach a peak following transmission and then to drop to a hypothesized set point, which has been shown to be associated with the time to disease progression (Mellors et al., 1996; O'Brien et al., 1996; Schacker et al., 1998). It has been shown that a single measurement 12 to 18 months post-HIV infection was the most predictive of disease progression (Lyles et al., 2000), although others have determined set point viral loads as a composite series of measurements starting earlier, around 6 months after infection (Fraser et al., 2007). In this chapter, viral set point was measured as a composite of three measurements around 12 months, although stable viraemia may never be reached as reported previously (Vidal et al., 1998). Comprehensive analysis of IFN-γ ELISPOT responses at 3-months post infection in this study showed that neither the magnitude nor the breadth of T cell responses to any region in the expressed subtype C genome had any predictive power for the viral set point at 12 months post infection. In this study, the breadth was defined as the number of peptide pools recognized. This implies that either the IFN-γ ELISPOT assay is not able to identify relevant early responses or the character of the initial immune responses that may dictate the course of viraemia is more complex than the magnitude and breadth of responses at a static measurement. This notion is consistent with recent data from the study of Richmond et al (Richmond et al., 2011), which has found divergent specificities of CD8+ T cell responses to be associated with different functional
readout or profiles in chronic HIV infection. In the same study, it was shown that the measurement of four functional parameters (IFN-γ, MIP-1β, IL-2 and TNF-α) and proliferation expanded the breadth of the detected CD8+ T cell responses by over 3.5 fold (Richmond et al., 2011). Data from HIV-1 infected individuals with persistently low viraemia in chronic infection further suggest that multifunctional CD8+ T cell responses, with the ability to mediate up to five different effector functions in response to HIV antigens, may form the basis of a more effective CD8+ T cell response (Betts et al., 2006; Harari et al., 2004). Another caveat for this lack of association is a possible viral sequence divergence from autologous epitopes and the mixture of consensus Gag and subtype C HIV vaccine-matched peptide sets used in this study. It is possible that using peptides based on the autologous infecting strains might have yielded stronger associations than found in this study. Additionally, it is tempting to speculate that increasing viral diversity during the first year of infection would render an early static T cell measurement redundant. It has been shown that in more than 75% of acutely subtype B and C infected individuals a single virus is transmitted (Abrahams et al., 2009; Keele et al., 2008). The ensuing viral diversification that occurs after transmission, as the disease becomes established, is likely shaped by immune pressure and CTL escape (Allen et al., 2005; Brumme et al., 2009b; Rousseau et al., 2008), resulting in diverse and variant epitope changes over time. More recently, a study by Goonetilleke et al. (Goonetilleke et al., 2009) identified the first T cell responses to the transmitted/founder virus and showed that these T cell responses rapidly select escape mutations concurrently with a decrease in viraemia in acute HIV-1 infection. A subsequent study by Streeck et al. (Streeck et al., 2009) also showed in the large cohort of individuals, identified during subtype B primary
HIV-1 infection, that immunodominant patterns of virus-specific CD8+ T cell responses are strongly associated with subsequent viral set point. In the aforementioned study, a panel of 222 HLA class I matched restricted optimal epitopes were used to screen immunodominance patterns of T cell responses during primary HIV infection. The discrepancy between Streeck et al. (Streeck et al., 2009) and the data presented in this chapter may also reflect the manner in which breadth was assessed and defined. Nevertheless, the data presented in this chapter are consistent with findings of several studies, which have found no association between the total frequency of HIV specific IFN-γ T cell responses and plasma viral load in early and chronic HIV-1 infection (Addo et al., 2003; Cao et al., 2003; Frahm et al., 2004; Novitsky et al., 2003). When stratified by disease profile, the frequency of IFN-γ ELISPOT responses did not differ significantly in rapid, intermediate and slow progressors. This is similar to the data of Richmond and colleagues (Richmond et al., 2011), showing no significant differences in the frequency of IFN-γ CD8+ T cell responses in long-term nonprogressors (LTNP) and normal progressors. In contrast, Pereyra et al (Pereyra et al., 2008) has shown in individuals who spontaneously control viraemia that the breadth and magnitude of HIV-1 specific CD8+ T cell responses was lower compared to chronic progressors, and that these individuals were distinguished from chronic progressors by the preferential targeting of Gag. Miura and colleagues (Miura et al., 2010) have also demonstrated that chimeric viruses isolated from these individuals who spontaneously control viraemia during acute/early stage of infection had a reduced viral replication capacity and harboured mutations associated with drug resistance and signature of transmitted HLA-B*57/58:01. In addition, a strong CD8+ T cell responses mounted against rare HLA-B*57 restricted TW10 CTL escape
variants were detected in elite controllers suggesting a dual mechanism for durable control of HIV replication encompassing CTL escape mutations and robust CTL responses arising from variant epitopes (Miura et al., 2009). Recently, a high frequency of perforin expression in HIV-1 specific CD8+ T cells was shown to be associated with HIV elite controllers (Hersperger et al., 2011; Hersperger et al., 2010), suggesting that the quality of CD8+ T cells is important in controlling disease. Taken together, these data suggest that the quality and specificity of virus specific CD8+ T cell responses, rather the magnitude might play a critical role in the control of viral replication. In support of this possibility, an association of early-differentiated HIV-specific and total CD8+ T cell central memory phenotypes with low viral set point was observed in the first year of infection within the same cohort (Burgers et al., 2009).

Several studies show an over-representation of certain HLA class I alleles, in particular HLA B*57, B*58:01, B*27, B*13, B*51 and B*81:01, in individuals who spontaneously control viraemia (Emu et al., 2008; Kiepiela et al., 2007; Pereyra et al., 2008). Analysis of the HLA class I allele distribution in the study cohort presented in this chapter revealed that none of the previously described protective HLA alleles were enriched in individuals with slow disease progression. Although very small numbers, it is tempting to speculate that the presence of known “protective alleles” are not sufficient to confer viral control. Consistent with these observations, Emu et al. (Emu et al., 2008) demonstrated that although the protective HLA class I alleles are associated with HIV-1 specific CD8+ T cells, their presence is neither necessary nor sufficient for viral control as one-third of HIV controllers do not carry these “protective” HLA class I alleles. Parallel to these
findings is the observation that HIV-1 disease progression in HLA-B*58:01 positive individuals is influenced by immune targeting of TW10 and escape mutations at T242N residues within TW10 epitope (Chopera et al., 2011).

In conclusion, the data presented in this chapter, bring into question the utility of using the IFN-γ ELISPOT assay at one time-point for assessing the impact of T cell immunity on viral set point. It is possible that measuring more specific immune responses at the single-peptide level may provide greater insight into epitope changes that may be associated with a high or low set point. In addition, the dominance of global HIV sequence diversity and continued accumulation of immune pressure-driven mutations during the earliest stage of infection may argue against equating epitope identity with viral control and disease progression. It is also tempting to conclude that IFN-γ alone does not reflect the status of T cells that are associated with viraemia, and delineating the phenotype and multifunctionality of virus specific CD8+ T cell responses and elucidating the features that influence viral set point and sustained viral control in acute HIV-1 infection might provide insights on the important functional correlate of viral control relevant to both vaccine design and evaluation. Nevertheless, the data in this chapter demonstrated that defining these responses at a static measurement is unlikely to provide the necessary immunological insight. The next chapter will extend these observations to identify the impact of T cell changes over time.
CHAPTER 4

KINETICS OF HIV-SPECIFIC T CELL RESPONSES: ASSOCIATIONS WITH DISEASE PROGRESSION

4.1 Introduction

The previous chapter showed that there was no association between the magnitude and breadth of HIV-1 specific IFN-γ T cell responses at 3-months post infection with viral set point at 12-months. This unpredictability of early T cell responses with subsequent viral control could be a result of HIV variability resulting in epitope escape from both humoral and T cell pressure (Allen et al., 2005; Borrow et al., 1997; Goulder and Watkins, 2004; Price et al., 1997). Consistent with this observation, a number of studies in HIV-1 and SIV infection have reported a selection of CTL escape variants during both primary and chronic infections (Allen et al., 2005; Bernardin et al., 2005; Goulder and Watkins, 2004; Li et al., 2007; O'Connor et al., 2002). Emerging data from HIV, SIV and Hepatitis C (HCV) longitudinal studies also suggest that CTL responses represent a major driving force for viral evolution, with more than 50% of sequence variations across the genome attributed to CTL mediated pressure in SIV and HIV (Allen et al., 2005; Kuntzen et al., 2007; O'Connor et al., 2004). More recently, the impact of CTL pressure on shaping viral diversity at a human population level has been observed through HLA imprinting (Bhattacharya et al., 2007; Brumme et al., 2007; Brumme et al., 2009b; Duda et al., 2009; Rousseau et al., 2008) and other studies have shown that certain selected escape variants can seriously diminish viral replicative fitness (Brockman et al., 2010; Chopera et al., 2008; Leslie et al., 2004; Liu et al., 2007; Martinez-Picado et al., 2006; Miura et al.,...
2009; Miura et al., 2010; Peyerl et al., 2004). However, in other studies the selection of escape variants in chronic HIV-1 and SIV infection can result in loss of immune control and disease progression (Barouch et al., 2002; Feeney et al., 2005; Goulder et al., 1997).

Delineating these events and understanding how virus specific CD8+ T cell responses evolve in relation to autologous viral escape within the first weeks and months after infection would provide a more comprehensive profile of T cell immunity to HIV-1. Building onto this work, the current chapter extends these observations and characterizes T cell responses from as early as 3 weeks post infection and relate these T cell recognition patterns with viral sequence evolution and disease progression over time.
4.2 Results

4.2.1 Evolving frequency of HIV-1 specific T cell recognition and association with viral load

To investigate the evolving frequency and hierarchy of T cell recognition patterns over time, this chapter will first report on the IFN-γ ELISPOT responses at 3-8 weeks post infection, at which the first PBMC samples were available from study. Figure 4.1 shows diverse patterns of T cell recognition across the expressed genome, with Nef, Pol and Gag emerging as immunodominant responses by 3-8 weeks post infection. There was greater than 50% recognition of Nef by 12 weeks and by 6-month, there was 100% of recognition to one or more of the Nef peptide pools. The hierarchy of T cell recognition patterns at 3-8 weeks post infection were to Nef (75%), Pol (50%), Gag and Env (47%), Vif (38%), Vpr (32%), Rev, Vpu and Tat (12%). Using a Generalized estimating Equation (GEE, see section 2.10.1 materials and methods) model to fit the frequency of recognition, there was a 23% chance of an increased response to Nef for every week post infection (p=0.0024) over the first 6-months, followed by a non-significant increase to both Pol (4.6%) and Gag (3.2%). Responses to Env and accessory proteins remained stable over the first 6 months of infection and collectively, these data show a distinct hierarchy of the rate of responses during acute infection, with responses to Nef evolving most rapidly.

The GEE model was used to determine whether a response to a specific region was associated with increased viral load. There was a significant (p=0.0042) odds ratio of 1.86 for responses to Env for every log_{10} increase in viral load, meaning that there was an
86% chance of having an Env response for every log_{10} increase in viral load. Conversely, there was a 35% chance of having a Gag response for each log_{10} viral load increase (p=0.0796); Figure 4.2A. These results show that although responses to Env appear to remain stable over the first 6 months of infection (Figure 4.2A), recognition of this region relates to a higher rate of viral load increase and recognition of Gag with a relatively lower rate of viral load increase. Noteworthy, responses to Nef and Pol, the earliest most immunodominant regions recognized during acute infection, were not associated with any increased or decreased rates of viral load increases. When using the same model to determine if responses to a specific region was associated with CD4 T cell counts (Figure 4.2B), there was no significant association, although there appeared to be an odds ratio of 0.925 of showing decreased Env specific T cell responses for every 50 CD4+ T cell/µl increase (p=0.0586).

![Graph showing frequency of T cell recognition across the expressed subtype C HIV-1 genome](image)

**Figure 4.1** Frequency of T cell recognition across the expressed subtype C HIV-1 genome. (A) Each dotted line represents response frequencies to Nef, Pol, Gag, Env, Vif, Vpr, Rev, Vpu and Tat at 6, 12 and 24 weeks post infection (range of weeks post infection for each time point are indicated in brackets). The two fine dotted horizontal lines represent cut-offs for immunodominant (>50%) and subdominant (<25%) responses.
Correlation of the frequency of IFN-γ ELISPOT responses with changes in viral load (A) and CD4+ T cell counts (B) over time. The GEE model was used to determine the association of the frequency of IFN-γ ELISPOT responses with viral load and CD4+ T cell counts. The fitted dotted horizontal lines in A and B represent an odd ratio of 1.

### 4.2.2 Evolving magnitude of HIV-1 specific T cell responses

In terms of magnitude, the immunodominance of Nef was further highlighted in Figure 4.3A, where a longitudinal analysis showed Nef to have a significantly higher magnitude of response over time than any other protein. As shown in chapter 3, responses directed against Nef made up more than 30% of the total cumulative T cell response relative to other proteins and were proportionally higher in magnitude than to other regions at any time point analysed (Figure 4.3B). As the magnitude of responses to each peptide region was not normally distributed, a negative binomial model was used (see section 2.10.1 materials and methods) to model the rate of change in the magnitude of each response over weeks post infection, while also adjusting for repeated measures. Figure 4.3C shows that the magnitude of Gag recognition was the only response which had a significant positive slope (p=0.0096), where a unit increase in weeks post-infection led to
a log 0.00364 increase in the slope of responses to Gag in the first 6 months post-infection.

Fig. 4.3 Magnitude, proportions and hierarchy of IFN-γ ELISPOT assay responses. (A) Cumulative magnitude of IFN-γ ELISPOT responses across the HIV-1 proteome at 6 (3-8), 12 (12-15) and 24 (20-26) weeks post infection. Data is represented as median ± Interquartile range limits for 33, 53 and 45 measurements at 6, 12 and 24 weeks respectively. (B) Pie charts depicting the relative contribution of Nef, Gag, Pol, Env and VVTRV (Vif, Vpr, Tat, Rev, Vpu) to the total magnitude of HIV-1 specific T cell responses at 6 (3-8), 12 (12-15) and 24 (20-26) weeks post infection. (C) Rate of change in the magnitude of HIV-1 specific T cell responses across the entire expressed genome over 3-26 weeks postinfection.
There was a non-significant increase in the response to Nef (p=0.0943), with a log 0.0218 increase in the slope of responses to Nef for every week post-infection increase. The magnitude of responses to other regions showed a decline or increase in responses over time, but none showed a significant rate of change over time. Taken together, although Nef was highly immunodominant and increased over 6 months, neither the frequency nor magnitude made any impact on the rate of viral load changes. However, the later emergence of Gag over time, both in frequency and magnitude, was associated with lower rates of viral load increases in this cohort. These data provide a unique insight into the dynamic nature of T cell responses, rather than static measurements in relation to determining the course of vireamia.

### 4.2.3 Recognition patterns of HIV-1 specific T cell responses over time

Investigating T cell recognition patterns in a subset of 34 individuals at more intensive time points immediately post infection led to the identification of three distinct response profiles: (i) Lost responses: defined as a drop of 80% or more in the magnitude of the peak response over two consecutive time points; (ii) New or emerging responses: defined by the appearance of detectable IFN-γ ELISPOT responses to a peptide pool at one time point which was not present at two previous consecutive time points; and (iii) Persistent responses: defined as those responses that persisted over time and were characterized by fluctuating recognition profiles. **Figure 4.4** displays representative examples for each profile, A) 36% of individuals showing Profile 1 (characterized by a rapid loss of HIV-1 specific IFN-γ T cell responses soon after primary infection); B) 22% showing Profile 2
characterized by responses that were new or emerging over time), and C) 42% of participants showing Profile 3 (characterized by responses that persisted over time).

Fig. 4.4 Longitudinal characterizations of HIV-1 specific T cells in a subset of 34 individuals over the year of infection. Tracking of IFN-γ ELISPOT responses over time showing three distinct profiles of T cell recognition: (A) lost (=29), (B) New or emerging (n=18) and (C) Persistent responses (n=34).
As shown in Figure 4.4 A, the earliest detectable IFN-$\gamma$ ELISPOT responses elicited in Profile 1, which is represented by CAP88, tended to peak early at 8 weeks post infection followed by a rapid decline in magnitude probably reflecting loss of cognate antigen recognition. In contrast, the IFN-$\gamma$ ELISPOT responses elicited in Profile 2 using a representative plot from CAP85 (Figure 4.4B) peaked at around 29 weeks post infection and persisted over time, possibly driven by the reversion of transmitted CTL escape mutations (Allen et al., 2004; Friedrich et al., 2004a; Leslie et al., 2004; Li et al., 2007). In Figure 4.4 C, although the majority of HIV infected individuals showed persistent responses (42%), virtually all these responses showed waxing and waning reactivities over time possibly reflecting fluctuating immunodominant profiles. Detail analysis of these profiles are shown in appendix 3A1-A3 (page 207-211). To discount the possibility of fluctuating responses being due to assay variability, the co-efficient of variation against CEF peptides in quality control samples was found to be 24%, and within two standard deviations of the mean IFN-$\gamma$ ELISPOT response (Figure 4.5A). The average percent fluctuation over time patient responses was 43% above the calculated assay CV. Of note, oscillations over time in the magnitude of non-HIV specific responses (i.e. CEF, PHA) were also observed in both HIV-uninfected (Figure 4.5B) and infected individuals (Figure 4.6). Moreover, in most HIV-1 infected participants, the fluctuating patterns of PHA and CEF responses mirrored those observed in HIV-specific responses (Figure 4.6 A and B). In CAP-255, a direct relationship was observed between the frequency of HIV specific IFN-$\gamma$ ELISPOT responses with PHA specific IFN-$\gamma$ ELISPOT responses ($r=0.869, p<0.0001$) and CEF specific IFN-$\gamma$ ELISPOT responses ($r=0.603, p=0.0081$, Figure 4.6A).
Fig. 4.5 Coefficient of variation of the IFN-γ ELISPOT assay. (A) Levy-Jennings plot showing variability of IFN-γ ELISPOT assay in 121 replicates, using a peptide pool of 9-mer Cytomegalovirus (CMV), Epstein Barr Virus (EBV) and Flu Virus (CEF) epitopes. The outer boundaries for out-range values was > 2SD’s. (B) A representative example of fluctuating IFN-γ ELISPOT responses to phytohaemagglutinin (PHA) in six HIV negative subjects over the 7 weeks period.

Similar results were also observed in CAP-206 where the frequency of HIV-specific IFN-γ ELISPOT responses was correlated with PHA and CEF specific IFN-γ ELISPOT responses (PHA: r=0.690, p=0.0031; CEF: r=0.425, p=0.10). Figure 4.6 C shows a significant correlation between HIV specific IFN-γ ELISPOT responses with responses against PHA in 34 HIV infected individuals (r=0.303, p<0.0001). Taken together, these data show that variations of HIV-specific T cell responses over time is likely to be a biological phenomena and suggests that fluctuations are a natural phenomenon, reflecting the flexible, non static nature of T cell responses that possibly reflects a waves of immunodominance (Liu et al., 2011; Turnbull et al., 2009).
Fig. 4.6 Representative examples of fluctuating IFN-γ ELISPOT responses to CEF peptide pools, phytohaemagglutinin (PHA) and HIV-1 specific pool responses over 20 weeks post-infection (A and B). Correlations showing a direct relationship between HIV-1 peptide pool responses and PHA (C).

To more closely investigate whether the three distinct T cell profiles observed in this study were confined to specific gene region across the HIV genome, the distribution of lost, persistent and new responses toward Gag, Pol, Env and Nef proteins over the first year of infection were compared (Figure 4.7). In total, there was an emergence of 18 new responses with concomitant loss of 29 different responses and analysis of these recognition profiles in Gag, Pol, Env and Nef showed that there was no statistically significant difference between these patterns. There was, however, a trend towards more responses to Nef peptide pools either persisting or being lost and more new responses to Pol and Env peptide pools. These data underscore that new or lost T cell recognition is
not confined to a specific protein region and that HIV-specific T cell responses are dynamic and fluid in nature.

![Proportion of IFN-γ ELISPOT responses that were lost, persistent and new to Nef, Gag, Pol and Env over the first year of infection. The profiles of T cell recognition were tracked over time and compared in Gag, Pol, Env and Nef. Fisher’s exact was used for statistical comparisons between the proportion of T cell recognition among the groups.](image)

**Fig. 4.7** Proportion of IFN-γ ELISPOT responses that were lost, persistent and new to Nef, Gag, Pol and Env over the first year of infection. The profiles of T cell recognition were tracked over time and compared in Gag, Pol, Env and Nef. Fisher’s exact was used for statistical comparisons between the proportion of T cell recognition among the groups.

### 4.2.4 The Dynamic nature of T cell recognition patterns and early disease progression

To determine whether lost, new or persistent responses had any biological meaning and impact on disease progression in the first 12 months post infection, study participants were grouped according to the defined disease progression categories (see section 3.2.7 for definition, page 78) (Figure 4.8). Individuals with rapid progression (n=9) possessed significantly less (p=0.0037) persistent responses (Profile 3) and a greater number (p=0.06) of new responses (Profile 2) when compared with intermediate
progressors (n=20). The frequency of lost responses (Profile 1) was equal between the intermediate and rapid progressors. No loss of IFN\(\gamma\) ELISPOT responses were detected in individuals showing early slow disease progression (n=5), where these subjects overwhelmingly possessed persistent responses (89%). These data would suggest that persistent responses over time, possibly recognizing invariant epitopes, might provide advantageous immune responses leading to sustained viral suppression and/or maintenance of CD4 counts. The loss of response appeared to provide no advantage to the host; while new responses, which emerged over time, and possibly driven by antigen load appears deleterious during early HIV infection.

![Bar chart showing proportion of lost, persistent, and new IFN-\(\gamma\) ELISPOT responses in rapid (n=9), intermediate (n=20), and slow progressors (n=5) over the first year of infection. Fisher's exact was used for statistical comparisons between the proportion of T cell recognition among the groups.]

Chapter 4: Kinetics of HIV-1 specific T cell responses: Association with disease progression
To understand whether a lost response may be due to disappearance of weak sub-dominant response, the peak magnitude of responses between lost and persistent responses were compared. Figure 4.9 shows that there was no preferential disappearance of sub-dominant responses and furthermore, the magnitude of peak responses for each recognition profile was unrelated to disease categories of rapid, intermediate and slow. Collectively, these data suggest that single peptide responses that are either lost, emerge as new responses or persist during the first year of infection are not associated with the magnitude of initial peak of responses during acute infection.

Fig. 4.9 Peak magnitudes of lost and persistent IFN-γ ELISPOT responses in rapid, intermediate and slow progressors. Horizontal lines depict the median values. There was no significant difference in the peak magnitude of lost and persistent responses among the different disease categories.
4.2.5 Identity of single peptide from pooled peptide responses

Given that the approach used to identify lost, persistent or new IFN-\(\gamma\) responses was based on peptide pool responses, it was important to verify that many of the pool responses were derived from recognition of single peptide responses. In this analysis, HIV-1 specific IFN-\(\gamma\) T cell responses were first evaluated by reacting PBMC with peptides arranged in a pool matrix-format to allow for the identification of candidate epitope peptides. Putative positive peptide specific T cell responses derived from deconvoluting pool-matrix responses were then retested in triplicate and confirmed in a second round of ELISPOT assays on selected participants. Figure 4.10 shows the correlation between the peptide pool responses initially identified and the single peptides used to confirm the responses. For Gag and Nef, there were highly significant correlations between peptide pool responses and confirmed single peptides, where the peptide confirmations falling into Profile 1 showed an \(r\) of 0.869 (\(p=0.0004\)) and 0.8154 (\(p<0.0001\)), for Gag and Nef respectively; for Profile 2 showed an \(r\) of 0.854 (\(p<0.0001\)) for Nef. The correlation of overall peptide pool responses with single peptides (regardless of the Profile of response), showed significant correlations for Gag (\(r=0.756; p<0.0001\)), Nef (\(r=0.8007; p<0.0001\)), Pol (\(r=0.7613; p<0.0001\)) and Env (\(r=0.5914; p<0.0002\)). These correlations show the reactivity in the peptide pools by a single peptide to be responsible for the IFN-\(\gamma\) ELISPOT T cell responses.
Fig. 4.10 Correlations between peptide pool responses and confirmed single peptides.
Spearman correlations between IFN-γ ELISPOT responses derived from the peptide pool versus single confirmed peptides within the pool for Gag, Nef, Pol and Env. The solid black symbols represent peptide pool vs single peptides for all responses; the solid red symbols represent peptide pool vs single peptides for peptides that showed evidence for autologous viral sequence change; the solid blue symbols represent peptide pool vs single peptides for peptides that showed persistent IFN-γ ELISPOT responses.
4.2.6 Recognition patterns associated with autologous viral escape

In order to understand the potential impact of the viral sequence evolution on T cell recognition profiles, the association of IFN-γ ELISPOT responses in relation to autologous epitope sequence variation in Gag and Nef were investigated. Tables 4.A and B shows the confirmed peptides identified in the peptide pool responses for Gag and Nef, and the likely epitopes within the peptide being targeted and which were associated with the profile of T cell responses: lost (profile 1), new (profile 2) or persistent (profile 3) responses (see appendix 3A for the profiles, page 207). The peak T cell responses in 14 T cell responses with Profile 1 at the single peptide level (range: 155 – 5653 sfu/10⁶ PBMC) and the timing of viral epitope escape (range: 2-28.5 weeks) are shown in Table 4.A. When the recognition profiles were overlayed with autologous viral sequence changes, it was evident that Profile 1 (lost responses) were observed with fixed viral escape over time (87.5%; 14/16) in Figure 4.11A. Escape occurred either in the putative epitope (E) or in the flanking region (F) (Table 4.A). Three of the fourteen peptides identified in Profile 1 were observed in individuals with rapid disease progression relative to eleven peptides seen in individuals with intermediate disease progression (Table 4.A). Fifty percent of Profile 2 (new/emerging responses) were associated with autologous sequence variation, however the low number of individuals (n=4) presenting new responses to Gag or Nef does not allow us to draw defined conclusions (Figure 4.11B). Profile 3 (persistent responses) were least associated with autologous viral escape, with 37% (7/19) showing epitope variation, or almost two-thirds of participants with invariant sequences (Figure 4.11C).
**Table 4.1A:** Confirmed peptide responses in 17 study participants in Gag and Nef, showing the profile of response over time, disease category, the region of viral variation within the putative epitope, peak IFN-γ ELISPOT response, time of autologous viral epitope escape and subsequent time to loss of peptide-specific responses.

<table>
<thead>
<tr>
<th>PID</th>
<th>Confirmed peptide responses</th>
<th>HLA background and restricting allele</th>
<th>Profile Disease Category</th>
<th>Viral variant position</th>
<th>Peak response (SFU/10^4 PBMC)</th>
<th>Time to viral escape (weeks)</th>
<th>Time to lost response (weeks)</th>
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<tbody>
<tr>
<td>CAP8</td>
<td>p17 GKHHYMLKHLVYW5A6REL</td>
<td>A<em>2301/-, B</em>0801/15:10, C*0701/1601</td>
<td>1 R</td>
<td>E</td>
<td>2695</td>
<td>11.5</td>
<td>30</td>
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<td>CAP37</td>
<td>Nef 6SKSITVGWPAVIRIRRTE</td>
<td>A<em>2301/2402, B</em>0702/5301, C*1701/-</td>
<td>1 R</td>
<td>F</td>
<td>2243</td>
<td>13</td>
<td></td>
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<tr>
<td>CAP37</td>
<td>Nef 129PGPGVRYPLTFGWCFKLVF</td>
<td>A<em>2301/2402, B</em>0702/5301, C*1701/-</td>
<td>1 R</td>
<td>F+E</td>
<td>5653</td>
<td>9.5</td>
<td>15</td>
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<tr>
<td>CAP85</td>
<td>Nef 111WVYHTQGFYFDQWNYTPGP</td>
<td>A<em>3002/-, B</em>0801/4501, C*0701/1601</td>
<td>1 I</td>
<td>F</td>
<td>1638</td>
<td>18.5</td>
<td>16</td>
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<tr>
<td>CAP88*</td>
<td>p15 357SHKARVVLEAMSOQANS</td>
<td>A<em>2902/6601, B</em>4501/5802, C*0602/-</td>
<td>1 I</td>
<td>F</td>
<td>465</td>
<td>10</td>
<td>26</td>
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<tr>
<td>CAP129</td>
<td>Nef 6EVGFVPVRQVPLRMYKKA</td>
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<td>1 I</td>
<td>E</td>
<td>155</td>
<td>2</td>
<td>13</td>
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<td>Nef 101IHSGKRRQDLIDLMVYHTQG</td>
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<td>1 I</td>
<td>F+E</td>
<td>1775</td>
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<td>13</td>
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<td>1 I</td>
<td>E</td>
<td>2190</td>
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<tr>
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<td>A<em>0202/2901, B</em>1503/5801, C*0210/0602</td>
<td>1 I</td>
<td>E</td>
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<td>1 I</td>
<td>E</td>
<td>2468</td>
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<td>1 I</td>
<td>E</td>
<td>1248</td>
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<tr>
<td>CAP229*</td>
<td>p24 234SDIAGTTSTLQEQ1AWMTSNPPVPV</td>
<td>A<em>0123/-, B</em>5801/-, C*0602/-</td>
<td>1 I</td>
<td>F</td>
<td>1188</td>
<td>18.5</td>
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<td>CAP255</td>
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<td>1 I</td>
<td>E</td>
<td>2688</td>
<td>24.5</td>
<td>51</td>
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<td>CAP264</td>
<td>Nef TREPMTYKAADFSLFFKKeKG</td>
<td>A<em>3601/6802, B</em>1510/5301, C*0401/0804</td>
<td>1 I</td>
<td>E</td>
<td>2725</td>
<td>17.5</td>
<td>20</td>
</tr>
<tr>
<td>CAP8</td>
<td>Nef 101IHSGKRRQDLIDLMVYHTQG</td>
<td>A<em>2301/-, B</em>0801/1510, Cw*0701/1601</td>
<td>2 R</td>
<td>F+E</td>
<td>2495</td>
<td>11.5</td>
<td>-</td>
</tr>
<tr>
<td>CAP256</td>
<td>Nef TREPMTYKAADFSLFFKKeKG</td>
<td>A<em>2901/6601, B</em>1503/5802, Cw*0401/0602</td>
<td>2 R</td>
<td>E</td>
<td>2935</td>
<td>21</td>
<td>-</td>
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<tr>
<td>CAP30</td>
<td>Nef 129RMPMTYKAADFSLFLLKEKG</td>
<td>A<em>0201/3402, B</em>4403/4501, Cw*0401/1601</td>
<td>3 I</td>
<td>E</td>
<td>313</td>
<td>8</td>
<td>-</td>
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<tr>
<td>CAP244</td>
<td>Nef 129PGPGVRYPQVNPWCFKLVF</td>
<td>A<em>2301/3004, B</em>4403/5802, Cw*0401/0602</td>
<td>3 I</td>
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<td>5735</td>
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<tr>
<td>CAP255</td>
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<td>E</td>
<td>4045</td>
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<td>-</td>
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<tr>
<td>CAP258</td>
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<td>3 R</td>
<td>E</td>
<td>4245</td>
<td>19.5</td>
<td>-</td>
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<tr>
<td>CAP261</td>
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<td>A<em>2911/4301, B</em>1302/1503, Cw*0602/-</td>
<td>3 I</td>
<td>F+E</td>
<td>300</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>CAP262*</td>
<td>Nef 6EVGFVPVRQVPLRMYKKA</td>
<td>A<em>0101/6602, B</em>4201/8101, Cw*06/1701</td>
<td>3 S</td>
<td>E</td>
<td>935</td>
<td>14</td>
<td>-</td>
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<tr>
<td>CAP268*</td>
<td>Nef 101IHSGKRRQDLIDLMVYHTQG</td>
<td>A<em>0205/2601, B</em>0705/5801, Cw*0701/0702</td>
<td>3 I</td>
<td>F+E</td>
<td>1543</td>
<td>4</td>
<td>-</td>
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</table>

Mean±SD 2313±1563 14±11 24±11
Table 4B: Confirmed peptide responses in 13 study participants in Gag and Nef, showing the Profile of response over time, disease category, peak IFN-γ ELISPOT response and time of autologous viral epitope escape.

<table>
<thead>
<tr>
<th>PID</th>
<th>Confirmed peptide responses</th>
<th>HLA background and restricting allele</th>
<th>Profile</th>
<th>Disease Category</th>
<th>Viral variant position</th>
<th>Peak response (SFU/10⁶ PBMC)</th>
<th>Time to viral escape (weeks)</th>
<th>Time to lost response (weeks)</th>
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</thead>
<tbody>
<tr>
<td>CAP174 Nef</td>
<td>77QVPLRPMYKAADFLE77</td>
<td>A<em>0301/7401; B</em>4901/5802; Cw*0602/0701</td>
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<td>R</td>
<td>-</td>
<td>923</td>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td>CAP229* Nef</td>
<td>77RPMTYKAADFDSLFFLKEKG35</td>
<td>A<em>0123/-; B</em>5801/-; Cw*0602/-</td>
<td>1</td>
<td>I</td>
<td>-</td>
<td>1800</td>
<td>15</td>
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</tr>
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<td>CAP210 p15</td>
<td>230KEFFRDYVDRFFKTLRAEQATQ321</td>
<td>A<em>6802/-; B</em>1510/-; Cw*0304/-</td>
<td>2</td>
<td>R</td>
<td>-</td>
<td>2068</td>
<td>-</td>
<td>-</td>
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<tr>
<td>CAP228 p24</td>
<td>157AFSPFEVIPMTALSEGA179</td>
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<td>S</td>
<td>-</td>
<td>320</td>
<td>-</td>
<td>-</td>
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<tr>
<td>CAP45 Nef</td>
<td>125PGQVRYPLTFGWFFKLVP147</td>
<td>A<em>2301/2902; B</em>1510/4501; Cw*0602/1601</td>
<td>3</td>
<td>S</td>
<td>-</td>
<td>2178</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CAP88* Nef</td>
<td>133NYYHTQGFPPDHQNYTGP131</td>
<td>A<em>2902/6601; B</em>4501/5802; Cw*0602/-</td>
<td>3</td>
<td>I</td>
<td>-</td>
<td>2677</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CAP206 Nef</td>
<td>129PGQVRYPLTFGWFFKLVP147</td>
<td>A<em>0301/3201; B</em>0702/4403; Cw*0210/0702</td>
<td>3</td>
<td>R</td>
<td>-</td>
<td>243</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CAP222 Nef</td>
<td>65EVGFQVPQVLPMYKA37</td>
<td>A<em>3001/3303; B</em>5301/8101; Cw*0401/-</td>
<td>3</td>
<td>S</td>
<td>-</td>
<td>2838</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CAP222 p24</td>
<td>178GTPO6LNTMLNTVGHH134</td>
<td>A<em>3001/3303; B</em>5301/8101; Cw*0401/-</td>
<td>3</td>
<td>S</td>
<td>-</td>
<td>1568</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CAP225* p24</td>
<td>178GTPO6LNTMLNTVGHH134</td>
<td>A<em>0101/3001; B</em>4202/8101; Cw*1701/1801</td>
<td>3</td>
<td>I</td>
<td>-</td>
<td>3327</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CAP228 Nef</td>
<td>125PGQVRYPLTFGWFFKLVP147</td>
<td>A<em>2301/2638; B</em>4403/5101; Cw*0303/0701</td>
<td>3</td>
<td>S</td>
<td>-</td>
<td>2915</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CAP257 p17</td>
<td>9GKHYYMLKHLWASREL16</td>
<td>A<em>2301/2902; B</em>4202/4403; Cw*1701/-</td>
<td>3</td>
<td>I</td>
<td>-</td>
<td>775</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CAP257 Nef</td>
<td>125PGQVRYPLTFGWFFKLVP147</td>
<td>A<em>2301/2902; B</em>4202/4403; Cw*1701/-</td>
<td>3</td>
<td>I</td>
<td>-</td>
<td>4928</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CAP262* p24</td>
<td>178GTPO6LNTMLNTVGHH134</td>
<td>A<em>0101/6602; B</em>4201/8101; Cw*06/1701</td>
<td>3</td>
<td>S</td>
<td>-</td>
<td>1335</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CAP268* p24</td>
<td>233TWITLQEQIAMTVSNPPVPV58</td>
<td>A<em>0205/2601; B</em>0705/5801; Cw*0701/0702</td>
<td>3</td>
<td>I</td>
<td>-</td>
<td>2325</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*a* = study participants who show a simultaneous profile of lost and persistent peptide-specific responses

E = mutation occurs within the targeted epitope

F = mutation occur within the flanking region of targeted epitope.

Chapter 4: Kinetics of HIV-1 specific T cell responses: Association with disease progression

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In Table 4.A, five of the seven persistent peptides identified in Profile 3 were more frequent in individuals with intermediate disease progression compared with one peptide identified in rapid and slow disease progression. In the representative example shown in Figure 4.11A, CAP217 possessed a peak IFNγ ELISPOT response of 2190 sfu/10^6 PBMC at 12 weeks post infection, recognizing the HLAB*5801 restricted TW10 (TSTLQEQIAW) epitope in p24 Gag (Table 4A). By 17 weeks post infection, a fixed T→N mutation occurred at position 3 and by 19 weeks post infection and the response fell below 80% of the peak response (Table 4A). For CAP88, there was a relatively weaker peak IFNγ ELISPOT response of 465 sfu/10^6 PBMC at 7 weeks post infection recognizing a probable HLA B*4501 restricted AS9 (AEAMSQANS) epitope in p15 Gag. There was a subsequent transient escape in the flanking region detected by 13 weeks post infection associated with reduction in responses to 138 sfu/10^6 PBMC. It was interesting to note that although there was a reversion back from valine to alanine at amino-acid position 374 at some point between 26 and 54 weeks post infection, there was no detectable IFNγ ELISPOT response during this time (Figure 4.11A). CAP256, showing a Profile 2 pattern, possessed a late peak IFNγ ELISPOT response (2935 sfu/10^6 PBMC) at 34 weeks post infection. It was evident from the autologous sequence change within the recognized epitopic region (probable Cw*0602-restricted AL9, AAFDLSFFL, Table 4A) that the initial non-recognition of the peptide used in the assay was due to sequence variation in the flanking region and within the epitope (Figure 4.11B).
Fig. 4.11 Profiles of T cell recognition in relation to autologous sequence variations in Gag and Nef. Representative examples of the three T cell recognition profiles in relation to autologous sequence variation, shown as the magnitude of IFN-γ ELISPOT responses over weeks post infection. Each example is a representative individual within the cohort, showing the high resolution HLA background for A, B and C alleles. The time to fixed epitope escape, where appropriate, is shown underneath each profile (with the putative epitope shaded grey). The hatched box shows the window of time in which autologous viral escape occurred.
By 30 weeks post infection, the infecting sequence (measured at 6 weeks post infection) changed to match the peptide sequence. Of note, in this individual, more than 80% of the peak IFNγ ELISPOT response was lost, regained and then lost despite no further variations in the autologous sequence (Figure 4.11B). CAP210 showed a very similar profile to CAP256, where there were two putative epitopes being recognized: HLA-B*1510-restricted YVDRFFKTL and HLA-Cw*0304-restricted RAEQATQD in p15 Gag (Table 4B). However, contrary to CAP256 there was no detectable autologous sequence variation despite showing a peak IFNγ ELISPOT response of 2068 sfu/10⁶ PBMC (Table 4B) at 30 weeks post infection (Figure 4.11B). Both CAP225 and 257 represented individuals with a Profile 3 pattern, showing persistent IFNγ ELISPOT responses in the absence of autologous sequence variation, despite high magnitude peak responses of several thousand sfu/10⁶ PBMC (Table 4B and Figure 4.11C).

4.2.7 Is entropy of epitope related to differing T-cell recognition patterns?

Previous studies suggest that viral sequence variability of CTL epitopes is more likely related to whether CTL epitopes will escape or mutate under T cell selection pressure (Frahm et al., 2004; Goulder et al., 2001a). To explore whether the degree of sequence variation could impact on the observed T-cell recognition patterns in this study, the degree of variability based on the average entropy scores of peptides identified in profile 1 and 3 on Table 4A and B were compared. The Shannon entropy scores were derived from the alignment of the 20 full-length HIV-1 subtype C genome from primary infection on the Los Alamos National HIV sequence database as described previously (Treurnicht et al., 2010). Lower entropies indicate lower variability and more conserved regions, which could be
related to stronger fitness constraints (Li et al., 2007) whereas higher entropies indicate higher variability and less conserved regions. Entropy of the peptide was defined as the mean of the entropies of each amino acid site in the peptide. As shown Figure 4.12, the entropy score between persistent (0.14±0.08) and lost (0.197±0.09) peptides IFN-γ T-cell responses did not differ significantly, suggesting that the entropy of a targeted CTL epitopes are unlikely to influence the recognition patterns observed in persistent and lost T cell responses.

![Fig. 4.12 Comparisons of peptide sequence variability between persistent and lost T-cell responses based on Shannon entropy.](image)

4.2.8 Decay of IFN-γ ELISPOT responses after epitope escape

The preceding results suggest that the majority of IFN-γ ELISPOT responses observed in Profile 1 arose from CTL-driven immune pressure resulting in autologous sequence variations either within targeted putative epitope or in the flanking region of epitope. From
a vaccine perspective, it is important to understand the fate of CTL escape mutations and the timing of loss of immune T cell responses for a better understanding of the mechanisms underlying effective control of HIV infection. In assessing the tempo of lost IFN\(\gamma\) ELISPOT responses in relation to autologous viral escape, Figure 4.13 shows a strong association with the estimated time of viral escape with the time to disappearance of the IFN\(\gamma\) ELISPOT response (\(p=0.019, r=0.61\)).

**Fig. 4.13** Relationship between the time to loss of IFN\(\gamma\) responses and the median time to viral escape or autologous sequence variation. Linear regression showing a linear correlation between the estimated median time of autologous epitope escape versus the time to loss of a IFN-\(\gamma\) ELISPOT response. The hatched lines indicate the 95% confidence intervals around the mean regression line. The estimated time of escape was calculated as the median time between the last time-point measured where the autologous sequence was wild type and the first time-point where the variant sequence was detected.
For the 10 individuals and 14 epitopes showing escape (Table 4A), the estimated time taken for the IFN$\gamma$ ELISPOT response to decay after the target epitope varied (Figure 4.13) was a mean 14 weeks (4.4-24 weeks, 95% confidence interval). These data indicate that once the presenting antigen was removed due to loss of cognate peptide recognition, there was likely no requirement for the continued survival of IFN$\gamma$-producing effector T cells, which dissipated between 1-6 months after epitope escape.
4.3 Discussion

In the previous chapter, comprehensive screening of HIV-1 specific IFN-γ T cell responses at 3-months post infection revealed a distinct hierarchical targeting of Nef, Pol and Gag, and showed that the breadth and magnitude of IFN-γ ELISPOT responses made at one time point was unrelated to viral set point at 12-months post infection. In the current chapter, this analysis was extended by investigating the kinetics of IFN-γ ELISPOT T cell responses over time and how these responses related to viral load and disease categories. Consistent with previously reported immunodominance patterns of T cell recognition, Nef, Pol and Gag were the most frequently recognized regions during the first 6 months post infection (Lichterfeld et al., 2004; Masemola et al., 2004a; Streeck et al., 2007), with a distinct hierarchy of evolving responses emerging as early as 3 weeks post infection. Identification of CTL epitopes and immunodominant patterns that are frequently and consistently targeted by CD8+ T cell responses during this earliest stage of infection is critical for the identification of immunogenic targets for HIV-1 vaccines since vaccines are likely to elicit epitope-specific CD8+ T cell responses that are relevant for contemporary circulating virus strains. It has been hypothesized that the earlier an epitope is presented by HLA class I molecules on infected target cells, the more likely it will serve as an effective target for CD8+ T cells (Gallimore et al., 1995; Loffredo et al., 2005b; van Baalen et al., 2002; Van Baalen et al., 1998). It has been shown that Gag specific CD8+ T cells recognize SIV-infected targets within 2 hours of infection, whereas Tat, Env and Nef specific CD8+ T cells only recognize virus infected target cells following de novo synthesis of viral proteins (Sacha et al., 2007a; Sacha et al., 2007b). In this thesis, Nef responses
were the most rapid to emerge over time, being dominant at all time points analysed and in close agreement with other studies (Lichterfeld et al., 2004; Masemola et al., 2004a; Streeck et al., 2007). However, despite this vigorous targeting of Nef, no association was found between anti-Nef specific T cell responses and the ability of the host to control viral load. Previous studies have demonstrated a direct correlation between Nef specific T cell responses and high viraemia in early and chronic HIV-1 infection (Kiepiela et al., 2007; Masemola et al., 2004a; Zuniga et al., 2006) suggesting that Nef-specific T cell responses may be deleterious for the infected individuals. Data shown in this chapter shows that T cell responses to Nef appeared to have no impact, either way, on the course of viraemia. However, what was clear from the data was a temporal relationship between Gag-specific T cell responses and viraemia. Anti-Gag specific T cells emerged more slowly, but was found to be associated with a decline in rates of viral load, supporting the hypothesis that the ability to target Gag in vivo contributes to viral control (Geldmacher et al., 2007a; Kiepiela et al., 2007; Masemola et al., 2004a; Streeck et al., 2007; Zuniga et al., 2006). Further support for Gag specific T cell responses in control of viral replication, have been shown following early escape mutations in Gag resulting in a fitness cost to the virus (Crawford et al., 2009; Liu et al., 2007; Martinez-Picado et al., 2006; Miura et al., 2010). Chopera et al. (Chopera et al., 2008) previously found that lower viraemia was noted for HIV-infected individuals after viral transmission with viruses containing footprints of B*57/58:01 escape mutations to HLA B*58:01-negative recipients, suggesting that mutations in the ISW9/TW10 Gag epitopes were associated with the transmission of less fit virus. The latter data were derived from some of the individuals examined in this chapter (Chopera et al., 2008). Another recent study by Miura et al. (Miura et al., 2009) suggest
that HLA B*57/B*5801 HIV-1 elite controllers select for rare Gag variants associated with reduced viral replication capacity and strong CTL recognition of these variant by CD8+ T cell responses. In addition, it was shown that Gag specific T cell epitopes could be recognized as early as 2 hours after SIV infection (Sacha et al., 2007a) and the early presentation of Gag may possibly represent the most efficient responses in vaccine induced immunity to clear possible viral infection in case of preventative vaccines. In agreement with previous findings, the frequency of Env specific T cell responses was found to be associated with higher viraemia (Kiepiela et al., 2007). Set against this finding, is the observation that targeting of CD8+ T cell Env epitopes presented by HLA B*5802 is associated with markers of HIV disease progression and lack of selection pressure (Ngumbela et al., 2008) and epitopes derived from this highly variable protein are expressed too late in the viral replication cycle close to the onset of progeny virus production (Sacha et al., 2007a). Together, these data suggest and support the hypothesis that CD8+ T cells recognizing different proteins of HIV vary in their respective antiviral efficiencies in early and chronic HIV-1 infection (Geldmacher et al., 2007a; Kiepiela et al., 2007; Masemola et al., 2004a; Zuniga et al., 2006). In this chapter, a sub-group of individuals were intensely followed longitudinally for HIV-1 specific IFN-γ ELISPOT responses and showed that the magnitude of T-cell responses fluctuated widely over the first year of infection. This highlighted the rapidity of T cell evolution and the often-unpredictable nature of T-cell recognition patterns. This analysis also revealed three distinct profiles of T-cell recognition, characterized by either lost, new, or persistent responses and showed that there was a tendency of lost and persistent responses to be clustered in Nef and emerging new responses to be clustered in Pol and Env. Almost 90%
of lost T-cell responses were coincidental with autologous sequence variation in either the putative or the flanking region of epitopes and was consistent with CTL immune pressure. In support of this, CTL-driven immune escape is considered to be one of the major factors limiting the effectiveness of virus-specific CTL responses (Allen et al., 2005; Bernardin et al., 2005; Goulder and Watkins, 2004) and has been demonstrated in the acute phase of SIV and HIV infection, suggesting a rapid selection or evasion of virus-specific CTL responses during the first few weeks of infection (Borrow et al., 1997; Goonetilleke et al., 2009; Li et al., 2007). This CTL-driven immune escape can interfere with intracellular epitope processing, abrogate peptide-HLA binding or disrupt recognition of the peptide/HLA complex by the T cell receptor (Allen et al., 2004; Draenert et al., 2004; Goulder and Watkins, 2004; Kelleher et al., 2001). Goonetilleke et al (Goonetilleke et al., 2009) identified the first T-cell responses to transmitted/founder virus and showed that these responses rapidly select escape mutations concurrently with a decrease in viral load during acute HIV-1 infection. These data provide insight into the contribution of CTL responses to the resolution of acute-phase viraemia. There is also strong inferential evidence supporting HLA class I-driven viral evolution across the HIV-1 genome in both subtype B and C HIV infection (Berger et al., 2010; Brumme et al., 2007; Brumme et al., 2009b; Duda et al., 2009; Kawashima et al., 2009; Rousseau et al., 2008; Treurnicht et al., 2010; Wang et al., 2009), where a high density of HLA-associated mutation sites can be found in Nef relative to Pol, Gag, and other regulatory proteins. This would suggest that CTL recognition exerts differential selection pressures in selected regions (Brumme et al., 2007; Rousseau et al., 2008; Wang et al., 2009). This high density of HLA-associated mutation sites in Nef along with other studies (Frahm et al., 2004; Gray et al., 2009;
Lichterfeld et al., 2004; Masemola et al., 2004a) have demonstrated the relative immunogenicity of this protein in the course of HIV-1 infection and might explain the high proportion of lost responses in Nef relative to Gag, Pol and Env observed in this chapter. Toggling selection sites, defined as a model of positive selection associated with immune escape and reversion, have also been observed for Nef, consistent with high density of HLA-associated polymorphisms (Delport et al., 2008), supporting the evidence of CTL-driven immune selective pressure in shaping HIV diversity. It is noteworthy, however, that the use of a mixture of consensus and subtype C vaccine-matched strains as reference peptides may have led to underestimating the frequency of the HIV-1 specific responses, especially to those found in highly variable regions of the HIV-1 genome. The use of autologous-based peptide sets may have improved the detection of T-cell responses in more variable regions of HIV-1 proteins as previously reported (Altfeld et al., 2003b). It is probable that the use of autologous peptides based on the transmitted infecting virus would avoid the potential underestimation of epitope escape. Nevertheless, the data presented in this chapter show that T cell responses vary widely and tend to oscillate. There is no doubt that a substantial proportion of regions across the expressed HIV-1 genome are under selection of immune driven CTL pressure and therefore, likely to accumulated escape mutations and hence epitope variation which may impact on the recognition of T cell responses over time. In this chapter, many oscillating T cell responses identified in Gag, Pol, Nef and Env were not associated with autologous peptide sequence variation. In agreement with a previous report (Turnbull et al., 2009), the relative proportion of autologous sequence variation within epitopic regions being targeted by persisting HIV-specific T cells was found to be low, suggesting that the fluctuations observed in many of
the persisting responses were due to mechanisms other than viral escape. Several mechanisms have been postulated for the temporal fluctuation of CTL responses that include: a) fluctuations in antigen load; b) divergence of the CD8+ TCR repertoire; and c) T cell exhaustion and/or apoptosis (Geldmacher et al., 2007b; Meyer-Olson et al., 2006; Turnbull et al., 2009). Other studies have reported perturbations of T cell receptor repertoire during primary HIV infection and hypothesized that this may be influenced by the clonal expansion and exhaustion of virus specific CTL clones during the initial burst of viraemia during acute infection (Gorochov et al., 1998; Pantaleo et al., 1997; Soudeyns et al., 2000). It has also been hypothesized that persistence of some epitopes are due to insufficient antiviral pressure from the corresponding CTL responses (Koibuchi et al., 2005). In this thesis, the peak magnitude of persisting T cell responses during the first year of infection were found to be similar to those of lost responses and this appeared to have no bearing on disease progression. These data are in agreement to the findings of Lui et al. (Liu et al., 2007) who found no differences in the peak magnitude of CTL responses between evolving and persistent epitopes. Furthermore, it was found that sites of CTL escape mutations were significantly associated with higher entropies (high variability) whereas epitopes persisting at higher frequencies lacked highly variable sites (Liu et al., 2007). Based on this observation, it was hypothesized that persistent CTL responses are directed against highly conserved epitopes. The three differing profiles of T cell recognition observed in thesis are likely a result of the structurally constrained nature of the epitopes targeted which may impose fitness cost to the virus. However, the likelihood that epitope variability is associated with differing profiles observed in this thesis is unclear, as no significant differences was observed in the average of entropy scores of peptides within
differing profiles. It has been shown that conserved epitopes were recognized by CD8+ T-cell responses with a probability similar to variable epitopes and continuously elicit subdominant CTL responses during both primary and chronic infection (Liu et al., 2009). Collectively, these data suggest that: a) the temporal nature of T cell fluctuations means that a static measurement in time is not a predictable or reliable marker of recognition and may have little meaning for disease progression; b) the three different response profiles are a likely result of the structurally constrained nature of the epitopes targeted and c) that persistent CTL responses over time, in the absence of sequence variation, might provide advantageous immune responses leading to sustained viral control. In conclusion, the significance and novelty of these results lie at several levels. First, that T cell epitope recognition is not a static event and there are temporal patterns of IFN-γ-based responses. Second, that persistent T cell responses to invariant epitopes are enriched in individuals who progress slowly. Third, it takes an average of 14 weeks for IFN-γ-based T cell responses to dissipate once the target epitope has escaped.

Collectively, the broader interpretation of these data are that continual invariant viral epitope presentation is required for persistent T cell immunity, which in turn, appears necessary for control of early disease progression. These data warrant further investigations on the specific impact of the qualitative nature of persistent CD8+ T cell responses on immune control in early HIV-1 infection and how this relates to disease progression. The next chapter builds on this work and investigates in more detail the impact of the polyfunctional nature of CD8+ T cells on selection of escape mutations.
CHAPTER 5
CO-EVOLUTION OF EPITOME-SPECIFIC CD8+ T CELL RESPONSES WITH VIRAL ESCAPE

5.1 Introduction

CD8+ T cell responses have numerous effector functions, most notably the ability to secrete multiple cytokines (for example IFN-γ, IL-2, CD107a and TNF-α) and chemokines (for example MIP-1β and MIP-1α) is referred to as “polyfunctionality.” Highly polyfunctional CD8+ T cells has been shown to associate with control of viral replication in chronic HIV-1 infection (Betts et al., 2006; Emu et al., 2008; Hersperger et al., 2010; Migueles et al., 2008; Pereyra et al., 2008). Emerging data also suggest that the memory maturation phenotype and proliferation of CD8+ T cells play a critical role in mediating virus control in HIV-1 infection (Addo et al., 2007; Burgers et al., 2009; Day et al., 2007; Saez-Cirion et al., 2007). Other studies suggest that the functional attributes of virus specific CD8+ T cell efficacy against HIV may be linked to the level of antigen sensitivity (Almeida et al., 2009). It has also been reported that the polyfunctionality of virus specific CD8+ T cells is dependent on antigen load and viral sequence diversification (Streeck et al., 2008). Whether the polyfunctionality of virus specific CD8+ T cell responses mounted prior to CTL escape can impact on viral sequence diversification remains to be clarified. Delineating the phenotype and function of virus specific CD8+ T cells that recognize variant and invariant epitopes is important to identify as this may provide clues to how T cells provide immune selective pressure. In chapter 4, some of earliest T cell responses
occurring during acute HIV-1 infection were described and how these evolved in relation to autologous viral escape and early disease progression (Mlotshwa et al., 2010). This chapter extends these observations and provides a more in-depth analysis of the polyfunctional profiles of virus specific CD8+ T cells and the relationship with CTL epitope selection pressure.

5.2 Results

5.2.1 Study participants

To assess the impact of polyfunctional profiles of virus specific CD8+ T cell responses on autologous viral escape, an in depth analysis of CD8+ T cells targeting a total of 22 variant epitopes were undertaken in a subsets of four individuals (Table 5.1). Kinetics of plasma viral loads and CD4+ T cell counts of the four studied participants (CAP45, CAP63, CAP210 and CAP239) are shown in Figure 5.1. Study participants were grouped according to defined disease progression (see section 3.2.7 for definition, page 78). Based on this definition, CAP63 and CAP210 were classified as rapid progressors; CAP45 as a slow progressor and CAP239 as an intermediate progressor.

Table 5. 1 Clinical characteristics of study participants

<table>
<thead>
<tr>
<th>PTID</th>
<th>HLA class I allele</th>
<th>Viral set point (copies/ml)</th>
<th>CD4 slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP45</td>
<td>A<em>23:01/29:02, B</em>15:10/45:01, Cw*06:02/16:01</td>
<td>2.75</td>
<td>2.557</td>
</tr>
<tr>
<td>CAP63</td>
<td>A<em>02:01/23:01, B</em>45:01/45:01, Cw*04:01/16:01</td>
<td>5.33</td>
<td>-8.423</td>
</tr>
<tr>
<td>CAP210</td>
<td>A<em>68:02/68:02, B</em>15:10/15:10, Cw*03:04/0304</td>
<td>5.48</td>
<td>-4.784</td>
</tr>
<tr>
<td>CAP239</td>
<td>A<em>01:23/29:02, B</em>42:01/58:01, Cw*06:02/17:01</td>
<td>5.19</td>
<td>-4.464</td>
</tr>
</tbody>
</table>
Fig. 5.1 Kinetics of plasma viral loads (A) and CD4+ T cell counts (B) during the first year of subtype C HIV-1 infection. The two horizontal dotted lines demarcate viral loads < 2000 and > 100000 RNA copies/ml (A); and CD4+ T cell counts < 350 cells/mm³ (B). Based on the clinical disease definition, CAP45 was classified as slow progressor, CAP63 and CAP210 as rapid progressors; and CAP239 as intermediate progressor.

Table 5.2 and Appendix 4 (page 212) shows details of the series of T cell epitopes selected for the study, where peptide epitopes underwent either early escape (EE) or late escape (LE) over time. Early escape epitopes was defined as sequence variation in the autologous transmitted viral sequence within a median time of less than 20 weeks post infection. Late escape was defined as sequence variation in the transmitted viral sequence at a median of more than 20 weeks post infection. The timing of escape was defined as the median time between the point where transmitted viral sequence mutants was first detected and the point where 100% of transmitted viral sequence mutants were last detected. Of eleven CTL epitopes identified, seven showed evidence of early escape and four were associated with late escape. Table 5.3 shows the evolution of CTL epitope sequences and their corresponding restricting HLA class I allele. The time of early escape epitopes
ranged from 3-18 weeks with a median of 8.5 weeks whereas those of late escape epitopes ranged from 40.5-64 weeks with a median of 44.2 weeks.

### 5.2.2 Do polyfunctional profiles of epitope-specific CD8+ T cells associate with epitope mutation?

To determine the impact of magnitude and polyfunctional profiles of virus specific CD8+ T cell-mediated immune selection pressure on viral sequence evolution, a series of CTL escape autologous epitope peptides listed in Table 5.2 were used to stimulate PBMC’s at sequential time points, ranging from 5-92 weeks post infection.

**Table 5.2** List of peptides used in the ICS assay identified in 4 acutely HIV-1 infected subjects.

<table>
<thead>
<tr>
<th>PTID</th>
<th>Epitope region</th>
<th>Epitope sequence</th>
<th>Weeks PI in ICS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP45</td>
<td>Gag&lt;sub&gt;11-20&lt;/sub&gt;</td>
<td>VHQPMSPRTL</td>
<td>9,16,18,22,65</td>
</tr>
<tr>
<td></td>
<td>Pol&lt;sub&gt;44-74&lt;/sub&gt;</td>
<td>THLEGKIIL</td>
<td>9,16,18,43</td>
</tr>
<tr>
<td></td>
<td>Rev&lt;sub&gt;52-60&lt;/sub&gt;</td>
<td>IHSISERIL</td>
<td>9,16,18,22,43,65</td>
</tr>
<tr>
<td></td>
<td>Nef&lt;sub&gt;63-73&lt;/sub&gt;</td>
<td>EEVGFVPVRPOV</td>
<td>9,16,43,65</td>
</tr>
<tr>
<td>CAP239</td>
<td>Vpr&lt;sub&gt;34-42&lt;/sub&gt;</td>
<td>FFRPWLHNL</td>
<td>5,10,15,45</td>
</tr>
<tr>
<td></td>
<td>Nef&lt;sub&gt;82-90&lt;/sub&gt;</td>
<td>KAAVDLSFF KGAVIDLSFF KAAFDLSFF KGAAPDLSFF</td>
<td>5,10,15,45,92</td>
</tr>
<tr>
<td></td>
<td>Gag&lt;sub&gt;108-117&lt;/sub&gt;</td>
<td>TSTLQEQQVWL TSNLQEQQTVL</td>
<td>5,10,15,45</td>
</tr>
<tr>
<td>CAP210</td>
<td>Gag&lt;sub&gt;11-20&lt;/sub&gt;</td>
<td>VHQAISPRTL VHQTISPRTL</td>
<td>5,6,7,8,14,42,50</td>
</tr>
<tr>
<td></td>
<td>Vif&lt;sub&gt;79-87&lt;/sub&gt;</td>
<td>WHLGHGSIE WHLGHAGIE</td>
<td>5,6,7,8,14,42</td>
</tr>
<tr>
<td></td>
<td>Gag&lt;sub&gt;164-172&lt;/sub&gt;</td>
<td>YVDRFFKTL</td>
<td>5,6,7,8,14,42,50</td>
</tr>
<tr>
<td>CAP63</td>
<td>Env&lt;sub&gt;814-822&lt;/sub&gt;</td>
<td>LLDSIAITV LLNSIAITV LLDSIAITLL</td>
<td>4,11,20</td>
</tr>
</tbody>
</table>

*Chapter 5: Co-evolution of epitope-specific CD8+ T cell responses with viral escape*
Table 5.3 Summary of selected early (EE) and late escape (LE) epitopes identified in 4 acutely HIV-1 infected subjects.

<table>
<thead>
<tr>
<th>PTID</th>
<th>CTL epitope and HLA restriction</th>
<th>Epitope sequence</th>
<th>Median time to escape (Weeks)</th>
</tr>
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<td>GQM/VHQPMSPRTLNAWVK</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>I</td>
<td></td>
</tr>
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<td>THLEKGII...</td>
<td>40.5 (LE)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V</td>
<td></td>
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<tr>
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<td></td>
<td>IF9 Rev_{52-60}</td>
<td>F</td>
<td></td>
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<tr>
<td></td>
<td>GV11 Nef_{63-73}</td>
<td>G</td>
<td></td>
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<td></td>
<td></td>
<td>L</td>
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<td>.G.F</td>
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<td></td>
<td>T821I LV9 Env_{814-822}</td>
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<td></td>
</tr>
</tbody>
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# Median time of escape was calculated as the median time between the latest time point where 100% of the transmitted mutant virus sequence were detected and the first time point where mutations were detected.

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Cells were assessed for the ability of epitope-specific CD8+ T cells to express up to six effector functions (IFN-γ, IL-2, TNF-α, MIP-1β, CD107a and perforin) as previously described (Hersperger et al., 2010). The gating strategy for dissection of polyfunctionality of virus specific CD8+ T cells is shown in Figure 5.2. To perform this analysis, cells producing different cytokine profiles in the presence of HIV peptides were characterized by the expression of CD45RO, CD27 and CD57 on CD4+ and CD8+ T cells, and gates for all six different functions were created as illustrated in Figure 5.2. These gates were subsequently combined by a Boolean gating strategy to generate the full array of possible cytokine combinations. The first level of analysis was to evaluate the frequency of epitope-specific CD8+ T cell responses to early and late escape epitopes from as early as 4-9 weeks post infection prior to the emergence of escape mutants. Figure 5.3A shows representative examples of flow cytometry plots showing the frequency of CD8+ T cells producing IFN-γ, IL-2, TNF-α, MIP-1β and CD107a following stimulation with no peptides, early escape Gag VL10 and late escape Rev IL9 epitopes. There appears to be a marked heterogeneity in the patterns of different cytokines produced by epitope-specific CD8+ T cells in both early Gag VL10 and late Rev IL9 escape epitopes, with CD107, MIP-1β and IFN-γ being the most dominant cytokines (Figure 5.3A). Figure 5.3 B shows the frequencies of epitope-specific CD8+ T cells recognizing early and late mutant epitopes with 1-, 2-, 3- and 4-functions. When the frequency of epitope-specific CD8+ T cells that produced various combination of cytokines towards early and late escaping epitopes were compared, there was a trend towards inducing 3-functions (IFN-γ, MIP-1β and CD107a) by early escaping epitopes (Figure 5.3B, p=0.066; also see Figure 5.4 for detailed analysis).
Fig. 5.2 Representative flow cytometry plots showing a gating strategy for identification of polyfunctionality of virus epitope-specific CD8+ T cell responses. PBMC from CAP0045 were stimulated with variant Rev IL9 epitope for six hours and then surface stained with lineage markers (CD14, CD19, CD3, CD4, CD8), memory maturation markers (CD27, CD45RO and CD57) along with six CD8+ T cell functions (IFN-γ, TNF-α, MIP-1β, IL2, CD107a and perforin). -); TD, terminal differentiated (CD45RO-/CD27-).
Fig. 5.3  Magnitude of total autologous virus specific CD8+ T cell responses to variant and invariant epitopes before escape mutation at 5-9 weeks post infection.  (A) Representative flow cytometric plots showing functional profiles (IFN-γ, TNF-α, CD107a, IL-2 and MIP-1β) in responses to no peptide stimulation (control), invariant Gag VL10 epitope and variant Rev IL9 epitope.  (B) The proportion of virus specific CD8+ T cells producing 1, 2, 3 and 4 functions to early and late escaping epitopes.  The pie charts represent the relative contributions of each function to the magnitude of virus specific CD8+ T cell responses to variant and invariant epitopes.  (C) Peak magnitude of the total epitope-specific CD8+ T cell responses to variant and invariant epitopes.
To determine whether the total magnitude of epitope-specific CD8+ T cells was associated with immune mediated selection pressure, the earliest peak magnitude of epitope-specific CD8+ T cells, before the emergence of early and late escape variants were compared. Figure 5.3 C shows that there was a trend of higher epitope-specific CD8+ T cells prior to early mutated epitopes when all the functions were grouped (p<0.067). The median proportions of epitope-specific CD8+ T cells at the peak responses prior to emergence of early and late mutant epitopes were 1.83% (range, 0.40 – 5.34%) and 0.24% (0.10 – 1.60%) respectively. Taken together, these data suggest that the total magnitude of epitope-specific CD8+ T cell responses may play an important role in mediating immune selection pressure of escape variants. This is consistent with recent observations in subtype B infection, which have demonstrated a significant association between the total magnitude of virus specific CD8+ T cells and selection of escape mutants (Ferrari et al., 2011).

Having shown that the total magnitude of epitope-specific CD8+ T cells at the earliest peak responses might impose sufficient pressure on variant epitopes to drive selection of escape mutants, the next level of analysis sought to determine whether this held true for the polyfunctional profile of epitope-specific CD8+ T cells. As shown in Figure 5.4, the polyfunctional profiles of virus epitope-specific CD8+ T cells prior to the emergence of viral escape mutants did not differ significantly between early or late mutated epitopes.
Fig. 5.4 Polyfunctional profiles of virus specific CD8+ T cells to early and late escape epitopes prior to escape mutation at 5-9 weeks post infection. The pie charts depict the proportion of the total CD8+ T cell responses, which produce 1, 2, 3, 4, and 5 functional responses to early and late escape epitopes. The x-axis denotes all the 32 possible combinations of virus specific CD8+ T cell responses for each functional parameter. The polyfunctional profiles of early and late escape epitopes are shown in the insert for CAP45, CAP210 and CAP239.
Of the six functions measured, expression of CD107a, IFN-γ and MIP-1β by epitope-specific CD8+ T cells were the most common functional combination, with CD8+ T cells mediating two (CD107a+MIP-1β+ or IFN-γ+MIP-1β+) or three (CD107a+MIP-1β+IFN-γ+) functional subsets more frequently to all epitopes, irrespective of whether the under went early or late escape. CD8+ T cells mediating one or four functional subsets was the least common function comprised of either CD107a+ or MIP-1β+ and CD107a+MIP-1β+IFN-γ+TNF-α+ for one and four functions respectively (Figure 5.4). There was no evidence of polyfunctional CD107a+MIP-1β+IFN-γ+TNF-α+IL2+ subsets preceding early or late mutant epitopes, and this mostly attributable to an absence of IL-2-producing CD8+ T cells. These results suggest that polyfunctional profiles of epitope-specific CD8+ T cells prior to mutations most likely had no bearing on variant escape mutations, being in agreement with recent findings (Ferrari et al., 2011).

5.2.3 Does the memory status of epitope-specific CD8+ T cells associate with epitope mutation?

Emerging data suggest that differences in memory maturational phenotype of virus specific T cells might play an important role in the control of HIV-1 viraemia (Addo et al., 2007; Burgers et al., 2009; Champagne et al., 2001; Ferrando-Martinez et al., 2012) and whether this is sufficient enough to impose pressure on the virus to drive the selection of escape mutants remains to be established. This part of the chapter focuses on comparing the memory maturational phenotypes of epitope-specific CD8+ T cells with early and late mutated epitopes at 4-9 weeks post infection prior to viral escape mutation for evidence of
escape mutant selection. **Figure 5.5 A** shows representative plots of the frequency of epitope-specific CD8+ T cells within early differentiated memory (ED, CD27+CD45RO+), late differentiated memory (LD, CD27-CD45RO+) and terminal differentiated (CD27-CD45RO-) memory CD8+ T cell subpopulations. **Figure 5.5 C** compares the frequency of epitope specific CD8+ T cells within ED-, LD- and TD-memory CD8+ T cell phenotype, showing no statistically significant differences between differentiation and early or late epitope escape. The median percentage of epitope-specific CD8+ T cells to early and late mutant epitopes within ED, LD and TD memory subpopulations were 62% (range, 32-72%) vs 54% (range, 42-65%), 19% (range, 7-41%) vs 28% (range, 19-41%) and 5% (range, 2-14%) vs 6% (3-10%) respectively (**Figure 5.5C**). Together, these data suggest that the memory maturation profiles of epitope-specific CD8+ T cells recognizing early and late mutant epitopes before mutation had no impact on the emergence of escape mutants. What of the levels of perforin? Representative plots showing the expression of perforin within ED, LD and TD memory CD8+ T cell subpopulations are shown in **Figure 5.5 B**. The majority of perforin expression within epitope-specific CD8+ T cells was found in terminally differentiated (TD) CD27-CD45RO- and late differentiated memory (LD) CD27-CD45RO+ CD8+ T cell memory subpopulations, and only within a small proportion on early-differentiated memory (ED) CD8+ T cells (**Figure 5.5B**). There were no statistically significant differences observed between the frequency of perforin expression on memory maturation profiles of epitope-specific CD8+ T cells recognizing epitopes which either were early and late mutants (**Figure 5.5D**). Although the levels of perforin were not related to epitope escape, it is noteworthy that the perforin expression on epitope-
specific CD8+ T cells increased with the maturation phenotype of CD8+ T memory cells, consistent with previous observations (Hersperger et al., 2010).

Fig. 5.5  The memory maturation profiles of virus specific CD8+ T cells to early and late escape epitopes before escape mutation at 5-9 weeks post infection. Representative flow cytometry plots showing a gating strategy for total cytokine production (A) and perforin expression (B) on memory maturation profiles. The frequency of total virus specific CD8+ T cells (IFN-γ, TNF-α, CD107a, IL-2 and MIP-1β) in (A) and perforin (B) shown as red dots were overlaid onto a density plots (black shading) of memory phenotype of total CD8+ T cell subsets. (C) The frequency of total cytokine production on memory CD8+ T cell subsets to early and late escape epitopes. (D) Perforin expression profile on memory maturation CD8+ T cell subsets to early and late escape epitopes. ED, early differentiated (CD45RO+/CD27+); LD, late differentiated (CD45RO+/CD27-); TD, terminal differentiated (CD45RO-/CD27-).
5.2.4 Comparison of the polyfunctional profiles and magnitude of epitope-specific CD8+ T cells before and after mutation.

Given that it has been shown that the first HIV specific CD8+ T cell responses in acute HIV-1 infection can exert the initial immune-mediated pressure that drives selection of escape mutants (Goonetilleke et al., 2009), it was deemed important to know the difference between epitope recognition before and after viral escape mutation. Such an analysis might shed light on which parameter plays a role in selection of escape. Figure 5.6 shows the total magnitude and polyfunctional profiles of CD8+ T cells recognizing epitopes before and after early escape mutations. As illustrated in Figure 5.6A, the magnitude of epitope-specific CD8+ T cells was significantly higher to epitopes prior to escape (2.27 ± 2.09% vs 0.39 ± 0.55 %, p=0.0343). This difference was made up of six of the seven (86%) early mutating epitopes being recognized considerably less by epitope-specific CD8+ T cells (Figure 5.6A), probably reflecting loss or diminished recognition of cognate antigen. In contrast, the Nef EV11 early mutating epitope appeared to be equally recognized by CD8+ T cells before and after escape mutations. This may be due to the fact that the Nef EV11 mutation may be well tolerated.

What of the polyfunctional profiles of epitope specific CD8+ T cells? Figure 5.6B shows the polyfunctional profiles of CD8+ T cells responding to epitope peptides prior to the emergence of viral escape mutations was similar to those elicited after the selection of escape mutants.
Fig. 5.6 Recognition of early wild-type mutating epitope-specific CD8+ T cells before and after escape mutation. A) Magnitude of virus epitope-specific CD8+ T cells to epitopes before and after viral escape mutations. (B) Polyfunctional profiles of early mutating epitopes before and after viral escape mutations. Responses were grouped and colour-coded according to the number of functions (■-1 function, ■-2 function, ■-3 function and ■-4 function). Polyfunction profiles and magnitude of epitope-specific CD8+ T cells were grouped according to the pattern of recognition (■-same magnitude, same polyfunctional cells; ■-lower magnitude, less polyfunctional cells; and ■-lower magnitude, same polyfunctional cells).
Moreover, an in-depth analysis of polyfunctional nature of CD8+ T cells in relation to the magnitude of epitope-specific CD8+ T cells before and after escape mutations also revealed a divergent pattern of epitope-specific CD8+ T cell recognition (Figure 5.6B). In one of the seven (14%) of early mutating epitopes analysed, epitope-specific CD8+ T cells a) showed the same magnitude and same polyfunctional profile, b) 43% (3/7) showed a lower magnitude and the same polyfunctional profile and c) 43% (3/7) showed a lower magnitude and a less polyfunctional profile of specific CD8+ T cells. Taken together, these data revealed distinct patterns of the polyfunctional nature and magnitude of CD8+ T cells, where the majority of epitope-specific CD8+ T cells showed diminished magnitude of recognition towards mutated epitopes, but the polyfunctional nature often remained unchanged.

5.2.5 Tracking the magnitude and polyfunction of CD8+ T cells following selection of CTL escape mutants.

It is widely known that responses to wild-type epitopes wane over time as a result of diminished antigenic drive and selection of escape mutations (Rehr et al., 2008) (Streeck et al., 2008) (Janbazian et al., 2012). In chapter 4 of this thesis, it was demonstrated that it takes an average of 14 weeks for IFN-γ based T cell responses to dissipate once the presentation of cognate antigen is lost as a result of escape mutations. To more closely examine this beyond the production of IFN-γ CD8+ T cell responses, the magnitude and polyfunctional profiles of CD8+ T cells prior to CTL escape mutation were compared over time following selection of CTL escape. **Figure 5.7** details evolving patterns of the magnitude and polyfunctional CD8+ T cells responding to early mutating epitopes.
Although dissection of epitope-specific CD8+ T cells revealed a marked heterogeneity in the recognition of early mutating epitopes following CTL escape mutation, there was an overall decline in the magnitude and polyfunctional profiles of epitope-specific CD8+ T cells recognizing these epitopes over time. With the exception of the B*45:01 restricted Nef EV1163-73 epitope which remained relatively unchanged following selection of CTL escape mutants, the majority of CD8+ T cells recognizing these epitopes (5/6; 83%) exhibited a significant reduction in the total magnitude after the selection of CTL escape mutants (p<0.0003, Figure 5.7).

Fig. 5. 7 Kinetics of virus epitope-specific CD8+T cells responding to early mutating epitopes over the first year of HIV-1 infection. Magnitude of epitope-specific CD8+ T cells to early mutating epitopes before and after escape mutation. Polyfunctional profiles of virus epitope specific CD8+ T cells are represented as pie charts and color coded according to the number of functions (-1 function, -2 function, -3 function and -4 function).
Most notably, A83G, T242N and A14T changes in KF-9 Nef_{82-90}, TW10 Gag_{108-117} and VL10 Gag_{11-20} respectively, appeared to result in the loss of CD8+ T cell recognition of these variant epitopes over time. It was also evident that selection of CTL escape mutations in KF-9 Nef_{82-90} and TW10 Gag_{108-117} occurred within the HLA binding anchor residues (Table 5.3). When an epitope prediction tool (www.immuneepitope.org) was applied to the data (which utilizes the predictors of proteasomal processing, TAP transport and MHC binding to produce an overall score of a particular epitope), the impact of the mutation in the B*58:01-restricted A83G-KF9 Nef_{82-90} epitope showed a reduction in the total epitope score compared to those of the transmitted epitope (0.75 vs -0.67, Table 5.3) suggesting that this mutation is likely to reduce the binding of the epitope to the HLA molecule. Analysis of the total epitope score revealed that the T242N mutant epitope had a slight higher total epitope score than the transmitted TW10 epitope (0.2 vs 0.05, Table 5.3). In the case of the HLA B*15:10 restricted VL10 Gag_{11-20} epitope, both wild-type transmitted and mutant epitopes exhibited a similar total epitope score (-1.01 vs -1.03, Table 5.3), but there was significant reduction in epitope-specific CD8+ T cells over time (Figure 5.7), suggesting that the lack of recognition following selection of CTL escape mutation was likely due to abrogation of TCR binding. When the polyfunctional profiles of CD8+ T cells was analysed over time, there was an overall reduction in the polyfunctionality of epitope-specific CD8+ T cells recognizing early mutating epitopes dominated by monofunctional cells suggesting that changes in epitope over time might have resulted in the concomitant loss of cognate antigen required for continued recognition by the T cell receptor (TCR) of CD8+ T cells or there were no priming for in vivo de novo T cell responses that could potentially recognized the emerging viral variants.
Table 5.4 Predictions of proteasome, TAP, MHC binding and processing scores of wild-type variant and mutant epitopes. An algorithm prediction tool (www.immuneepitope.org) was used to assess predicted scores for proteasome, TAP, MHC binding and processing of autologous wild-type variant and mutant epitopes. HLA binding motifs highlighted in red for each 9- to 11-mer optimal epitopes were predicted using the online HLA motif scanner tool (www.hiv.lanl.gov/content/immunology/motif_scan/motif_scan).

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<th>Epitope sequence</th>
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<th>TAP score</th>
<th>MHC binding score</th>
<th>Processing score</th>
<th>Total score</th>
<th>MHC IC50</th>
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<td>. . . . . . . .</td>
<td>1.25</td>
<td>-0.26</td>
<td>-2.38</td>
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<td>-2.25</td>
<td>1.05</td>
<td>-1.20</td>
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</tr>
<tr>
<td>V822A</td>
<td></td>
<td>. . . . . . . .</td>
<td>1.10</td>
<td>-0.3</td>
<td>-1.97</td>
<td>0.80</td>
<td>-1.17</td>
<td>93.08</td>
<td></td>
</tr>
</tbody>
</table>
5.3 Discussion

While it is well established that HIV specific CD8+ T-cell responses play a critical role in the containment of HIV-1 (Borrow et al., 1994; Koup et al., 1994) as well as select for CTL escape mutants that may impact on the fitness cost of the virus (Crawford et al., 2009; Goulder and Watkins, 2004; Leslie et al., 2004; Martinez-Picado et al., 2006; McMichael et al., 2009), little is known about the impact of the polyfunctional nature of CD8+ T cells on viral sequence evolution. Polyfunctionality is defined as the ability of CD8+ T cells to exert multiple effector functions (IFN-γ, TNF-α, MIP-1β, CD107 and IL-2) and has been shown to associate with control of viral replication (Almeida et al., 2007; Betts et al., 2006; Julg et al., 2010; Precopio et al., 2007), however, there is also evidence that this is largely dependent on the level of antigen and viral sequence diversification (Janbazian et al., 2012; Migueles et al., 2009; Rehr et al., 2008; Streeck et al., 2008). In this chapter, the magnitude and polyfunctional nature of epitope specific CD8+ T cells were analysed to examine the impact on the selection of viral escape mutations. It is shown that: (1) the magnitude of the total functional epitope-specific CD8+ T cells prior to selection of early escape was higher when compared with late escape mutants, (2) the polyfunctional nature of virus epitope-specific CD8+ T cells was not associated with selection of early escape mutants, (3) neither the memory maturational profiles nor perforin expression of epitope-specific CD8+ T cells differed significantly between early and late mutating epitopes, (4) there was diminished recognition of epitopic specific CD8+ T cells following selection of escape mutants and (5) there was a marked degree of
heterogeneity in the patterns of magnitude and polyfunctionality of epitope specific CD8+ T cells over time following a selection of escape mutants.

The findings that the total magnitude of epitope specific CD8+ T cells was higher towards early mutating epitopes compared to those of late mutating epitopes is in agreement with recent data (Ferrari et al., 2011) and suggest that the magnitude of HIV specific CD8+ T cells is likely to be important for the selection of escape mutants. The reasons for this apparent relationship between early selection of escape mutants and magnitude of functional CD8+ T cells is unclear but may reflect that CD8+ T cells can exert immune selection pressure on the virus (Allen et al., 2005; Borrow et al., 1997; Henn et al., 2012; Li et al., 2007; Liu et al., 2011; Price et al., 1997). Consistent with this, it has been shown that epitope-specific CD8+ T-cell responses can rapidly select for escape mutations concurrent with declined viral load in acute HIV-1 infection (Goonetilleke et al., 2009). Furthermore, recent data from Henn et al (Henn et al., 2012) also illustrated that the majority of early low frequency adaptive mutations across the HIV-1 proteome in acute HIV-1 are associated with CD8+ T cell responses. Taken together, these data along with the results presented in this chapter highlight a temporal link between a strong immune driven CD8+ T cell pressure and selection of escape mutants in early HIV-1 infection.

The results in this chapter show that the polyfunctional profiles of CD8+ T cells in response to early and late mutating epitopes were similar, suggesting that functional profile of CD8+ T cells measured had no association with selection of epitope escape. This finding is in agreement with recent study by Ferrari et al (Ferrari et al., 2011) and is
further corroborated by several studies which have shown that the polyfunctional profiles of epitope-specific CD8+ T cells may be linked more with antigen load (Janbazian et al., 2012; Migueles et al., 2009; Rehr et al., 2008; Streeck et al., 2008) and sensitivity (Almeida et al., 2009). The lack of relationship between the polyfunctional nature of CD8+ T cells and selection of escape mutants could also be attributed to a limited sample size used in this study. Similar findings were observed when the memory maturational profiles and perforin expression of epitope specific CD8+ T cells were analysed suggesting that neither the memory profiles nor perforin expression were associated with early or late selection of escape mutants. This lack of association between memory maturation and early selection of escape is also in agreement with Ferrari et al (Ferrari et al., 2011) and is probably related to functional exhaustion of virus epitope specific CD8+ T cells (Day et al., 2006; Streeck et al., 2008; Trautmann et al., 2006; Yamamoto et al., 2011). Moreover, the majority of epitope-specific CD8+ T cells recognizing early and late mutating epitopes that expressed perforin in the present study were terminally differentiated (TD, CD27-CD45RO-) T cells, which is in agreement with previous findings that examined for the presence of perforin expression on various human CD8+ T cell memory subsets (Chattopadhyay et al., 2009; Hersperger et al., 2010; Takata and Takiguchi, 2006). Although perforin expression on virus specific CD8+ T cells have been shown to be inversely associated with low viral load (Hersperger et al., 2010; Migueles et al., 2008), the present study found no association between the level of perforin expression on epitope-specific CD8+ T cells and appearance of CTL escape mutants. This would suggest that perforin per se may have no bearing on selection of epitope mutants. It was shown in chapter 4 that rapid disease progression may in fact be related to escape (see

Chapter 5: Co-evolution of epitope-specific CD8+ T cell responses with viral escape
As to be expected, the overall magnitude of early mutating epitopes following the selection of CD8+ T cell epitope mutation was found to be significantly lower than those prior to the selection of escape mutants. This is in agreement with the recent studies (Ferrari et al., 2011; Liu et al., 2011), which have demonstrated that CD8+ T cell responses were strongest against founder virus epitopes following selection of escape mutations and that there was a subsequent contraction of these responses. This is compatible with the findings shown in chapter 4, where IFN-γ responses diminished after epitope escape and is likely due to loss of cognate antigen.

A more in-depth analysis of CD8+ T cell polyfunctionality upon targeting mutant epitopes following the selection of CTL escape variants revealed three distinct profiles: (1) 14% showed the same magnitude and polyfunctional profile; (2) 43% showed a lower magnitude and same polyfunctional profile and (3) 43% showed a lower magnitude and a less polyfunctional profile of epitope-specific CD8+ T cells. It is thus clear that the magnitude and polyfunctional nature of CD8+ T cells to mutant epitopes varies substantially after the selection of CTL escape variants and this finding is in agreement with prior data that have demonstrated a high degree of heterogeneity of functional profiles of epitope-specific CD8+ T cell responses in HIV infection (Almeida et al., 2007; Emu et al., 2008; Pereyra et al., 2008; Streeck et al., 2008; Tang et al., 2010). Additional support for this finding comes from studies that have shown that epitope-specific CD8+ T cells are often comprised of a single dominant and various subdominant clonotypic populations that can respond variably to changes in viraemia and that these clonotypes have different abilities to recognize epitope variants (Meyer-Olson et al., 2006; Simons et
Emerging data also suggest that dominant and subdominant T cell clones have phenotypic and functional characteristics linked to antigen sensitivity further suggesting that the specificity of individual T cell clones might play an important role in the evolution of epitope-specific CD8+ T cell responses (Conrad et al., 2011; Janbazian et al., 2012). Immune mediated selective pressure is believed to be an important driving force of HIV-1 evolution and accumulation of escape mutants leads to a loss of CD8+ T cell epitope specific responses in the individual’s viral population (Allen et al., 2005; Friedrich et al., 2004b; Goulder and Watkins, 2004; Liu et al., 2007; Liu et al., 2011; Turnbull et al., 2006). It is also well established that CD8+ T cell responses to wild-type epitopes wane over time as a result of diminished antigen drive and emergence of viral escape mutations (Allen et al., 2005; Goulder and Watkins, 2004; Turnbull et al., 2009). Although the findings presented in this chapter suggest that the overall magnitude and polyfunctional of CD8+ T cells to wild-type mutant epitopes are substantially varied and in some instances reduced over time following the selection of escape mutants, is in agreement with previous data (Allen et al., 2005; Goulder and Watkins, 2004; Turnbull et al., 2009), but contrast with those of recent studies which have illustrated that viral sequence diversification plays a major role in the emergence of polyfunctional CD8+ T cells (Janbazian et al., 2012; Streeck et al., 2008). The discrepancy between the data presented in the current study and those of Streeck et al (Streeck et al., 2008) and Janbazian et al (Janbazian et al., 2012) is likely due to differences in cohorts, type of antigenic stimulation (i.e peptides corresponding to previously described CD8+ T cell epitopes) and HLA class I diversity. Although one cannot exclude the causal role of antigen load on the emergence of polyfunctional CD8+ T cells as reported previously (Janbazian et al., 2012; Rehr et al.,
the limited sample size precluded the current study from addressing this issue.

Collectively, this chapter provides evidence that there is limited recognition of mutant epitopes by CD8+ T cells following the selection of CTL escape mutants. There was no supporting evidence for the role of polyfunctional CD8+ T cells on the early appearance of CTL escape mutants raising a question of whether polyfunctionality may simply reflect reduced in vivo antigen exposure rather than being a direct mediator of viral selection. This finding also highlights a diverse pattern of epitope-specific CD8+ T cells targeting mutant epitopes following the selection of escape mutants and demonstrates that the magnitude and polyfunctional of CD8+ T cells to mutant epitopes are reduced over time after the selection of escape mutants. The next chapter of this thesis will look at the apoptosis as an alternative evasion strategy used by HIV-1 to evade immune recognition.
CHAPTER 6
ASSOCIATION OF PLASMA MEDIATORS OF APOPTOSIS AND IMMUNE ACTIVATION

6.1 Introduction

Over the past years, considerable progress has been made in understanding the mechanisms employed by HIV to evade the immune system. One of the strategies developed by HIV is to activate intrinsic and extrinsic apoptotic pathways that cause effector cell death and hence inadequate immunity (Gougeon, 2003; Petrovas et al., 2005). Increased plasma levels of apoptosis mediators such TRAIL; CD95/Fas and TNF-RII have also been shown in HIV infection and suggested that this pathway contributes to CD4+ T cell depletion during progression to AIDS (de Oliveira Pinto et al., 2002; Gasper-Smith et al., 2008; Gougeon, 2003). In vitro studies demonstrate that CD4+ and CD8+ T cells from HIV infected subjects are susceptible to spontaneous apoptosis, activated induced cell death (AICD), CD95/Fas, TNF-related apoptosis inducing ligand (TRAIL), TNF-receptor (TNRF) I and II mediated apoptosis as compared to uninfected subjects (de Oliveira Pinto et al., 2002; Gougeon, 2003; Petrovas et al., 2007b). In addition, HIV-specific CD8+ T cells were shown to exhibit reduced levels of anti-apoptotic proteins, Bcl-2 and Bcl-xl and to be more prone to CD95/Fas induced apoptosis (Petrovas et al., 2007a; Petrovas et al., 2004). Furthermore, apoptosis of HIV-specific CD8+ T cells were found to be associated with increased mitochondrial mass and reduced Bcl-2 level expression compared to CMV-specific CD8+ T cells from HIV-infected individuals. Several proteins including Env, Nef, Vpr, Vpu, Vif and Tat have also been shown to play an important role in the
apoptotic induction of CD4+ and CD8+ T cells (Arnoult et al., 2004; Buenz and Badley, 2004; Gougeon, 2003). Increasing evidence also suggests that some of these apoptotic events that occur in HIV infection are further exacerbated by chronic activation of the immune system, partly mediated by persistent exposure to HIV antigens (Badley et al., 2000; Grossman et al., 2002; Hazenberg et al., 2000). Furthermore, the intensity of activation-induced apoptosis in both CD4+ and CD8+ T cells was found to correlate with immune activation and disease progression in chronic HIV infection (Gougeon et al., 1996). Additional evidence further supporting the role of apoptosis in HIV disease progression stems from studies of SIV-infected macaque where apoptosis of T cells was found to be associated with pathogenicity of lentiviral infection (Estaquier et al., 1994; Gougeon, 2003). It has also been shown that HIV specific T cells were skewed toward an activated phenotype, which was associated with increased susceptibility to spontaneous and CD95/Fas induced apoptosis (Chun et al., 2004). Despite this evidence, supporting the role of apoptosis in HIV infection, the mechanisms that govern increased propensity of T cells to undergo apoptosis and the phenotype of T cells that undergo apoptosis remains to be defined. Whether chronic immune activation is the mechanism that governs whether T cells undergo apoptosis is largely unknown. The main hypothesis of this study is that early differentiated CD4+ and CD8+ memory T cells are more likely to undergo apoptosis resulting in a skew towards terminal differentiated effector T cells. This study sought to explore the levels of apoptosis markers in early HIV infection and relate these findings with immune activation and further characterize the sensitivity of different memory CD4+ and CD8+ T cell subsets to spontaneous, CD95/Fas and TNF-RII induced apoptosis.

Chapter 6: Association of plasma mediators of apoptosis and immune activation
6.2 Results

6.2.1 Increased plasma levels of soluble TRAIL and TNF-RII in HIV-1 infected subjects.

To determine the dynamic range of soluble death receptor levels, quantitation of plasma Fas, TRAIL and TNF-RII were first undertaken in chronically HIV infected and HIV uninfected subjects recruited in the CAPT001 cohort of serodiscordant couples (see section 2.1.2 for cohort description, page 53). HIV infected individuals were stratified into groups based on plasma viral load > 10.000 RNA copies/ml (n=13) and < 10.000 RNA copies/ml (n=7). Figure 6.1 shows the comparisons of plasma FAS (A), TRAIL (C) and TNF-RII (E) levels in HIV-infected subjects with viral load greater or less than 10.000 RNA copies/ml in HIV-infected subjects. Plasma Fas levels were significantly higher in HIV-infected subjects with viral load > 10.000 RNA copies/ml (670 ± 336 pg/ml versus 389 ± 145 pg/ml, p=0.0467) compared with HIV-uninfected subjects and similar in HIV-infected subjects with viral load< 10.000 RNA copies/ml (p=NS). Although there was a trend toward increased plasma Fas levels in HIV-infected subjects with viral load < 10.000 RNA copies/ml compared with uninfected subjects (611 ± 133 pg/ml versus 389 ± 145 pg/ml), this did not reach statistical significance (p>0.05). In contrast, plasma TRAIL levels were markedly increased in HIV-infected subjects with viral load greater or less than 10.000 RNA copies/ml compared with uninfected subjects (p<0.0001, Figure 6.1 C, Table 6.1). Similarly, HIV-infected subjects with viral load > 10.000 RNA copies/ml exhibited a significantly increased plasma level of TNF-RII compared with HIV uninfected controls (p<0.0001, Figure 6.1E, Table 6.1). There was also a non-significant
increase in levels of TNF-RII relative to HIV-infected individuals with a viral load <10,000 RNA copies/ml (p>0.05, Figure 6.1E, Table 6.1). To investigate whether increased levels of these soluble death receptors in HIV-1 infection were associated with viraemia, the levels of plasma Fas, TRAIL and TNF-RII were correlated with viral load in HIV-infected subjects (Figure 6.1B, D and F).

**Fig. 6.1** Plasma levels of Fas (A), TRAIL (C) and TNF-RII (E) in HIV-infected with VL>10,000 RNA copies/ml (n=13), HIV-infected with VL<10,000 RNA copies/ml (n=7) and HIV-uninfected subjects (n=9). Correlations of plasma soluble Fas (B), TRAIL (D) and TNF-R-II (F) levels with viral load in HIV-1 infected subjects.

Chapter 6: Association of plasma mediators of apoptosis and immune activation
Comparisons of plasma Fas, TRAIL and TNF-RII in HIV infected and uninfected subjects.

<table>
<thead>
<tr>
<th></th>
<th>HIV+ (VL&gt;10000 copies/ml)</th>
<th>HIV-</th>
<th>HIV+ (VL&lt;10000 copies/ml)</th>
<th>HIV-</th>
</tr>
</thead>
<tbody>
<tr>
<td>sFAS</td>
<td>670.7±93.20*</td>
<td>389.6±48.36</td>
<td>611.8±133.4</td>
<td>389.6±48.36</td>
</tr>
<tr>
<td>sTRAIL</td>
<td>2428±235.9***</td>
<td>574.5±72.00</td>
<td>2484±346.3***</td>
<td>574.5±72.00</td>
</tr>
<tr>
<td>sTNF-RII</td>
<td>818.1±207.9***</td>
<td>79.17±38.59</td>
<td>304.5±84.66</td>
<td>79.17±38.59</td>
</tr>
</tbody>
</table>

* p<0.05, ***p<0.0001

Relating plasma Fas levels to viral load showed that there was negative trend with viral load <10,000 RNA copies/ml (r=-0.3011, p=NS) and positive trend with viral load >10,000 RNA copies/ml (r=0.3567, p=NS). However, these correlations did not yield any statistical significance (Figure 6.1B). Among HIV-infected subjects with VL>10,000 RNA copies/ml, there was a non-significant positive trend (r=0.4462, p=NS) between plasma TRAIL and viral load whereas no association was observed between plasma TRAIL and viral load <10,000 RNA copies/ml (Figure 6.1.D). In contrast, plasma TNF-RII exhibited a non-significant positive relationship between viral load <10,000 RNA copies/ml (r=0.300, p=NS) and viral load >10,000 RNA copies/ml (r=0.4636, p=NS) (Figure 6.1.F). However, this association only reached statistical significance when all the viral loads were correlated with plasma level TNF-RII (r=0.600, p=0.006). Comparative analysis of plasma Fas and TRAIL with all viral load showed no meaningful association (r=-0.085, p=NS, Fas, r=0.1187, p=NS, TRAIL). Collectively, these data suggest that plasma Fas, TRAIL and TNF-RII are up-regulated in chronic HIV infection and that the level of TNF-RII associates with viral load.
6.2.2 Longitudinal analysis of plasma Fas, TRAIL and TNF-RII in acute HIV infection and association with viral load.

Having demonstrated that there is an up-regulation of Fas, TRAIL and TNR-RII in chronic HIV-1 infection; this analysis was extended to the CAPRISA 002 Acute HIV-1 infection cohort. Here, the kinetics of these plasma markers were analysed over the first year of infection and associated with changes in plasma viral load and absolute CD4+ T cell counts. In this analysis, a generalized estimating equation (GEE, see 2.10.1 materials and methods for description) was fitted for plasma Fas, TRAIL and TNF-RII in a subset of 21 participants identified within the first 5 weeks of HIV-1 infection to model the slopes of these plasma markers over 53 weeks. Figure 6.2A, B and C shows the slopes of plasma Fas, TRAIL and TNF-RII, where there was no significant change in Fas, TRAIL and TNF-RII levels (slope=-0.0016, p=0.1748; slope=-0.0011, p=0.3550 and slope=-0.0014, p=0.2595 respectively, Figures 6.2A, B and C). Using the same model to discern whether longitudinal changes in these plasma markers could be related to changes in viraemia over time, it was found that for every one log_{10} increase in HIV RNA copies/ml there was an odds increased in plasma TNF-RII and TRAIL of 1.82 and 0.81 log_{10} respectively (Table 6.2). Using the adjusted GEE model, only changes in plasma TNF-RII with vireamia remained significant (p<0.0001, Table 6.2). In contrast, longitudinal analysis of plasma Fas with vireamia over time using the same model yielded no significant association (adjusted, p=0.5159); unadjusted, p=0.1167). Collectively, these data show of the three apoptotic markers, TNF-RII was significantly associated with viraemia, where for every log_{10} viral load there was a 1.77 log_{10} increase in TNF-RII.
Fig. 6.2 Longitudinal analysis of plasma soluble Fas (A), TRAIL (B) and TNF-RII (C) levels over the first year of infection in the CAPRISA 002 acute HIV-1 infected subjects (n=21). A Generalized Estimating Equation (GEE) modeling fit slopes in the log scale of Fas, TRAIL and TNF-RII are shown with standard error (SE) on each graph.
### Table 6.2
Longitudinal changes in plasma markers of apoptosis and association with viraemia over time.

<table>
<thead>
<tr>
<th>Plasma markers</th>
<th>Unadjusted GEE model</th>
<th>Adjusted GEE model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate</td>
<td>Standard error</td>
</tr>
<tr>
<td>Log_{10} Fas</td>
<td>0.4904</td>
<td>0.3126</td>
</tr>
<tr>
<td>Log_{10} TRAIL</td>
<td>0.8069</td>
<td>0.3682</td>
</tr>
<tr>
<td>Log_{10} TNF-RII</td>
<td>1.8218</td>
<td>0.3503</td>
</tr>
</tbody>
</table>

#### 6.2.3 Plasma Fas, TRAIL and TNF-RII levels at 13 weeks post infection are not associated with the course of vireamia in the first 12 months.

Increased plasma markers of apoptosis such as Fas ligand, TRAIL and TNF-RII have been reported in acute HIV-1 infection as markers of induced cell death soon after viral transmission (Gasper-Smith et al., 2008). However, the relationship between the course of these markers of apoptosis as possible predictors of disease progression remains to be established. To assess whether the course of plasma Fas, TRAIL and TNF-RII levels during early HIV-1 infection are related to viral set point, the levels of these plasma markers at 13 weeks post infection were correlated with viral set point. Viral set point was defined as an average log_{10} RNA copies/ml at three time points around 52 weeks post infection (Appendix 3A, page 208). In Figure 6.3 A, B and C, correlation of plasma Fas and TRAIL at 13 weeks post infection with viral set points revealed no significant associations (Fas, r=-0.248, p=0.3053; TRAIL, r=-0.189, p=0.423). Although there was a trend of a positive relationship between plasma TNF-RII at 13 weeks post infection and viral set point, this association did not yield any statistical significance (r=0.349,
Collectively, these results suggest that plasma markers of apoptosis are elevated during acute HIV-1 infection and that the levels of sFas and TRAIL plasma markers during the earliest stage of HIV-1 infection in this study were associated with viral set point. Additionally, the finding of a positive trend between sTNF-RII and viral set point highlights an important role of the TNF-RII pathway in HIV-1 pathogenesis and is consistent with prior studies that have found sTNF-RII as predictive markers of disease progression (Aukrust et al., 1994; Godfried et al., 1994; Stein et al., 1997).

**Fig. 6.3** Correlation of plasma Fas (A), TRAIL (B) and TNF-RII (C) at 13 weeks post infection with viral set point. Viral set point is defined as an average of log10 viral load at three time points around 52 weeks, ranging from 48 to 56 weeks post infection.
6.2.4 Lack of association between CD4+ T cell count and plasma Fas, TRAIL and TNF-RII.

CD4+ T cell depletion, one of the hallmarks of HIV-1 pathogenesis, has been shown to be associated with spontaneous and activation-induced apoptosis, partly mediated by death receptors (Gougeon, 2003; Petrovas et al., 2005). To investigate whether the levels of soluble death receptors were related to CD4+ T cell depletion, a generalized estimating equation (GEE) was fitted for plasma Fas, TRAIL and TNF-RII to model the slopes and associates this with changes CD4+ T cell counts over the first 53 weeks post infection. Table 6.3 shows the association between changes in plasma Fas, TRAIL and TNF-RII with CD4+ T cell counts. In both adjusted and unadjusted GEE models, correlative analysis of plasma Fas, TRAIL and TNF-RII over time with CD4+ T cell counts revealed that none of these plasma markers of apoptosis bore any relationship with changes in CD4+ T cell counts. However, it is worthwhile to note that quantitation of surface markers of death cell receptors on CD4+ T cells were not assessed due to limited availability of peripheral blood mononuclear cells. Such data would have provided a more direct and useful analysis to link surface expression of death receptors on CD4+ T cells and CD4+ T cell depletion. Relating the plasma markers of apoptosis at 13 weeks post infection with CD4 slopes to determine whether increased levels of plasma Fas, TRAIL and TNF-RII in early HIV-1 infection could predict loss of CD4+ T cells count, also showed that these plasma markers of apoptosis had no bearing on CD4+ T cell depletion (Figure 6.4).
Table 6.3  Longitudinal changes in plasma markers of apoptosis and association with CD4+ T cell counts over time.

<table>
<thead>
<tr>
<th>Plasma markers</th>
<th>Unadjusted GEE model</th>
<th>Adjusted GEE model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate</td>
<td>Standard error</td>
</tr>
<tr>
<td><strong>Log₁₀ Fas</strong></td>
<td>-49.13</td>
<td>73.79</td>
</tr>
<tr>
<td><strong>Log₁₀ TRAIL</strong></td>
<td>-183.55</td>
<td>124.45</td>
</tr>
<tr>
<td><strong>Log₁₀ TNF-RII</strong></td>
<td>-181.80</td>
<td>119.73</td>
</tr>
</tbody>
</table>

Fig. 6.4 Correlation between the levels of plasma Fas (A), TRAIL (B) and TNF-RII (C) at 13 weeks post infection and the slopes of CD4.

6.2.5 Association of plasma Fas, TRAIL and TNF-RII with CD4+ and CD8+ T cell activation.

HIV-1 induces a chronic generalized activation of the immune system, which is considered to be a major driving force of HIV pathogenesis (Deeks et al., 2004; Giorgi et al., 1999; Hazenberg et al., 2003; Paiardini et al., 2009; Silvestri et al., 2007; Sousa et al.,...
2002, Hunt et al., 2008). This chronic activation is believed to be a major contributor of T cell sensitization to apoptosis (Badley et al., 2000; Hazenberg et al., 2003; Petrovas et al., 2005). However, the mechanism underlying this sensitization of T cells to apoptosis by chronic immune activation remains to be elucidated. The aim of this chapter was to examine whether longitudinal changes in plasma Fas, TRAIL and TNF-RII levels could be related to changes in immune activation. This was measured by expression of CD38 and HLA-DR on CD4+ and CD8+ T cells at 5, 13 and 23 weeks post infection using multicolour flow cytometry on the CAPRISA 002 cohort samples. Figure 6.5A and B show the lack of significant correlations of plasma Fas and TRAIL with the percentages of CD4+ and CD8+ T cells expressing CD38+ and HLA-DR+ at 5, 13 and 23 weeks post infection. However, changes in plasma TNF-RII was significantly associated with the percentage of CD4+ and CD8+ expressing CD38+ and HLA-DR+ T cells 5, 13 and 23 weeks post infection (Figure 6.5C). Relating longitudinal changes in plasma TNFR-RII to the percentage of CD4+ CD38+ HLA-DR+ expressing T cells revealed a direct and significant relationship at 5 (r=0.589, p=0.010), 13 (r=0.7626, p=0.0015) and 23 (r=0.467, p=0.067) weeks post infection (Figure 6.5C). Similarly, there was a direct correlation between plasma TNF-RII and the percentage of CD8+ CD38+ HLA-DR+ expressing T cells at 5, 13 and 23 weeks post infection, reaching statistical significance at 13 weeks post infection (r=0.6615, p=0.010). Taken together, these findings show that the up-regulation of TNF-RII levels in plasma is directly related to T cell activation, which is corroborated by several studies reporting that TNF-RII is a marker of soluble immune activation (Aukrust et al., 1994; Godfried et al., 1994; Stein et al., 1997).
Chapter 6: Association of plasma mediators of apoptosis and immune activation

Fig. 6.5 Correlations of plasma Fas, TRAIL and TNF-RII with CD4+ and CD8+ T cell activation defined by the expression of CD38 and HLA-DR at 5 (n=19), 13 (n=14) and 23 (n=16) weeks post infection.
6.2.6 Sensitivity of CD4+ and CD8+ T cells to spontaneous, CD95/Fas and TNF-RII induced apoptosis

The role of apoptosis in CD4+ T cell depletion during HIV and SIV infection is well documented (Badley et al., 2000; Petrovas et al., 2005), but the mechanisms that govern increased propensity of T cells to undergo apoptosis and the phenotype of T cells that undergo apoptosis remain unresolved. Thus far, this chapter has shown that soluble TNF-RII levels in plasma associate directly with viral load and T cell activation. This section now seeks to characterize and delineate the sensitivity of activated CD4+ and CD8+ T cell subpopulations to spontaneous, C95/Fas-, TNF-RII and Etoposide-induced apoptosis. For these experiments, cryopreserved peripheral blood mononuclear cells from HIV-infected (n=6) and uninfected (n=6) subjects in the CAPT001 cohort were stimulated in the absence or presence of CH-11 (anti-CD95/Fas), TNF-RII (anti-TNF-RII) and Etoposide. Induction of CD4+ and CD8+ T cell apoptosis was measured by the expression of active caspase-3 (see material and methods) and etoposide was used as a positive control as it induces DNA strand breaks by inhibiting topoisomerase (the enzyme that induces transient double strand breaks as part of its enzymatic mechanism) (Champoux, 2001; Fortune and Osheroff, 2000). Figure 6.6 shows representative plots of the gating strategy employed for measuring active caspase-3 within CD4+ and CD8+ T cell memory subsets. Memory T cell subsets were measured using differential expression of CD45RA, CD27 and CCR7 as described previously (Burgers et al., 2009; Champagne et al., 2001; Mueller et al., 2001). Based on this expression, CD8+ and CD4+ T cells were classified into six and five distinct subpopulations respectively; Naïve (CD45RA+CD27+CCR7+), central memory (CM, CD45RA-CD27+CCR7+), transitional memory (TM, CD45RA-CD27+CCR7-),
intermediate (Tinter, CD45RA+CD27+CCR7-), effector memory (EM, CD45RA-CD27-CCR7-) and effector (Teff CD45RA+CD27-CCR7-) T cells. The intermediate memory T cell subset was a unique population within CD8+ T cells and has been previously shown to be distinct from naïve and effector cells based on the levels of CD127 and CD57 (Burgers et al., 2009).

Fig. 6.6 Gating scheme for dissection of CD4+ and CD8+ T cell apoptosis in HIV infection measured by expression of active caspase-3. Initial gate was placed on the singlets, followed by lymphocytes with a subsequent exclusion of monocytes/B cells (CD14/19). Sequentially live CD3+, CD4+ and CD8+ events were then defined and further gated on memory CD4+ and CD8+ T cells based on the expression of CD45RA, CCR7 and CD27 for identification of active caspase-3.
As a first step, analysis of active caspase-3 within total CD4+ and CD8+ T cells were compared in HIV infected and uninfected subjects in the CAPT001 cohort. Representative flow cytometry plots showing examples of spontaneous apoptosis, CD95/Fas, TNF-RII and Etoposide induced apoptosis in one HIV infected and uninfected control subject are illustrated in Figure 6.7.

![Flow cytometry plots](image)

**Fig. 6.7** Representative plots for spontaneous apoptosis, TNF-RII, CD95/Fas and Etoposide induced apoptosis in CD4+ and CD8+ T cells are shown for one subject in each group. Apoptosis sensitivity of CD4+ and CD8+ T cells was evaluated based on the simultaneous gating live/dead fixable violet fluorescent reactive dyes and active caspase-3. The percentages are shown in each quadrant for the expression of active caspase-3.
The proportion of spontaneous, TNF-RII, CD95/Fas and etoposide-induced apoptosis on CD4+ T cells were markedly increased in the HIV-1 infected individuals compared with the HIV control (Figure 6.7). Similarly, HIV-1 infected individuals exhibited a significantly increased level of spontaneous, TNF-RII, CD95/Fas and Etoposide induced apoptosis on CD8+ T cells compared to the HIV-1 uninfected control. This is shown collectively in Figure 6.8A, where statistical analysis of CD4+ T cells from six HIV infected subjects revealed a significant increase in spontaneous apoptosis (22.0±1.9% versus 9.4±2.2%, p=0.0043), CD95/Fas (34.2±2.8% versus 18.1±3.3%, p=0.0173), TNF-RII (21.9±2.6% versus 9.0.1±2.2%, p=0.0173) and Etoposide (53.7±1.6% versus 35.9±3.7%, p=0.0095) induced apoptosis as compared with HIV uninfected controls. A similar trend was observed when the induction of spontaneous, CD95/Fas, TNF-RII and Etoposide-induced apoptosis were analysed on CD8+ T cells (Figure 6.8B). Overall, CD8+ T cells from HIV-infected subjects represented 34.8±5.2% versus 14.4±2.2%, 42.9±6.6% versus 19.6±2.5%, 33.9±6.4% versus 15.6±2.5%, and 76.2±4.3% versus 55.4±4.8% for spontaneous apoptosis (p=0.002), CH-11/CD95 (p=0.0043), TNF-RII (p=0.0173) and Etoposide-induced apoptosis (p=0.0190) compared to HIV uninfected controls respectively. Furthermore, the levels of spontaneous, CD95/Fas-, TNF-RII and Etoposide-induced apoptosis were higher on CD8+ T cells compared to CD4+ T cells in both HIV uninfected controls and HIV infected subjects (Figure 6.8C and D). However, this was only significant for spontaneous apoptosis (for both HIV uninfected controls and HIV infected subjects, p=0.041), TNF-RII (for HIV uninfected subjects, p=0.026) and Etoposide induced apoptosis (for HIV uninfected subjects, p=0.0171 and HIV infected subjects, p=0.0286). Together, these findings clearly demonstrate that CD4+ and CD8+ T
cells from HIV infected subjects are more sensitive to spontaneous apoptosis, CD95/Fas, TNF-RII and Etoposide-induced apoptosis. This further corroborates several studies that have demonstrated a higher propensity of CD4+ and CD8+ T cells from HIV infected subjects to undergo apoptosis compared with uninfected subjects (de Oliveira Pinto et al., 2002; Mueller et al., 2001; Petrovas et al., 2007a; Petrovas et al., 2004).

**Fig. 6.8** Susceptibility of CD4+ and CD8+ T cells to spontaneous apoptosis, CD95- and Etoposide-induced apoptosis. Proportion of CD4+ (A) and CD8+ (B) T cells expressing active caspase-3 in HIV-1 infected and uninfected subjects in response to spontaneous, CD95-, TNF-RII and Etoposide induced apoptosis. Comparison of spontaneous, CD95-, TNF-RII and Etoposide induced apoptosis in CD4+ and CD8+ T cells in HIV uninfected (C) and infected (D) subjects.
These data also suggest that CD8+ T cells are more prone to undergo apoptosis compared with CD4+ T cells in both HIV-1 uninfected controls and HIV infected subjects. Apoptosis of CD4+ and CD8+ T cells in HIV infection are likely to be differentially regulated by distinct mechanisms and that this mechanism is Fas-independent and in agreement with prior observations (de Oliveira Pinto et al., 2002; Holm et al., 2004; Homann et al., 2001; Teh et al., 1996).

6.2.7 Sensitivity of activated CD4+ and CD8+ T cells to spontaneous, CD95/Fas and TNF-RII induced apoptosis

The observation that CD4+ and CD8+ T cells from HIV-1 infected subjects are more prone to undergo spontaneous, CD95/Fas and TNF-RII induced apoptosis led to the next stage of the investigation: to study whether apoptosis of CD4+ and CD8+ T cells are related to activation status, as measured by surface expression of HLA-DR and CD38. It has been hypothesized that persistent immune activation in HIV-1 infection is a major mechanism of T cell sensitization to apoptosis (Grossman et al., 2002; Hazenberg et al., 2000). To test this hypothesis, the levels of spontaneous, CD95/Fas, TNF-RII and Etoposide-induced apoptosis were analysed on CD4+ and CD8+ either expressing HLA-DR+, CD38+ or/and HLA-DR+CD38+ cells. Figure 6.9A shows representative flow cytometry plots that delineate the level of apoptosis (gated on violet reactive fluorescent dyes and active caspase-3) and activation (based on the expression of CD38+ and HLADR+) on CD4+ and CD8+ T cells. Further characterization of the levels of apoptosis on CD4+ and CD8+ T cells were assessed by overlaying HLADR+, CD38+, HLADR+CD38+ and HLADR-CD38- CD4+ and CD8+ T cells dot plots (red) on
live/dead v/s active caspase-3 density plots (grey) as shown in Figure 6.9B. The levels of apoptosis were more abundant on CD4+ and CD8+ T cells that expressed either HLA-DR+ or HLA-DR+CD38+.

Fig. 6. 9 Representative flow cytometry plots and gating strategy showing the levels of apoptosis on activated CD4+ and CD8+ T cells. (A) The level of apoptosis and activation were first analysed on CD4+ and CD8+ T cells by gating on live/dead versus active caspase-3 and HLA-DR versus CD38 respectively. (B) Representative density plots depicting the level of apoptosis gated on live/dead versus active caspase-3 (grey) with overlaid HLA-DR+, CD38+HLADR+, CD38+ and CD38-HLADR-CD4+ and CD8+ T cells (red dots).
Figure 6.10 displays increased levels of spontaneous, CD95/Fas, TNF-RII and Etoposide-induced apoptosis on activated CD4+ and CD8+ T cells that either expressed CD38 or HLA-DR. The median percentages of spontaneous apoptosis on CD4+ T cells expressing CD38+ and HLA-DR+ were significantly higher in HIV-infected subjects compared to HIV-uninfected controls (19.9±5.5% versus 11.9±5.5%, p=0.069 for CD38+, 51.9±10.6% versus 35.1±6.4% for HLA-DR+, p=0.0173, Figure 6.10A). Similar results were observed when the median CD38+ and HLA-DR+ co-expression upon CD95/Fas and TNF-RII induced apoptosis on CD4+ T cells were compared between HIV-infected subjects and HIV uninfected controls (Figure 6.10 C and E). Within the CD8+ compartment, HIV-infected subjects also exhibited significantly increased levels of spontaneous; CD95/Fas and TNF-RII induced apoptosis on CD38+ and HLA-DR+ CD8+ T cells (p<0.05, Figure 6.10 B, D and F). When the same analysis was performed on highly activated CD4+ and CD8+ T cells defined by the co-expression of CD38+ and HLA-DR+, no significant differences were observed between HIV-infected subjects and HIV uninfected controls (Figure 6.10A-F, Table 6.4A and B). Additional analysis of spontaneous, CD95/Fas and TNF-RII induced apoptosis on non-activated CD4+ and CD8+ T cells, defined by the lack of CD38 and HLA-DR expression, revealed a significantly increased percentage of apoptosis in HIV-infected subjects compared to HIV uninfected controls (Figure 10A-F, Table 6.4A and B). In Figure 6.10 G and H, the frequency of etoposide-induced apoptosis on activated and non-activated CD4+ and CD8+ T cells also showed a similar trend to those of spontaneous, CD95/Fas and TNF-RII induced apoptosis. Taken together, these results suggest that although activation might play an important role in the selective and sensitivity of CD4+ and CD8+ T cells to
undergo apoptosis, a substantial proportion of T cells that apoptose in HIV infection are not solely linked with activation status. These data indicate that factors other than activation alone contribute to the priming of CD4+ and CD8+ T cells to undergo apoptosis.

Fig. 6. 10 Susceptibility of activated (HLADR+, CD38+ and HLADR+CD38+) and non-activated (HLADR-CD38-) CD4+ and CD8+ T cells to spontaneous (A and B), CD95 (C and D), TNF-RII (E and F) and Etoposide (G and H) induced apoptosis in HIV-1 infected (HIV+, n=6) and uninfected (HIV-, n=6) subjects. Statistical comparisons were performed using Man-Whitney two-tailed test.


**Table 6.4A** Level of spontaneous, CD95/Fas, TNF-RII and Etoposide induced apoptosis on activated CD8+ T cells.

<table>
<thead>
<tr>
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<th>DR+</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-</td>
<td>58.8±5.4</td>
<td>12.±6.1</td>
<td>15.8±5.5</td>
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<tr>
<td>HIV+</td>
<td>63.5±4.7</td>
<td>32.4±14.0*</td>
<td>33.6±12.3*</td>
<td>60.3±5.7*</td>
</tr>
<tr>
<td>Spontaneous&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.7±6.1</td>
<td>17.6±5.9</td>
<td>20.4±7.5</td>
<td>55.2±8.1</td>
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<td>CD95/Fas&lt;sup&gt;a&lt;/sup&gt;</td>
<td>69.3±5.7</td>
<td>39.9±16.4*</td>
<td>43.4±16.1*</td>
<td>66.8±6.27*</td>
</tr>
<tr>
<td>TNF-RII&lt;sup&gt;a&lt;/sup&gt;</td>
<td>61.9±6.8</td>
<td>30.8±16.7*</td>
<td>31.9±13.7*</td>
<td>45.9±8.3</td>
</tr>
<tr>
<td>Etoposide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>83.9±4.3</td>
<td>52.2±13.5</td>
<td>63.8±10.8</td>
<td>63.6±5.2</td>
</tr>
</tbody>
</table>

* Data are expressed as mean± standard deviation. *, p<0.05 is calculated by Mann Whitney non-parametric test.

**Table 6.4B** Level of spontaneous, CD95/Fas, TNF-RII and Etoposide induced apoptosis on activated CD4+ T cells.

<table>
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<th>DR+</th>
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<td>HIV-</td>
<td>51.7±11.1</td>
<td>6.6±4.8</td>
<td>11.9±5.5</td>
<td>35.1±6.4</td>
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<td>HIV+</td>
<td>53.1±11.3</td>
<td>15.3±5.3*</td>
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<td>51.9±10.6*</td>
</tr>
<tr>
<td>Spontaneous&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.7±7.0</td>
<td>30.8±8.7*</td>
<td>27.3±5.8*</td>
<td>72.2±3.5</td>
</tr>
<tr>
<td>CD95/Fas&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59.6±10.2</td>
<td>14.5±7.1</td>
<td>62.4±9.6</td>
<td>45.9±9.7</td>
</tr>
<tr>
<td>TNF-RII&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46.4±7.7</td>
<td>12.3±5.4</td>
<td>34.4±7.8</td>
<td>73.6±2.6*</td>
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<td>Etoposide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.0±61.3</td>
<td>29.8±9.5</td>
<td>44.8±7.8</td>
<td>63.0±5.9</td>
</tr>
</tbody>
</table>

* Data are expressed as mean± standard deviation. *, p<0.05 is calculated by Mann Whitney non-parametric test.
6.2.8 Sensitivity of CD4+ and CD8+ T cell memory subpopulation to spontaneous, apoptosis

To precisely define which phenotype of CD4+ and CD8+ T cell memory subset is more sensitive to apoptosis, levels of active caspase-3 were measured within CD4+ and CD8+ T cells expressing variations of CD45RA, CD27 and CCR7 surface markers (see Figure 6.6 in section 6.2.5). Figure 6.11A shows the proportion of spontaneous apoptosis as measured by expression of active caspase-3 increased within the different CD4+ and CD8+ T cell memory subpopulations. Of note, the proportion of spontaneous apoptosis on CD4+ and CD8+ T cells increased with the differentiation status of memory CD4+ and CD8+ T cells in both HIV infected subjects and HIV uninfected controls. Furthermore, the level of spontaneous apoptosis on CD4+ and CD8+ T cells trended to be higher on EM and Teff cells in HIV infected subjects and HIV uninfected controls. Comparisons of EM and Teff CD4+ and CD8+ memory T cell subpopulations revealed that HIV infected subjects had a significantly higher proportion of CD4+ EM, CD8+ EM and CD8+ Teff that were prone to spontaneous induced apoptosis. Table 6.5A and B shows the level of spontaneous apoptosis was significantly increased in CM, TM and EM CD4+ and CD8+ T cells in HIV-infected compared to uninfected subjects. There was no statistically significant difference observed in the proportion of spontaneous apoptosis on naïve and Teff CD4+ T cells, and naïve and Tinter CD8+ T cells in HIV-infected compared to uninfected subjects.
Fig. 6.11  Susceptibility of CD4+ and CD8+ memory T cell subsets to spontaneous (A), CD95 (B), TNF-RII (C) and Etoposide-induced apoptosis (D) in HIV-1 infected (HIV+, n=6) and uninfected (HIV-, n=6) subjects. CD45RA, CD27 and CCR7 expression were used to delineate Naive (CD45RA+CD27+CCR7+), CM (CD45RA-CD27+CCR7+), TM (CD45RA-CD27+CCR7-), Tint (CD45RA-CD27+CCR7-), EM (CD45RA-CD27-CCR7-) and Eff (CD45RA+CD27-CCR7-).
6.2.9 Sensitivity of CD4+ and CD8+ T cell memory subpopulation to Fas and TNF-RII induced apoptosis

CD95/Fas-induced apoptosis on CD4+ and CD8+ T cells resulted in increased ex vivo sensitivity of CM, TM and EM to apoptosis in HIV-infected compared with HIV uninfected controls (p<0.05, Figure 11B). Apoptosis of CD4+ T cells induced by CD95/Fas on CM, TM and EM was significantly higher in HIV-infected individuals compared with HIV uninfected controls (Table 6.5A). Similar results were observed when the frequency of CD95/Fas induced apoptosis were measured on CM, TM and EM CD8+ T cells in HIV-infected individuals compared with HIV-infected controls (Table 6.5B). Analysis of TNF-RII-induced apoptosis on CD4+ and CD8+ T cells also showed a greater ex vivo sensitivity to apoptosis in CM, TM and EM in HIV-infected compared with HIV uninfected controls (Figure 6.11C). Although the frequency of Etoposide-induced apoptosis on CD4+ and CD8+ T cell subsets mirrored those of CD95/Fas and TNF-RII-induced apoptosis, no significant differences were observed in HIV-infected and uninfected subjects. Collectively, these data indicate that CD4+ and CD8+ T cell memory subsets exhibit different capacities to undergo spontaneous, CD95/Fas and TNF-RII-induced apoptosis with greater ex vivo sensitivity displayed by EM and Teff. This greater sensitivity of late differentiated CD4+ and CD8+ T cell memory subsets to apoptosis may contribute to the skewing of memory maturation of CD4+ and CD8+ T cells. This may explain why, in several studies, there is a lack of more late differentiated memory T cells in HIV infection, in contrast to CMV infection (Champagne et al., 2001; Gougeon, 2003; Mueller et al., 2001; Petrovas et al., 2005; Riou et al., 2012).
Table 6.5A: Apoptosis of CD4+ T cell subsets in HIV-infected and uninfected subjects.

<table>
<thead>
<tr>
<th></th>
<th>HIV-</th>
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<th>HIV-</th>
<th>HIV+</th>
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<tbody>
<tr>
<td>N</td>
<td>0.94 ± 0.62</td>
<td>1.01 ± 0.33</td>
<td>1.14 ± 0.68</td>
<td>0.97 ± 0.34</td>
<td>3.7 ± 2.55</td>
<td>2.7 ± 0.52</td>
<td>0.88 ± 0.25</td>
<td>1.48 ± 0.36*</td>
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<tr>
<td>CM</td>
<td>1.17 ± 0.58</td>
<td>3.48 ± 1.60*</td>
<td>2.16 ± 0.80</td>
<td>8.59 ± 5.4*</td>
<td>10.7 ± 5.87</td>
<td>22.1 ± 7.3</td>
<td>1.07 ± 0.35</td>
<td>3.34 ± 1.42*</td>
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<tr>
<td>TM</td>
<td>1.72 ± 0.70</td>
<td>5.34 ± 1.80*</td>
<td>5.52 ± 1.49</td>
<td>15.8 ± 6.6*</td>
<td>7.56 ± 1.66</td>
<td>23.4 ± 4.78</td>
<td>2.08 ± 0.89</td>
<td>5.84 ± 2.45*</td>
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<tr>
<td>EM</td>
<td>19.1 ± 8.10</td>
<td>39.3 ± 3.70*</td>
<td>38.9 ± 7.52</td>
<td>56.8 ± 4.2*</td>
<td>55.2 ± 7.16</td>
<td>71.1 ± 3.4*</td>
<td>18.81 ± 8.90</td>
<td>38.9 ± 4.87*</td>
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<tr>
<td>Teff</td>
<td>22.7 ± 13.7</td>
<td>22.4 ± 3.40</td>
<td>24.9 ± 16.7</td>
<td>29.2 ± 9.2</td>
<td>62.4 ± 8.1</td>
<td>60.5 ± 4.95</td>
<td>17.43 ± 6.31</td>
<td>20.03 ± 1.31</td>
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* Data are expressed as mean ± standard deviation. *, p<0.05 is calculated by Mann Whitney non-parametric test.

Table 6.5B: Apoptosis of CD8+ T cell subsets in HIV-infected and uninfected subjects.

<table>
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<td>N</td>
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<td>1.4 ± 0.72</td>
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<td>3.9 ± 1.416</td>
<td>0.75 ± 0.13</td>
<td>2.25 ± 0.80*</td>
</tr>
<tr>
<td>CM</td>
<td>2.5 ± 1.6</td>
<td>15.5 ± 1.6*</td>
<td>2.9 ± 1.70</td>
<td>20.9 ± 19.8*</td>
<td>13.9 ± 9.5</td>
<td>37.2 ± 21.2</td>
<td>6.34 ± 2.36</td>
<td>11.09 ± 9.06</td>
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<tr>
<td>TM</td>
<td>3.2 ± 1.7</td>
<td>9.6 ± 4.1*</td>
<td>6.4 ± 2.80</td>
<td>18.9 ± 11*</td>
<td>23.8 ± 7.39</td>
<td>50.6 ± 18.9</td>
<td>1.77 ± 1.11</td>
<td>12.8 ± 15.6*</td>
</tr>
<tr>
<td>Tint</td>
<td>5.3 ± 2.8</td>
<td>7.3 ± 2.90</td>
<td>6.3 ± 3.5</td>
<td>9.4 ± 6.0</td>
<td>35.5 ± 9.36</td>
<td>42.0 ± 9.07</td>
<td>4.30 ± 2.3</td>
<td>11.05 ± 5.64</td>
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<tr>
<td>EM</td>
<td>30.4 ± 4.4</td>
<td>59.3 ± 12.6*</td>
<td>39.9 ± 8.4</td>
<td>65.8 ± 12.6*</td>
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<td>83.8 ± 5.77</td>
<td>30.31 ± 4.6</td>
<td>59.24 ± 13.6*</td>
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<tr>
<td>Teff</td>
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<td>18.6 ± 10.1</td>
<td>33.8 ± 15.9*</td>
<td>64.7 ± 9.43</td>
<td>77.7 ± 7.27</td>
<td>15.18 ± 7.60</td>
<td>26.16 ± 13.38</td>
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</table>

* Data are expressed as mean ± standard deviation. *, p<0.05 is calculated by Mann Whitney non-parametric test.
6.2.10 Distribution of activation markers on CD4+ and CD8+ T cell memory subpopulations

The preceding data indicate that CD4+ and CD8+ T cell memory subsets exhibit different capacities to undergo spontaneous, CD95/Fas and TNF-RII induced apoptosis with greater ex vivo sensitivity displayed by EM and Teff. Whether this differential sensitivity of CD4+ and CD8+ T cell memory subpopulations to apoptosis could be influenced by their activation status remains largely undefined. In an attempt to discern this relationship, the expression of CD38 and HLA-DR on memory CD4+ and CD8+ T cells were analysed to investigate whether differential sensitivity of CD4+ and CD8+ T cell memory subsets to apoptosis were related to their activation status. **Figure 6.12** shows the distribution of CD38+ and/or HLA-DR+ among naïve and memory CD8+ T cell subsets following stimulation with or without CD95/Fas and Etoposide in HIV-infected and uninfected subjects. The proportion of CD38+ and/or HLA-DR+ on memory CD8+ T cell subsets trended to be higher in HIV infected subjects compared to HIV uninfected controls. In HIV-infected subjects, Naïve CD8+ T cells had the lowest proportion of CD38+ or CD38+HLA-DR+ expression whereas CM exhibited a non-significant higher expression of HLA-DR+ or CD38+HLADR+ compared to other CD8+ T cell subpopulations (**Figure 6.12A and C**). In contrast, comparable percentages of HLA-DR+ or CD38+HLADR+ were observed among TM, Tinter, EM and Teff CD8+ T cell subpopulations in HIV infected subjects and the same trend was also seen in HIV-uninfected subjects. Furthermore, expression of CD38+ appears more uniformly distributed among naïve and different memory CD8+ T cell subpopulations in both HIV infected and uninfected subjects (**Figure 6.12B**). Within the CD4+ T cell compartment, the proportion of CD38+
HLA-DR+ did not differ significantly among Naïve and different CD4+ T cell memory subpopulations in HIV-infected subjects and similar trend was also observed in HIV-uninfected subjects (Figure 6.13A). The same profile was also observed when the proportion of CD38+ or HLA-DR+ was compared on Naïve and different CD4+ memory T cell subsets in HIV infected and uninfected subjects (Figure 6.13B and C). Overall, the data infer that CD38+ and/or HLA-DR+ expression are distributed relatively evenly in memory CD4+ and CD8+ T cell subpopulations.

**Fig. 6.12 Activation profiles of CD8+ memory T cells in HIV-infected and uninfected subjects.** The median percentages of HLA-DR+CD38+ (A), CD38+ (B) and HLA-DR+ (C) are shown. Bars indicate the interquartile range for the median value.
Fig. 6.13 Activation profiles of CD4+ memory T cells in HIV-infected and uninfected subjects. The median percentages of HLA-DR+CD38+ (A), CD38+ (B) and HLA-DR+ (C) are shown. Bars indicate the interquartile range for the median value.

6.2.11 Apoptosis profiles of activated memory CD4+ and CD8+ T cell subpopulations

To further delineate whether the sensitivity of memory CD4+ and CD8+ T cell subpopulations to apoptosis is related to immune activation, the levels of spontaneous, CD95/Fas, TNF-RII and Etoposide induced apoptosis were analysed on highly activated (defined by co-expression of CD38+ and HLA-DR+) and non-activated (defined by the lack of CD38- and HLA-DR- expression) CD4+ and CD8+ T cell subsets. As shown in Figure 6.14A, the level of spontaneous induced apoptosis were predominantly on highly activated CD4+ T cell subpopulations (Naïve, CM, TM, EM and Teff) relative to those of...
non-activated CD4+ T cell subpopulations in HIV-infected subjects and HIV uninfected controls (p<0.05). This pattern was also observed when the profile of CD95/Fas and TNF-RII induced apoptosis on highly activated and non-activated CD4+ T cell subpopulations in HIV-infected subjects and HIV uninfected controls were analysed (Figure 6.14B and C). This only reached statistical significance for CD95/Fas and TNF-RII induced apoptosis on naïve, CM, TM and EM highly activated CD4+ T cell subpopulations in HIV-uninfected controls and naïve, CM and Teff highly activated CD4+ T cell subpopulations in HIV-infected subjects. Similarly, induction of apoptosis by Etoposide in HIV-infected subjects and HIV-uninfected controls followed the same trend. This induction of apoptosis was found to be significantly higher in naïve, CM, EM and Teff highly activated CD4+ T cell subjects in HIV-uninfected subjects and naïve and Teff highly activated CD4+ T cell subpopulations in HIV-infected subjects (Figure 6.14D). Within the CD8+ T cell compartment, discernible patterns similar to CD4+ T cell subsets was observed with highly activated CD8+ T cell subpopulations displaying greater *ex vivo* sensitivity to spontaneous, CD95/Fas, TNF-RII and Etoposide induced apoptosis compared to non-activated CD8+ T cell subpopulations in HIV infected subjects and HIV uninfected subjects (Figure 6.14A, B, C and D). Together, these data suggest that the majority of CD4+ and CD8+ T cell subpopulations co-expressing CD38+ and HLA-DR+ were more prone to undergo spontaneous, CD95/Fas and TNF-RII induced apoptosis in HIV infected subjects and HIV uninfected controls. 
Fig. 6.14 Apoptosis profiles of activated and non-activated CD4+ T cell subsets in HIV-infected and uninfected subjects. The median percentages of spontaneous (A), CD95/Fas (B) and Etoposide (C) induced apoptosis are shown. Statistical comparisons were performed using Man-Whitney two-tailed test.
Fig. 6.15  Apoptosis profiles of activated and non-activated CD8+ T cell subsets in HIV-infected and uninfected subjects. The median percentages of spontaneous (A), CD95/Fas (B) and Etoposide (C) induced apoptosis are shown. Statistical comparisons were performed using Man-Whitney two-tailed test.
6.3 Discussion

In SIV and HIV infection, excessive induction of apoptosis has been suggested as one of the major mechanisms responsible for CD4+ T cell depletion (Badley et al., 2000; Cummins and Badley, 2010; Gougeon, 2003; Petrovas et al., 2005). It has been reported that some of the apoptotic events that occurs in HIV infection are further exacerbated by chronic immune activation mediated by persistent exposure to HIV antigens (Cummins and Badley, 2010; Grossman et al., 2002; Hazenberg et al., 2000). While the mechanisms of immune mediated cell death in early HIV-1 infection remains to be resolved, induction of cell death pathways by HIV Env, Nef, Vpr, Vpu, Vif and Tat have been reported in chronic HIV-1 infection (Arnoult et al., 2004; Buenz and Badley, 2004; Cummins and Badley, 2010; Gougeon, 2003; Petrovas et al., 2005). This chapter reports on the levels of plasma markers of apoptosis mediators in early and chronic HIV infection and how these markers associate with changes in viraemia, CD4+ T cell counts and immune activation markers over time. The results show that plasma levels of TRAIL, TNF-RII and Fas are significantly increased in HIV-1 infection and that TRAIL and TNF-RII are directly associated with changes in viraemia over time. Consistent with these findings, elevated levels of plasma sTRAIL and sTNF-RII have been shown in HIV infection (de Oliveira Pinto et al., 2002; Herbeuval et al., 2005a; Herbeuval et al., 2009; Lederman et al., 2000; Ostrowski et al., 2006). A study by Gasper-Smith (Gasper-Smith et al., 2008) also demonstrated that increased levels of plasma sTRAIL appear 7.2 days before the peak of viraemia and that up-regulation of sTNF-RII levels coincide with peak viraemia at the acute phase of HIV-1 infection. In support of this, studies in SIV and HIV infection have
shown that depletion of gut CD4+ T cells occurs as early as 7 days after infection (Picker et al., 2004; Veazey et al., 1998; Viollet et al., 2006) and other studies have reported depletion of gut CD4+ T cells during the first month of HIV infection (Brenchley et al., 2004; Guadalupe et al., 2003). Additional support for the role of TRAIL in HIV-1 infection comes from the study of Herbeuval et al (Herbeuval et al., 2005a) which has demonstrated that plasmacytoid dendritic cells from HIV-1 infected patients with high viral loads express higher levels of TRAIL and are able to induce selective apoptosis of HIV uninfected CD4+ T cells (Cummins and Badley, 2010; Herbeuval et al., 2005b). Increased expression of TRAIL and DR5 in lymphoid tissues from HIV infected patients have also been reported and plasma TRAIL levels appear to be directly correlated with viral load suggesting this pathway contributes to CD4+ T cell depletion during infection (Herbeuval et al., 2005a; Herbeuval et al., 2009). Consistent with these studies (de Oliveira Pinto et al., 2002; Gasper-Smith et al., 2008; Herbeuval et al., 2005a; Herbeuval et al., 2009), changes in the slope of TRAIL reported in this chapter was directly associated with viraemia. There was also a significant increase in plasma levels of sTNF-RII and the changes in the slope of TNF-RII over the first year of HIV infection was directly related to changes in viral load. Plasma levels of TNF-RII are thought to reflect TNF-α activity and increased concentration of soluble TNF-RII in HIV infection are associated with increased immune activation and the decline of CD4+ T cells (Aukrust et al., 1994; Godfried et al., 1994; Look et al., 2000; Stein et al., 1997; Zangerle et al., 1994a; Zangerle et al., 1994b). In contrast, the longitudinal data presented in this chapter, found no association between changes in sTNF-RII and decline in CD4+ T cell counts over time, which is also in agreement with the studies of Bilello et al. (Bilello et al., 1996)
and Hober et al. (Hober et al., 1999). They found no associations between the levels of sTNF-RII and the decrease in CD4+ T cell counts using multivariate analysis in individuals with chronic HIV infection. The data presented in this chapter is unique in that longitudinal changes of sTNF-RII and CD4+ T cell are reported during the earliest stage of HIV infection, which may account for some of the differences seen in other studies (Aukrust et al., 1994; Godfried et al., 1994; Zangerle et al., 1994a). Although, several studies have suggested a role of TRAIL in the apoptosis of CD4+ T cells in HIV-1 infection (Herbeuval et al., 2005b; Lum et al., 2001; Yang et al., 2003a), up-regulation of plasma TRAIL found in this chapter was also not associated with decreased CD4+ T cell counts. Data from in vitro studies also suggest that CD4+ T cells exposed to HIV-1 undergo apoptosis by a TRAIL-dependent mechanism, which is inhibited by anti-IFN-α antibodies (Herbeuval et al., 2005b). This would suggest that IFN-α plays an important role in HIV-1 induced TRAIL expression by CD4+ T cells. Increased expression of TRAIL on CD4+ T cells have been reported in individuals with HIV-1 infection (Herbeuval et al., 2005b) and TRAIL has been shown to induces selective apoptosis of uninfected CD4+ T cells in HIV-1 infected human peripheral blood lymphocyte-non-obese diabetic-severe combined immunodeficient (hu-PBL-NOD-SCID) mice (Miura et al., 2001). Collectively, the data presented in this chapter show that plasma levels of sTRAIL and sTNF-RII, when closely measured over the first of year of infection, associate with viral load but have no bearing on CD4+ T cell counts. The mechanism of CD4+ T cell depletion, a hallmark of HIV pathogenesis, is largely undefined and emerging data has proposed chronic immune activation as a major mechanism of CD4+ T cell sensitization to apoptosis (Badley et al., 2000; Gougeon, 2003; Grossman et al., 2002;
Hazenberg et al., 2000; Petrovas et al., 2005). In addition, studies by Deeks et al (Deeks et al., 2004) and Sousa et al (Sousa et al., 2002) in HIV-1 infection have observed a direct linked between loss of CD4+ T cells and immune activation suggesting that elevated immune activation in HIV-1 infection predicts subsequent CD4+ T cell decline. The objective of this chapter was to identify whether up-regulation of plasma markers of apoptosis mediators in early HIV-1 infection are linked to immune activation. The data presented here suggest that levels of plasma sTNF-RII over the first 6 months of HIV-1 infection were directly associated with immune activation, defined by CD4+ T cells co-expressing CD38+ and HLA-DR+. These data support and extend the findings of several studies, which have demonstrated a direct link between increased serum levels of sTNF-RII and immune activation in HIV-1 infection (Aukrust et al., 1994; Look et al., 2000; Zangerle et al., 1994a). Of considerable interest in relation to these finding is the observation of decreased percentages of membrane-bound TNF-RII in monocytes and lymphocytes, and increased circulating levels of soluble TNF-RII in individuals with advanced HIV-1 disease (Hestdal et al., 1997). Increased spontaneous release of sTNF-RII by PBMCs after HIV-1 infection in vitro has also been reported suggesting an enhanced shedding of sTNF-RII in PBMCs from HIV-1 infected individuals (Hestdal et al., 1997). This observation is further corroborated by a study of de Oliveira et al. (de Oliveira Pinto et al., 2002) which has shown that increased susceptibility of CD4+ and CD8+ T cells to TNF-RI and TNF-RII in HIV infection was not related to up-regulation of their membrane-bound receptors expression on CD4+ and CD8+ T cells. Although, early in vitro studies by Katsikis et al. (Katsikis et al., 1995) showed no differences between the levels of spontaneous and TNF-R-II apoptosis in HIV-1 infection similar to the present
study, a study by de Oliveira et al. (de Oliveira Pinto et al., 2002) observed an increased ex vivo sensitivity of CD4+ and CD8+ T cells to TNF-RI and TNF-RII induced apoptosis. This was shown to be linked to in vivo down regulation of Bcl-2 and not related to membrane-bound TNF-RI and TNF-RII expression on CD4+ and CD8+ T cells or altered expression of adapter proteins TRADD, TRAF-2 and RIP in lymphocytes from HIV-1 infection (de Oliveira Pinto et al., 2002). Increased sensitivity of CD8+ T cells and HIV-1 specific CD8+ T cells to spontaneous and CD95/Fas-induced apoptosis has also been shown to inversely correlate with down-regulation of Bcl-2 levels in HIV-1 infection suggesting that down-regulation of this anti-apoptotic molecule might be responsible for the enhanced apoptosis sensitivity and short survival of CD8+ T cells in HIV-1 infection (Petrovas et al., 2004). In agreement with previous studies (de Oliveira Pinto et al., 2002; Mueller et al., 2001; Petrovas et al., 2007a; Petrovas et al., 2004), the present study also found a significant increased ex vivo sensitivity of CD4+ and CD8+ T cells to spontaneous, CD95/Fas and TNF-RII induced apoptosis in HIV-1 infected subjects compared to HIV uninfected controls. Furthermore, activated CD4+ and CD8+ T cells were found to be more sensitive to spontaneous, CD95/Fas and TNF-RII induced apoptosis compared to non-activated CD4+ and CD8+ T cells in both HIV-1 infected subjects and HIV uninfected controls. These data along with other studies (Badley et al., 2000; Hazenberg et al., 2003; Petrovas et al., 2005) suggest that immune activation might play an important role in sensitizing T cells to apoptosis. In support of this concept, Petrovas et al. (Petrovas et al., 2007a) showed a significant correlation between ex vivo expression of CD38+ CD8+ T cells, and spontaneous and CD95/Fas-induced apoptosis in HIV-1 infection. Another study by Chun et al. (Chun et al., 2004) revealed that HIV-1
specific CD8+ T cells were skewed toward a CD38+ CD8+ T cells phenotype, characterized by *ex vivo* sensitivity to spontaneous and CD95/Fas induced apoptosis. It has also been speculated that increased susceptibility of CD4+ and CD8+ T cells to spontaneous apoptosis during HIV-1 infection correlates with immune activation and disease progression, further supporting a critical role of immune activation in priming CD4+ and CD8+ T cells to undergo apoptosis (Gougeon et al., 1996). Several lines of evidence also suggest that total and HIV-1 specific CD8+ T cells are associated with markedly reduced levels of anti-apoptotic molecules Bcl-2 and Bcl-xL, indicating an intrinsic defect or dysregulation of extrinsic apoptosis pathway in HIV-1 infection (Petrovas et al., 2004). It has been shown that the binding of HIV gp120 to CD4 receptor that is expressed on activated T cells can induce the down-regulation of Bcl-2, thereby promoting the release of cytochrome c and induction of apoptosis (Arnoult et al., 2004; Buenz and Badley, 2004). The Tat protein has also been shown to induce apoptosis by down-regulation of Bcl-2, and up-regulation of caspase-8 and CD95/CD95L on HIV-1 infected T cells (Arnoult et al., 2004; Gougeon, 2003). Collectively, these data suggest that HIV can indirectly induce apoptosis of CD4+ and CD8+ T cells through down-regulation of Bcl-2 and up-regulation CD95/CD95L. Despite increasing evidence that supports the role of Bcl-2 in sensitizing CD8+ T cells to apoptosis, comparable levels of Bcl-2 on CD4+ T cells have also been reported in both HIV-1 infected and uninfected individuals (Petrovas et al., 2004) suggesting that different intracellular pathways might be involve in the regulation of CD4+ and CD8+ T cell apoptosis. It has been shown *in vitro* that activated CD4+ T cells that express FasL can kill Fas expressing CD8+ T cells independent of antigen recognition (Arnoult et al., 2004; Piazza et al., 1997 #100). This
mechanism might explain increased \textit{ex vivo} sensitivity of CD8+ T cells to spontaneous, CD95/Fas and TNF-RII induced apoptosis compared to CD4+ T cells in both HIV-1 infected and uninfected individuals reported in this study.

Up-regulation of CD95L/FasL on the surface of infected macrophages have been shown to interact with up-regulated CD95/Fas expressed on the surface of uninfected CD4+ T cells further supporting the role of soluble factors in enhancing CD4+ T cell apoptosis and depletion (Badley et al., 2000). Although, these findings support the role of HIV-1 mediated acceleration of T cell destruction in HIV-1 infection, the phenotype of the T cells that are prone to undergo apoptosis has not been extensively characterized. This chapter has identified that effector memory (EM) CD4+ and CD8+ T cells were more sensitive to spontaneous, CD95/Fas and TNF-RII induced apoptosis in both HIV infected subjects and HIV uninfected controls in agreement with previous studies (Estaquier et al., 1995; Katsikis et al., 1995; Meynard et al., 1992; Mueller et al., 2001; Petrovas et al., 2007a; Viollet et al., 2006). Further support of this finding are the studies of Gougeon \textit{et al} (Gougeon et al., 1996) and McCloskey \textit{et al} (McCloskey et al., 1998) which have found an increased \textit{ex vivo} sensitivity of CD45RO$^{High}$ CD8+ T cells (predominantly effector memory T cells) to CD95/Fas induced apoptosis in HIV-1 infected subjects. In another study by Mueller et al (Mueller et al., 2001) it was demonstrated that accumulation of HIV specific CD8+ T cells in the CD45RA-CD62L- compartment was associated with increased \textit{ex vivo} sensitivity to spontaneous and CD95/Fas induced apoptosis again supporting the findings presented in this chapter. Furthermore, it is demonstrated in this chapter that the level of activation was evenly distributed among different memory CD4+
and CD8+ T cell subpopulations and that activated memory CD4+ and CD8+ T cell subpopulations were sensitive to spontaneous, TNF-RII and CD95/Fas induced apoptosis indicating that immune activation might contribute to the selective apoptosis sensitivity of CD4+ and CD8+ T cells in HIV-1 infection. Consistent with this observation, increased level of spontaneous and CD95/Fas induced apoptosis on activated total and HIV specific CD8+ T cells has been reported in HIV infection and suggested as a possible mechanism for T cell sensitization to apoptosis (Badley et al., 2000; Hazenberg et al., 2000). Several studies have also reported a strong correlation between the levels of spontaneous and CD95/Fas induced apoptosis with ex vivo expression of CD38 on HIV specific CD8+ T cells (Chun et al., 2004; Petrovas et al., 2007a) again supporting an increased sensitivity of activated CD4+ and CD8+ T cells to apoptosis observed in this chapter. In addition, a study by Appay et al (Appay et al., 2002) found an inverse relationship between the levels of immune activation and Bcl-2 expression on HIV specific CD8+ T cells during acute HIV infection suggesting that the Bcl-2 anti-apoptotic molecule is likely to sensitized HIV specific CD8+ T cells to apoptosis. Together, these data explain the differential sensitivity of CD4+ and CD8+ T cell memory subpopulations to apoptosis observed in this chapter and highlight the important role of apoptosis in the pathogenesis of HIV-1 infection.

In summary, the findings presented in this chapter show that the levels of plasma sFas, sTRAIL and sTNF-RII are significantly up-regulated in HIV infection and that changes in plasma sTNF-RII levels in acute HIV infection associate with viral load and immune activation. Furthermore, the levels of spontaneous, CD95/Fas and TNF-RII induced apoptosis were significantly higher in CD4+ and CD8+ T cells from HIV infected subjects.
highlighting the important role of apoptosis during HIV infection. The findings of high levels of spontaneous, CD95/Fas and induced apoptosis on activated CD4+ and CD8+ T cells in HIV infection suggest that chronic immune activation is likely to prime CD4+ and CD8+ T cells to apoptosis. The present data also indicate that CD4+ and CD8+ effector memory and effector T cells are more prone to spontaneous, CD95/Fas and TNF-RII induced apoptosis and suggest that apoptosis may be a critical mechanism that alters the balance of T cell memory differentiation in HIV infection.
CHAPTER 7

CONCLUDING REMARKS

Considerable progress made over the last decade has provided some insight on the role of the earliest immune responses to HIV-1 infection. Throughout this thesis, we have attempted to investigate the influence of CD8+ T cell responses on viral set point and subsequently progression to AIDS. In the first part of this thesis in Chapter 3, we described some of the earliest T cell responses that occurs within the first 3 months of HIV-1 subtype C infection and their association with viral set point at 12 months post infection. This emanated from the hypothesis that the magnitude and breadth of early T cell responses at 3 months post infection would determine the course of viraemia and disease progression.

Herein, we identified a distinct hierarchical targeting of IFN-γ T cell responses at 3 months post infection in the order of cumulative T cell responses in Nef, Pol, Gag, Env, Vif, Vpr, Rev, Vpu and Tat; and also demonstrated that none of this early hierarchical targeting of T cell responses were related to the course of viraemia and disease progression in acute subtype C HIV-1 infection. These data suggest that either the IFN-γ ELISPOT assay is not able to identify relevant early T cell responses that may predict disease outcome or the nature of the initial immune responses that may dictate the course of viraemia is more complex when measured at static time point. Building on this prior work, the analysis in Chapter 4 extended these observation and tracked T cell responses from as early 3 weeks post infection and related this to disease progression and viral
sequence evolution over time. It was shown using a GEE model that Gag and Env were associated with decrease and increase viral load over time, respectively. Collectively, these data highlight the important role of anti-Gag T cell responses in control of viraemia and further supports the hypothesis that the ability to target Gag in vivo contributes to viral control. We further showed that the magnitude of T cell responses fluctuated widely over the first year of infection and this was associated with three distinct profiles T cell recognition, characterized by lost, new or persistent T cell responses. When these profiles of T cell recognition patterns were related to disease categories, it was shown that individuals with rapid progression had a significantly less persistent and a greater number of new T cell responses compared with intermediate and slow progressors. In contrast, individuals with early slow disease progression appeared to possess a greater number of persistent and no loss of T cell responses compared with those showing rapid disease progression. Furthermore, the tempo of lost T cell responses was significantly associated with estimated time of viral escape (95% confident interval (CI): 14 weeks, range: 4.4 - 24). Collectively, the broader interpretations of these data are that the temporal patterns of T cell fluctuations reflects the rapidity of T cell evolution and the unpredictable nature of T cell recognition patterns during acute and early stage of infection, and means that a static measurement in time is not a reliable marker of recognition and has a little meaning for disease progression.

In chapter 5, an in-depth analysis of the polyfunctional profiles of CD8+ T cells and the relationship with CD8+ T cell epitope selection pressure was made. It was shown that the total magnitude of HIV specific CD8+ T cell was associated with early selection of escape
mutants and that neither the polyfunctional nature, memory maturational profiles nor perforin expression of CD8+ T cells were associated the selection of epitope. These data highlight a temporal link between a strong immune driven CD8+ T cell pressure and selection of escape mutants in early HIV-1 infection.

In the last chapter, the impact of soluble markers of apoptosis on viral set point and disease progression showed that the levels of soluble Fas, TRAIL and TNF-RII at 3 months post infection were not associated with the course of viraemia and CD4 T cell counts in the first 12 months. When the changes in these plasma markers of apoptosis were modelled over time, there was a direct association between the levels of TRAIL and TNF-RII with viraemia suggesting the important role of TRAIL and TNF-RII pathway in HIV-1 pathogenesis. In addition, plasma sTNF-RII over the first 6 months of HIV-1 infection was also directly associated with immune activation. Furthermore, T cells from HIV-1 infected subjects were highly sensitivity to spontaneous, Fas/CD95 and TNF-RII-induced apoptosis compared to control subjects, and the level of apoptosis were predominantly on highly activated CD4+ and CD8+ memory T cell subpopulation in both HIV-1 infected and control subjects. This would suggest that activation is likely to prime T cells to apoptosis and specifically those with a more differentiated memory phenotype.

Overall, the work presented in the PhD raises has shed light onto (i) the character of T cell immunity that associates with viral replication in acute HIV-1 infection, (ii) the use of the IFN-γ ELISPOT assay as a immunological readout to assess the impact of T cell immunity on viral set point and disease progression, (iii) the temporal nature of T cell fluctuations.
that associates with disease progression and (iv) the impact of the polyfunctional nature of CD8+ T cells on the early selection of CTL mutants. Translation of this knowledge to understanding correlates of T cell mediated immunity to future HIV vaccines will provide valuable insight into strategies for reducing the global AIDS epidemic, most notably in Southern Africa.
APPENDICES

Appendix 1 A: Ethical clearance certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49  Mr Mandla D Mlotshwa

CLEARANCE CERTIFICATE  M121001

PROJECT
Characterization of HIV-1 Specific T Cell Responses during HIV-1 Subtype C Infection

INVESTIGATORS
Mr Mandla D Mlotshwa.

DEPARTMENT
School of Pathology/Aids Research Unit

DATE CONSIDERED
26/10/2012

DECISION OF THE COMMITTEE*
Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 26/10/2012  CHAIRPERSON

(Professor PE Cleaton-Jones)

*Guidelines for written ‘informed consent’ attached where applicable
cc: Supervisor : Prof Lynn Morris

DECLARATION OF INVESTIGATOR(S)
To be completed in duplicate and ONE COPY returned to the Secretary at Room 10004, 10th Floor,
Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned
research and I/we guarantee to ensure compliance with these conditions. Should any departure to be
contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the
Committee. I agree to a completion of a yearly progress report.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...
Appendix 1B: Ethical clearance certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49  Gray, et al

CLEARANCE CERTIFICATE

PROJECT
CAPRISA 002 Viral Set Point and
Clinical Progression in HIV-1 subtype C
infection: The Role of Immunological and
viral factors during acute and early infection

INVESTIGATORS
Prof C Gray, et al

DEPARTMENT
National Institute for Communicable Diseases

DATE CONSIDERED
n/a

DECISION OF THE COMMITTEE*

Approved unconditionally
Re-certification of Protocol M040202
for a further 5 years (Version 4.00
dated 19 June 2007)

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon
application.

DATE 08.04.25

CHAIRPERSON
(Professor P E Cleary Jones)

*Guidelines for written ‘informed consent’ attached where applicable

cc: Supervisor:

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10004, 10th Floor,
Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned
research and I/we guarantee to ensure compliance with these conditions. Should any departure to be
contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the
Committee. I agree to a completion of a yearly progress report.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES
Appendix 1C: Ethical clearance certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 de Bruyn

CLEARANCE CERTIFICATE

PROJECT

INVESTIGATORS
Dr G de Bruyn

DEPARTMENT
Perinatal HIV Research Unit

DATE CONSIDERED
07.03.02

DECISION OF THE COMMITTEE*
APPROVED UNCONDITIONALLY

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 07.04.22

CHAIRPERSON (Professors PE Cleaton-Jones, A Dhai, M Vorster, C Feldman, A Woodiwiss)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor:

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10005, 10th Floor, Senate House, University.

I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. I agree to a completion of a yearly progress report.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES
Appendix 1D: Design of peptide pool matrix format and whole genome ELISPOT screening plate layout.
Appendix 1E: Autologous virus sequencing

RNA was isolated from plasma samples using the Magna-Pure Compact Nucleic Extractor (Roche Diagnostics Corporation, Indianapolis, USA) and reverse transcribed using the Invitrogen Thermoscript Reverse Transcription Kit (Invitrogen, Paisley, United Kingdom) and the primer, Gag D reverse (5'-AAT TCC TCC TAT CAT TTT TGG-3'; HXB pos 2382-2402) and Nef O reverse (5'-AGG CAA GCT TTA TTG AGG -3'; HXB pos 9608-9625) for nef as previously described (Chopera et al., 2008). Limiting dilution nested PCR was carried out by serial end-point dilution of the cDNA (Rodrigo et al., 1997). The first round PCR primers were Gag D forward (5'-TCT CTA GCA GTG GCG CCC G-3'; HXB pos 626-644) and Gag D reverse (5'-AAT TCC TCC TAT CAT TTT TGG-3'; HXB pos 2382-2402). The second round PCR primers were Gag A forward (5'-CTC TCG ACG CAG TCG GCT T-3'; HXB pos 683-704) and Gag C reverse (5'-TCT TCT AAT ACT GTA TCA TCT GC-3'; HXB pos 2334-2356). For nef, the first round PCR primers were SQ15F (5'-GAG AGC GGT G CT TCT-3'; HXB pos 8561-8578) and Nef O reverse. The second round PCR primers were Nef forward (5'-CCT AGA AGA ATA AGA CAG GGC TT-3'; HXB pos 8754-8776) and Nef reverse (5'-CCT GGA ACG CCC CAG TGG-3'; HXB pos 9443-9461). PCR products were either directly sequenced or cloned using the pGEM-T Easy vector system (Promega). Sequencing was carried out using an ABI PRISM dye terminator cycle-sequencing kit (Applied Biosysytems) and the primers Gag A forward, Gag A reverse (5'-ACA TGG GTA TCA CTT CTG GGC T-3'; HXB pos 1282-1303), Gag B forward (5'-CCA TAT CAC CTA GAA CTT TGA AT-3'; HXB pos 1226-1246), Gag B reverse (5'-CTC CCT GAC ATG CTG TCA TCA T-3'; HXB pos 1825-1846), Gag C forward (5'-CCT TGT TGG TCC AAA ATG CGA-3'; HXB pos 1748-1768) and Gag C reverse for direct sequencing. Sequences were assembled using ChromasPro (www.technelysium.com.au/chromas.html) and aligned using ClustalW (with default settings; (Thompson et al., 1994)).
Appendix 1F: Standard curves and duplicate correlation of sFas, sTRAIL and sTNF-RII.

**A** sFas Standard curve

**B** sTRAIL standard curve

**C** sTNF-RII Standard curve

**D** sFas

**E** sTRAIL

**F** sTNF-RII
### Appendix 1 G: Custom Designed BD LSR II detectors configuration

<table>
<thead>
<tr>
<th>Laser</th>
<th>Detector Name</th>
<th>Flurochrome detected</th>
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<th>BP Filter</th>
</tr>
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<td>FSC</td>
<td>Forward Scatter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blue 488nm</td>
<td>A</td>
<td>PE Blue</td>
<td>685LP</td>
<td>710/40</td>
</tr>
<tr>
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<td>B</td>
<td>FITC, CFSE, GFP</td>
<td>505LP</td>
<td>515/20</td>
</tr>
<tr>
<td>Blue 488nm</td>
<td>C</td>
<td>Side scatter</td>
<td>488LP</td>
<td></td>
</tr>
<tr>
<td>Green 532nm</td>
<td>A</td>
<td>Cy7PE, Alexa 750PE</td>
<td>740LP</td>
<td>780/40</td>
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<td>Green 532nm</td>
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Appendix 3 A1: Representative of T cell recognition in Profile 1: Lost responses
Appendix 3 A1: Representative of T cell recognition in Profile 1: Lost responses
Appendix 3 A2: Representative of T cell recognition in Profile 2: New or emerging responses
Appendix 3 A3: Representative of T cell recognition in Profile 3: Persistent responses
Appendix 3 A3: Representative of T cell recognition in Profile 3: Persistent responses
## Appendix 4: Summary of selected variant and invariant epitopes identified in 4 acutely HIV-1 infected subjects.

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Appendix 4: Summary of selected variant and invariant epitopes identified in 4 acutely HIV-1 infected subjects.

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### Appendix 4: Summary of selected variant and invariant epitopes identified in 4 acutely HIV-1 infected subjects.

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<th>PTID</th>
<th>Weeks</th>
<th>PI</th>
<th>Median time of escape</th>
<th>CTL epitope and HLA restriction</th>
<th>Epitope sequence</th>
<th>No. of sequences</th>
<th>% Variant</th>
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<td>D816N LV9 Env&lt;sub&gt;814-822&lt;/sub&gt;</td>
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<td>100%</td>
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<td>................V..............</td>
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<td>9%</td>
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