UNIVERSITY OF KWAZULU-NATAL

THE ROLE OF HLA-C RESTRICTED CD8+ T CELL RESPONSES IN THE CONTROL OF HIV REPLICATION

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PREFACE

The experimental work described in this dissertation was performed in the HIV Pathogenesis Programme (HPP) Laboratory, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban from January 2008 to December 2009. This work was supervised by Professor Thumbi Ndung'u. These studies represent original work by the author and have not been submitted in any form for any degree or diploma to any other university. When the work of others has been used, it is clearly acknowledged in the text.

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ABBREVIATIONS

AIDS	-Acquired immunodeficiency syndrome
HIV	-Human Immunodeficiency Virus
WHO	-World Health Organisation
ELISPOT	-Enzyme-linked immunospot assay
IFN-γ	-Interferon- gamma
TNF-α	-Tumor necrosis factor- alpha
MIP-1β	-Macrophage inflammatory protein- 1 beta
IL-2	-Interleukin- 2
PCR	-polymerase chain reaction
ICS	-Intracellular Cytokine Staining
PBMC	-Peripheral blood mononuclear cells
HLA	-Human Leukocytes Antigen
MHC	-Major Histocompability Complex
HAART	-Highly active antiretroviral therapy
CTL	-CD8+ T lymphocytes
Env	-Envelope protein
Gp41, 120, 140	-Glycoprotein 41, 120, 140
CA	-Capsid
Nef	-Negative factor
Pol	-Polymerase
RT	-Reverse Transcriptase
TBE	-Tris base, Boric acid, EDTA
TB	-Tuberculosis
Tat	-Trans-Activator of Transcription

Vpr	-Viral Protein R
Vpu	-Viral Protein U
Vif	-Virion Infectivity factor
Rev	-Regulator of Virion
RRE	-Rev Response Element
SIV	-Simian Immunodeficiency Virus
NLS	-Nuclear localization sequence
NES	-Nuclear export sequence
RNA	-Ribonucleic acid
DNA	-Deoxyribonucleic acid

ABSTRACT

Certain HLA-B-restricted CD8+ T cell responses are associated with control of viremia whereas HLA-Cw* restricted responses, including Gag epitopes are associated with high viremia. To better understand the role of HLA-Cw* restricted epitopes in viral control, HLA-Cw* restricted epitopes were optimally defined. Seventy eight study subjects from a cohort of 451 chronically infected participants had HLA-Cw* restricted CD8+ T cells responses as quantified by intracellular cytokine staining assessing IFN-γ secretion. Fine mapping and HLA restriction of the optimally defined HLA-Cw* restricted epitopes were performed using ELISPOT assay. Functional avidity of responses was assessed by peptide dilution in an ELISPOT assay. Two novel HLA-Cw* restricted epitopes Cw*04-TF10 (in reverse transcriptase) and Cw*08-RM9 (in gp120) were optimally defined. A previously described epitope, Cw*07- KY11 (Nef) was the most frequently targeted epitope in this cohort (30/78) and has high functional avidity compared to other HLA-Cw restricted CD8+ T cell responses.

The polyfunctionality of HLA-B*57/5801-restricted Gag-specific HIV-1 CD8+ T cell responses and HLA-Cw*07-KY11 restricted CD8+ T cell responses within the same study subject was determined. Polyfunctionality of CD8+ T cell responses to HLA-B*57/5801 and HLA-Cw*07 restricted epitopes were determined in nine study subjects assessing IFN-γ, TNF-α, IL-2, MIP-1β, and CD107a by multicolour flow cytometry.

Additionally *gag* and *nef* genes were sequenced from plasma. HLA-B*57/5801-restricted IFN-γ-producing CD8+ T cell responses were of lower magnitude than HLA-Cw*07 responses (p=0.0012) for the nine subjects. The majority of responses were monofunctional (75%), irrespective of HLA restriction. HLA-B*57/5801 and HLA-Cw*07 restricted CD8+ T cells did not differ significantly in polyfunctionality (p=0.84). Possession of ≥3 functions correlated positively with CD4+ T cell counts (r=0.85; p=0.006). The percentages of monofunctional CD8+ T cells inversely correlated with CD4+ T cell counts (r=-0.79; p=0.05). There was no correlation between polyfunctionality and viral load and sequence variation within targeted epitopes did not impact polyfunctionality. These results suggest that polyfunctionality of HIV-1-specific CD8+ T cells is associated with disease progression independent of restricting HLA alleles, and that loss of these polyfunctional cells correlates with increased in the frequency of monofunctional virus-specific CD8+ T cells. In addition, sequence variation does not appear to significantly impact CD8+ T cell polyfunctionality in chronic HIV infection.

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CHAPTER 1. INTRODUCTION

1.1. HIV AND IMMUNE SYSTEM

The human immunodeficiency virus (HIV) is the causative agent for the most destructive pandemic worldwide since it was first reported in 1981. There are about 33.4 million reported cases of people living with HIV infection worldwide and 22.4 million live in sub-Saharan Africa (UNAIDS, 2009). South Africa has the highest rate of HIV infection in the world with the province of KwaZulu-Natal leading the HIV prevalence in South Africa. Currently, there is no cure for HIV or an effective vaccine (Hahn et al., 2000, Goulder and Watkins, 2008). However, the introduction of highly active antiretroviral therapy (HAART) has transformed the management of HIV in developed countries. The increasing number of new HIV infections, indicate the need to develop an effective vaccine to protect against HIV infection. Traditional vaccine strategies have so far failed to protect against HIV infection with a high degree of efficacy (Pantaleo and Koup, 2004, Barouch et al., 2000, Barouch and Letvin, 2000). The high mutation rate and complexity of HIV has complicated the development of effective HIV vaccines.

The HIV infects important cells in the human immune system such as CD4 T cells, macrophages and dendritic cells. The number of circulating CD4+ cells is greatly reduced in HIV-1 infection (Deeks and Walker, 2004, Ho et al., 1995). Thus HIV infected individuals will usually present over time with significantly reduced CD4+ T cells in their blood (Douek et al., 2003, Douek et al., 2002). Human immunodeficiency virus depletes CD4+ T cells by direct killing of virus infected cells, increasing CD4+ T cell

turnover and killing of CD4+ T cells by HIV-1 specific CD8+ T cells (Rowland-Jones et al., 1998, Wong et al., 2010, Yang et al., 1997a). Low CD4+ T cells in the blood results in the loss of cell-mediated immunity and the body becomes susceptible to opportunistic infections (Douek et al., 2003). The drastic loss of CD4+ T cells in the blood eventually results in acquired immunodeficiency syndrome (AIDS). This syndrome is characterized by an impaired immune system, leading to opportunistic infection such as tuberculosis (TB), pneumonia and others (Deeks and Walker, 2004).

HIV infection can be divided into four stages; incubation stage, acute stage, chronic stage and full blown AIDS which leads to death. During the incubation stage, the virus infects the immune system cells (macrophages, dendritic cells and CD4+ T cells); this stage is usually asymptomatic (Kahn and Walker, 1998, Mehandru et al., 2004). The incubation period, also known as window period lasts for 2 to 3 weeks, and during this period HIV antibodies are beginning to be produced (Deeks and Walker, 2007). At this stage HIV tests will not detect the HIV antibody unless sensitive techniques are used (Feinberg and McLean, 1997, Piatak et al., 1993). The second stage of acute infection is characterized by high viral loads and low CD4+ T cell counts in the blood (Pantaleo, 1997, Deeks and Walker, 2004). In the acute stage, there is an activation of CD8+ T cells which kill the infected cells, resulting in antibody production(Appay et al., 2008a). CD8+ T cells are thought to be important in controlling viremia in the acute stage as the CD4+ T cells rebound (Pantaleo, 1997). This stage lasts for approximately 28 days, and includes clinical symptoms such as lymphadenopathy, fever, pharyngitis (sore throat), rash, myalgia (muscle pain), malaise and mouth sores (Kahn and Walker, 1998). The third

stage is chronic stage or latency, characterized by few or no symptoms and can last for two weeks to twenty years or more. During this stage, HIV is active in the lymphoid organs and in the body organs that are rich in CD4+ T cells (Siliciano et al., 2003). Large amounts of the virus become trapped in the follicular dendritic cell networks (Siliciano et al., 2003, Burton, 2002). The final stage of HIV infection is AIDS and this is usually accompanied by symptomatic opportunistic infections (TB, pneumonia, etc). In this stage, the CD4+ T cells decline rapidly and fall below 200 cells per µl, and this stage is usually described as full blown AIDS (Appay and Sauce, 2008a, Douek et al., 2002).

1.2. HIV TRANSMISSION AND PREVENTION

Human immunodeficiency virus is transmitted through infected blood, body fluids such as semen, vaginal fluids and breast milk (Ammaranond et al., 2003, Smith et al., 2004). There are four main routes of HIV infection: unsafe sex, contaminated needles, breast milk and mother to child transmission (Coovadia and Coutsoudis, 2007). However, the most common route of HIV transmission is through unsafe sex. The HIV infected sexual secretions of one partner come into contact with the genital, oral or mucous membranes of the uninfected partner (Smith et al., 2004). In Southern Africa most transmission occurs between heterosexual partners whereas in Western countries homosexual transmission is thought to be more common (Boily et al., 2009). Sexual transmission of HIV can be prevented by correct and consistent use of latex condoms. It was first reported in 1999 that in sub-Saharan Africa male circumcision may reduce the risk of HIV infection (Weiss et al., 2000). Subsequently, randomized clinical trial studies conducted in South Africa, Kenya and Uganda showed that men circumcised in sterile conditions and given counseling had reduction of HIV transmission for heterosexual sex by 60%, 53% and 51% respectively (Bailey et al., 2007, Siegfried et al., 2005).

HIV can also be transmitted through mother-to-child transmission and by multiple infection or super infection (Coovadia, 2004, Smith et al., 2004). HIV positive pregnant women may transmit the virus to the child during pregnancy (in utero), during child birth (intrapartum) or via breastfeeding (Coovadia, 2004). However, the provision of

antiretroviral therapy given during pregnancy reduces the rate of mother-to-child transmission to 1%. Initial studies showed that avoidance of breastfeeding after giving birth may reduce the transmission of the virus to the child (De Cock et al., 2000). In contrast, recent studies report that mothers who are taking antiretroviral drugs may safely exclusively breastfeed their children for up to twelve months (Coovadia and Bland, 2007, Thior et al., 2006, Smith et al., 2004). Prior to these findings (WHO) developed a policy to state that women on ARV may exclusively breastfeed the child for 12 months, believing this will reduce mother-to-child transmission (Coovadia et al., 2007).

1.3. STRUCTURE OF HIV AND LIFE CYCLE

HIV has a spherical structure with a diameter of 120 nm, approximately 60 times smaller than a red blood cell (Chan and Kim, 1998, Freed, 1998). HIV has two copies of positive single stranded RNA that codes for nine genes. The single stranded RNA (ribonucleic acid) is located inside the nucleocapsid protein, p7 (Mannioui et al., 2005, Freed, 1998). Also contained within p7 are the enzymes required for the development of the virion. These are reverse transcriptase, protease, ribonuclease and integrase (Freed, 1998). The matrix protein also known as p17 is the product of HIV *gag* gene. It assists in anchoring the gp41/gp120 spike(Chan and Kim, 1998). The envelope protein (Env) is situated on the surface of the virus, consisting of three molecules called glycoproteins (gp41, gp120 and gp160) which assist the virus in anchoring on the cell membrane (Chan and Kim, 1998, Chan et al., 1997) (Fig. 1). The glycoprotein of the envelope proteins helps the

virus to attach and fuse with the host cells and initiate replication (Chan et al., 1997, Kuiken et al., 2008). They also promote the fusion of infected cells with neighbouring uninfected cells forming synctia (Chan and Kim, 1998). The envelope glycoproteins are the targets of fusion inhibitor drugs and have been used in trial vaccines (Chan and Kaur, 2007).

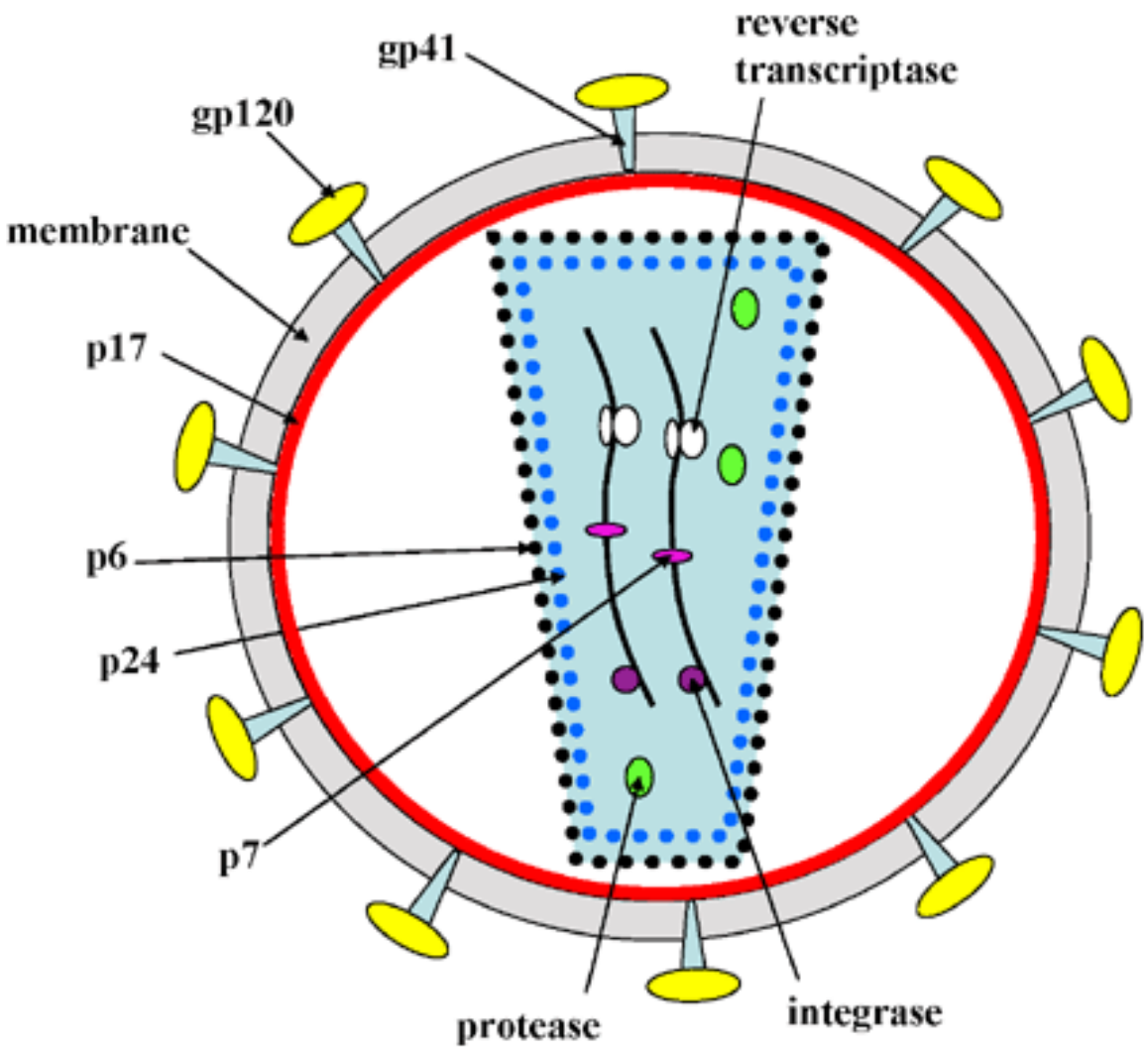

Figure 1. Structure of Human Immunodeficiency virus.Showing the location of different proteins (Gag, Env glycoprotein and the pol-encoded enzymes IN, RT and PR (Freed, 1998).

The HIV genome consists of nine genes which encode 19 proteins. These genes are *gag, pol, env, tat, rev, nef, vif, vpr and vpu* (Subbramanian and Cohen, 1994, Cullen, 1992). The regulatory proteins of HIV are Tat and Rev. Nef, Vif and Vpr are accessory proteins that confer the ability on HIV to infect cells and to produce new viral copies thereby causing disease (Ranki et al., 1994, van Baalen et al., 1997, Hunt, 2010, Miller and Sarver, 1997, Trono, 1995). Tat (trans-activator of transcription) consists of 86 to 101 amino acids depending on the virus subtype (Kuiken et al., 2008, Allen and Altfeld, 2003). Tat accelerates the production of more HIV virions. HIV strains that do not have Tat protein in their genome fail to replicate. Tat induces chromatin remodeling and recruits elongation-competent transcriptional complexes on the viral LTR (Zheng et al., 2005, Gibbs et al., 1995). Tat inhibits LFA-1 mediated Ca^{2+} influx through the binding of L-type Ca^{2+} channel and impairs NK cell cytotoxicity (Majumder et al., 2009). Rev, another regulatory protein, consists of 116 amino acids including nuclear localization sequence (NLS) and nuclear export sequence (NES)(Zheng et al., 2005). The Rev protein regulates the expression of HIV proteins by controlling the export rate of mRNAs (Cullen, 1998). Rev proteins are involved in shuttling RNA from the nucleus and the cytoplasm by binding to RRE RNA (Pond et al., 2009, Pollard and Malim, 1998) while the Vif protein counteracts the functions of APOBEC3G (a cell protein which deaminates DNA: RNA hybrids and/or interferes with Pol proteins (Zheng et al., 2005, Gandhi et al., 2008).

Negative factor (Nef) is an accessory protein which is synthesized early in the HIV life cycle and is important in disease progression (Lichterfeld et al., 2004b, Collins et al.,

1998, Page et al., 1997) . The Nef (negative factor) protein manipulates the host's cellular machinery and thus allows infection, survival or replication of the pathogen (Stumptner-Cuvelette et al., 2001, Page et al., 1997). Nef protein also promotes the survival of infected cell by down modulation the expression of Major Histocompability complex –I (MHC-1) and CD4 T cells. Nef protein assists the virus to maintain its viral loads and to overcome host immune defense resulting in AIDS progression (Sinclair et al., 1997). In addition, HIV-1 Nef downregulates CD4+ T cell and MHC proteins which help the virus to evade host immune responses (Stumptner-Cuvelette et al., 2001, Cohen et al., 1999, Adnan et al., 2006). Gag is one of the most important genes found in all retroviruses and is required for assembly of viral proteins (Freed, 1998). It consists of 1,500 nucleotides and encodes four separate proteins which are the building blocks for the viral core. HIV-1 Gag protein has diverse functions in the life cycle of the virus, including viral assembly. The Gag protein consists of a subunit called p24, which is a component of the virus that is relatively highly conserved. Immune responses against p24 may give strong protection since the virus can only avoid these responses at significant fitness cost (Goepfert et al., 2008, Goulder and Watkins, 2008). Gag and Pol (Polymerase) are next to each other in the HIV genome. The *pol* gene, which is translated as a combined *gag-pol* reading frame encodes for reverse transcriptase and integrase (Freed, 1998).

The viral protein R (Vpr) consists of 96 amino acids and is found in HIV-1, HIV-2 and SIV (Le Rouzic and Benichou, 2005). The role of the Vpr protein in AIDS pathogenesis is to import viral DNA into the nucleus as a component of pre-integration complex and it also plays a role in cell-cycle progression, regulating apoptosis and transactivation of the

HIV-LTR as well as host cell genes (Gibbs et al., 1995, Altfeld et al., 2001a, Trono, 1995, Vodicka et al., 1998). Vpr dysregulates immune system functions by disarming both adaptive and innate immune system and also mediates dysregulation of signal transduction (Majumder et al., 2009, Le Rouzic and Benichou, 2005, Goh et al., 1998). Vpr is normally found in the sera and the cerebrospinal fluid of AIDS patients indicating that it may exert biological functions in varied manners. In addition, previous studies conducted in monkeys indicate that *vpr* deletion in SIV results in low viral loads and rarely do infected monkeys progress to AIDS (Hadian et al., 2009, Gibbs et al., 1995). These results suggest a major role of the *vpr* gene in disease progression in HIV infection (Altfeld et al., 2001a, Emerman and Malim, 1998). Viral protein U (Vpu) is an accessory protein with 81 amino acids in size and its promotes the release of new virus particles from the infected cells, downmodulation of CD4+ T cells in the endoplasmic reticulum and it is also involved in envelope maturation (Pollard and Malim, 1998, Le Rouzic and Benichou, 2005, Kuiken et al., 2008, Neil et al., 2008).

1.4. HIV LIFE CYCLE

The HIV life cycle begins when the envelop glycoprotein gp120 binds the CD4 T cell receptor and subsequently interacts with other coreceptors such CCR5 (chemokine receptor 5) and CXCR4 (C-X-C chemokine receptor type 4) (Freed, 1998, Chan and Kim, 1998). Transmembrane gp41 induces the membrane fusion reaction that occurs between the lipid bilayer of the virion and the host cell plasma membrane. This process leads to the release of the viral core into the host cell cytoplasm (Chan and Kim, 1998, Wyatt and

Sodroski, 1998). The virus uncoats inside the cell cytoplasm. During this process the capsid (CA) is lost, while the matrix (MA), nucleocapsid and other polymerase encoded enzymes (integrase and reverse transcriptase) are retained (Zheng et al., 2005, Chan et al., 1997, Freed, 1998) (Fig. 2). During the uncoating process, reverse transcription takes place, whereby viral RNA reverse transcription to double strand DNA is largely completed. Mutations often take place during the reverse transcription process and these may lead to drug resistance and allow the virus to evade the immune system (Freed, 1998). A high-molecular weight complex is then formed and is referred to as the pre-integration complex. It is then transported into the nucleus across the nuclear membrane. Inside the nucleus, the viral DNA is integrated into the host DNA; this is catalyzed by integrase (Zheng et al., 2005) (Fig. 2). The provirus or integrated DNA will then serve as templates for mRNA synthesis (Zheng et al., 2005).

The integrated DNA provirus is transcribed into mRNA and then spliced into small pieces. The mRNA is transported into the cytoplasm and envelope glycoproteins are synthesized in the endoplasmic reticulum (Chan et al., 1997). They are transported to the plasma membrane through the secretory pathway. Gag and Gag-pol polyprotein precursors are transported by an unknown mechanism into the plasma membrane (Freed, 1998). After transportation into the plasma membrane, Gag precursor recruits two copies of ssRNA which interact with the Gag-pol precursors. They are then assembled into structures which are visible by electron microscopy (EM) structures leading to the formation of buds. After budding, viral particles cleave the Gag and Gag-pol region polyprotein precursor into mature proteins (Freed, 1998) (Fig. 2). Structural proteins Gag

and Env are produced from the full length mRNA. The final stage of the viral cycle is the assembly of new HIV-1 virions which begins at the plasma membrane (Freed, 1998). These pieces are transported into the cytoplasm where they are translated into regulatory proteins (Tat) and (Rev) (Pollard and Malim, 1998, Pond et al., 2009). The mature virus is then able to infect another cell (Zheng et al., 2005, Freed, 1998).

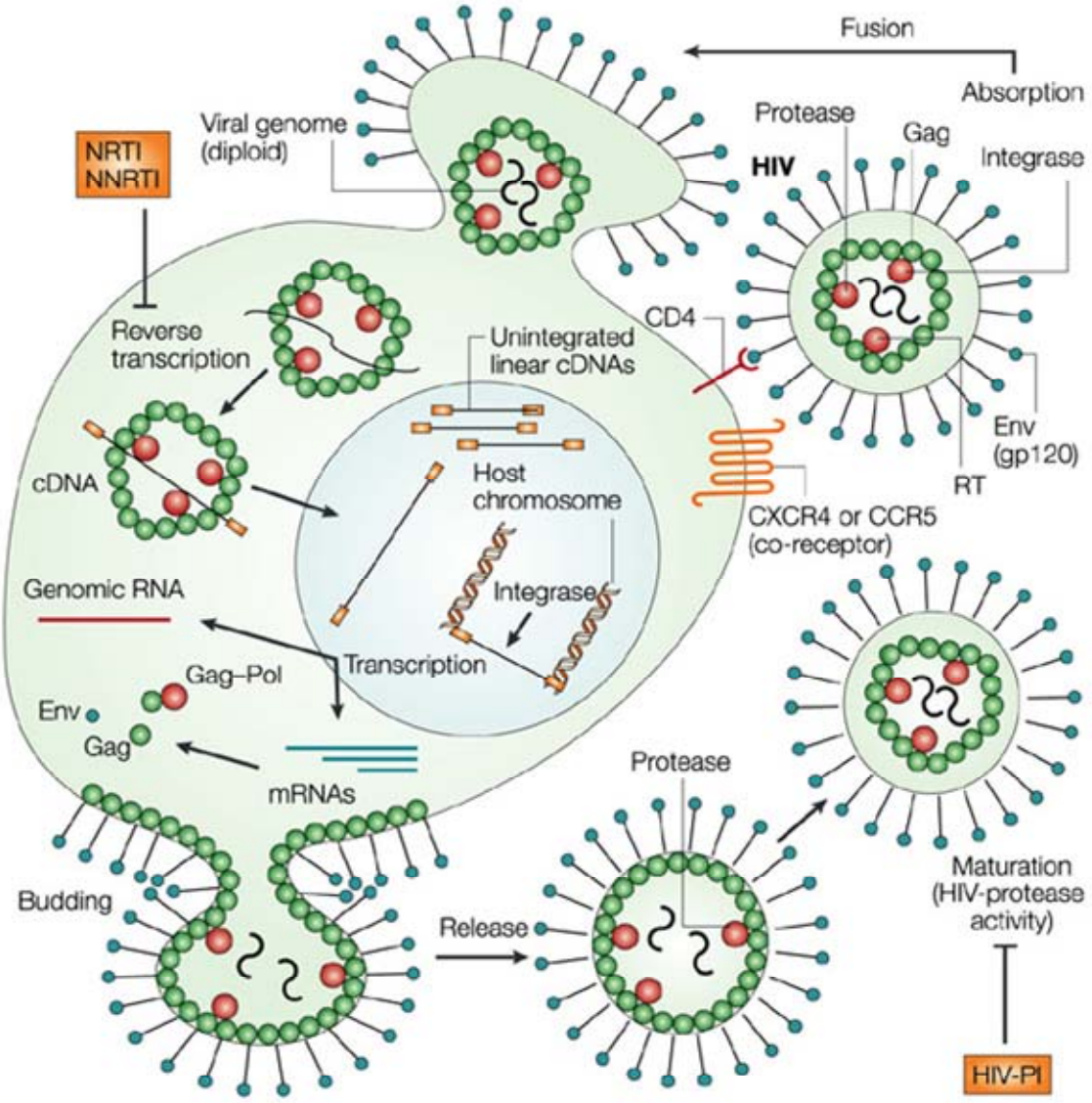

Figure 2. The HIV replication cycle**.**

HIV infects the CD4 T cells using gp120 of the envelope, HIV RNA is then released inside the host cells. HIV RNA is reverse transcribed into DNA which is then integrated into the host's DNA. Viral DNA is then transcribed into viral RNA followed by protein translation inside the cytoplasm and new virus is released and infects more cells (Monini et al., 2004).

1.4. IMMUNE RESPONSE TO VIRAL INFECTION

The immune system is the protective mechanism for the body against any foreign particles that enters into the body. It consists of the innate (non-specific) and adaptive (specific) immune system. The adaptive immune system comprises of B lymphocytes and T lymphocytes. B lymphocytes mature into memory B cells which then become plasma cells that produce antibodies, also known as immunoglobulins. T lymphocytes consist of CD4 T cells (helper cells) and CD8 T cells (killer cells). Cytotoxic T cells mature into memory T cells which express perforins that lyse the target APC. They also produce cytokines and chemokines which kill virus infected cells (Hickling, 1998).

The innate immune system includes the skin, mucosa, complement system, natural killer cells and intrinsic immunity. NK cells are important for tumor surveillance and they can secrete several cytokines (IFN-γ, IL-1, TNF-α). NK cells secreting IFN-γ can influence the immune system by promoting differentiation of type 1 T cell and of type 2 cells.

Natural killer cells are thought to be the most important component of innate immunity, which is also thought to play a crucial role in control of HIV (Martin et al., 2002). Host genetic studies indicate that individuals who co-express KIR3DS1 (a regulatory receptor on the surface of NK cells) and HLA-Bw4-081 (HLA-alleles that presumably binds to KIR3DS1 and activates NK cells are associated with low viral loads and slow disease progression (Martin et al., 2002). It was then reported that HIV controllers are enriched

with certain NK cells receptors which suggests that NK cells play a role in disease outcomes (Vieillard et al., 2010).

T lymphocytes are very important in the immune system, especially CD8 T cells because they kill the viral infected cells using either cytotoxic or non-cytotoxic pathway (Borrow et al., 1994). CD8 T cells recognize antigens that are presented by MHC-I molecules while CD4 T cells recognize antigen presented on MHC- II molecules (Hickling, 1998). CD8 T cells are responsible for destroying viral infected cells. The major histocompability complex (MHC) is divided into two classes, MHC class I and II. Class I molecules are found in all nucleated cells, except red blood cells whereas class II molecules are found on antigen presenting cells (APC), including dendritic cells, macrophages and B cells (Simpson, 1988, Groothuis et al., 2005). Studies indicate that MHC genes are involved in controlling both humoral and cell-mediated immune responses (Groothuis et al., 2005).

Major Histocompability Complex-I (MHC-I) molecules are glycoproteins with two non-covalently associated polypeptide chains known as the light chain and heavy chain (Kosc et al., 1998, Simpson, 1988). The light chain is commonly known as β2 microglobulin and the heavy chain is known as α chain. The two α chains (α1 and α2) fold up to form a peptide binding groove, however MHC-I may bind peptides 8-10 amino acids long while MHC-II binds longer peptides (Kosc et al., 1998, Simpson, 1988, Groothuis et al., 2005). Human beings may have a maximum of six different MHC-I products and slightly more for MHC-II. A MHC molecule has one binding site and can only bind one peptide at a

time (Groothuis et al., 2005). A peptide must be associated with a given MHC of the individual or else an immune response is not initiated.

MHC molecules are encoded for by several genes in humans known as human leucocyte antigens (HLA gene) and the most well researched of these are HLA-A, B, C, DPAI, DPBI, DQAI, DQBI, DRA, DRBI (Groothuis et al., 2005, Mallal et al., 1990). They are divided according to the MHC classes; HLA-A, B, C belong to class I and D belongs to class II. The HLA molecules are located on the surface of antigen presenting cells (APC). HLA-A and HLA-B restricted epitopes play a significant role in controlling HIV replication (Goulder and Watkins, 2008, Kiepiela et al., 2007). However, the role of HLA-C restricted CD8+ T cell is not well studied and is possibly underestimated in HIV control. This may be due to less expression of HLA-C alleles on the cell surface compared to HLA-A and HLA-B (Goulder et al., 1997a). Studies have suggested that low surface expression of HLA-C alleles may result from weak binding of β2 microglubulin (β2m) (Zemmour et al., 1992). Linkage disequilibrium of HLA-C with other HLAs may also result in underestimating its role in the control of HIV (Goulder et al., 1997a, Thomas et al., 2009).

1.5. ROLE OF CYTOTOXIC T CELLS RESPONSES IN HIV PATHOGENESIS.

HIV-1 specific CD8+ T cells play a vital role in the control of HIV replication and this has been proven by the decline in HIV/SIV viral loads after HIV acute infection, coinciding with an increase in HIV specific CD8+ T cells (Hess et al., 2004, Evans et al., 1999, Borrow et al., 1994, Klatt et al., 2010). However, differences exist in their antiviral effectiveness based on their HLA restriction, epitope specificity, functional epitope avidity, targeted viral protein (Kiepiela et al., 2007, Altfeld et al., 2006). CD8+ T cell depletion performed in animal models indicates that absence of CD8+ T cell is associated with high viral load (Schmitz et al., 1999, Fauce et al., 2007). Most recent studies have demonstrated that CD8 T cell depletion results in the rise in viral loads to its acute infection peak (Wong et al., 2010, Klatt et al., 2010).

In addition, there is also consistent association between certain HLA-class 1 molecules and the control of viral replication (Migueles et al., 2003, Kiepiela et al., 2004, Altfeld et al., 2006). These findings have supported the view that effective HIV-1 specific CD8+ T cell immunity controls viral replication. There are many studies that have shown significant evidence that CD8+ T cells play a crucial role in both HIV and SIV replication (Yang et al., 1997a, Walker and Burton, 2008, Koup et al., 1994). However, there was no significant association between the number of HIV-1 specific CD8+ T cells

and virological control when responses are measured by ELISPOT or intracellular cytokine staining (ICS) assay (Addo et al., 2003, Altfeld et al., 2001b).

The mechanism of how CD8+ T cells inhibit viral replication is still under investigation. In addition, HIV infection leads to the decline of CD4+ T cell and the virus infects and is associated with disease progression (Vasan et al., 2007, Appay et al., 2008b). Previous studies reported that CD8+ T cells kill viral infected cells either via cytolytic or non-cytolytic pathways (Yang et al., 1997b, Rowland-Jones et al., 1998). The protective role of HLA-B*27 and HLA-B*57 alleles in human as well as Mamu-B*17 and Mamu-A*01 in macaques is well established and the epitopes restricted by these alleles are well defined (Migueles et al., 2003, Mothe et al., 2003, Maness et al., 2008). HLA-B*57 alleles in HIV controllers have an ability to present a broad number of HIV peptides for T cell recognition and also generate high magnitude of CD8+ T cell responses (Altfeld et al., 2003a, Migueles et al., 2003).

Many HIV positive individuals can control viremia for a long time without progressing to AIDS while others progress faster to disease or others maintain undetectable viral loads for long time, these individuals may provide important information in vaccine design strategy (Migueles and Connors, 2001, Deeks and Walker, 2007, Friedrich et al., 2004). There is a group of individuals who are known as elite HIV controllers, this group presents with viral load <50 copies/ml and high CD4 T count after long time of HIV infection. Some elite HIV controllers may be infected with replicative-incompetent virus (Blankson et al., 2007, Bailey et al., 2009, Lassen et al., 2009). Blankson *et al* (2007)

used a highly sensitive co-culture assay to isolate the virus successfully from elite controllers. Studies performed by Blankson et al where two viruses were isolated and sequenced; one virus from the plasma and the other one from the resting CD4+ T cells (archived virus) revealed that the plasma virus contained mutations in HLA-B*57 restricted epitopes and very rarely on archived virus (Bailey et al., 2006b, Blankson et al., 2007). In addition, the same group of researchers showed that CD8+ T cells mediated selective pressure on plasma virus in elite controllers and suggested that there must be ongoing HIV-1 replication in spite of very low viral loads (Bailey et al., 2009).

HIV controllers have more functional CD8+ T cells compared to non-controllers and they are also likely to target the conserved proteins of HIV (Altfeld et al., 2003b, Addo et al., 2007). HLA-B*27 and HLA-B57 alleles are overrepresented in the HIV controllers or long-term nonprogressors indicating a major role for MHC-I molecules in controlling HIV replication. The multiepitopic and *de novo* CD8+ T cells response of HIV controllers have an ability to suppress viral replication despite escape mutations (Bailey et al., 2006a). Likewise, in a cohort of 578 chronic HIV infected subjects, it was demonstrated that only Gag-specific CD8+ T cell responses were associated with lower HIV viremia, including those who have HLA-B*57 (Kiepiela et al., 2004, Sacha et al., 2007). Even in the acute phase of infection HLA-B*57 allele was associated with viral control (Migueles et al., 2003). This demonstrates that host genetic factors may influence HIV pathogenesis and may provide a functional link between HLA-B*57 and viral immune control (Altfeld et al., 2003a, Migueles et al., 2003).

Previous studies reported that mutations within Gag may induce a viral fitness defect while mutations within highly variable region like envelope (Env) may impact less on viral growth kinetics (Leslie et al., 2005, Goulder et al., 2001c, Crawford et al., 2009). Changes in epitope sequences may affect the recognition and also interfere with the peptide processing and affect their presentation on the cell surface of SIV or HIV infected cells (Koup et al., 1994, Leslie et al., 2004). Studies have reported that changes in epitope amino acid sequences especially in immunodominant Gag-restricted epitopes may result in the loss of recognition of the virus by cytotoxic T cell responses (Barouch and Letvin, 2002, Gao et al., 1999). Moreover, escape mutations may decrease the fitness of the virus which may facilitate disease progression. However, other studies show that there is significant correlation between the number of Gag escape mutations targeted by specific HLA-B-restricted allele and reduced viral loads, therefore transmission of HIV-1 Gag but not Nef immune escape mutations is associated with reduced viral loads (Goepfert et al., 2008).

However, cohorts of HLA-B5701+ patients show that mutations within the p24 Gag peptide presented by the HLA-B5701 allele reduce the replicative capacity, but these do not lead to virologic rebound (Migueles et al., 2003). When a HLA-B*5701 positive individual transmits the virus to a non-B*5701 individual mutations may revert and the *gag* mutations may increase the viral fitness (Leslie et al., 2006). Additionally, Gag restricted epitopes are recognized by CD8+ T cells after 2 hours post infection, before integration and protein synthesis, while Env-derived epitopes were recognized after 18 hours (Sacha et al., 2007).

Genomic studies have reported the association of HLA-C locus and other genetic variants with an ability to control HIV (Fellay et al., 2007). Recent genome wide studies conducted in HIV infected individuals from nine cohorts identified allelic polymorphisms associated with viral set point (HCP-5 and 5' HLA-C) or disease progression (RNF-39 and ZNR1) (Fellay et al., 2007, Thomas et al., 2009, Han et al., 2008). In addition, a population-based association study revealed complete linkage disequilibrium between HCP5 and HLA-B*5701/Cw*06 (Thomas et al., 2009, Han et al., 2008). However, other studies reported a crucial contribution of sub-dominant HLA-C restricted CTL responses to HIV replication when the linkage disequilibrium with other HLAs has been corrected.

Additionally, surface expression increased and HIV/AIDS control is associated with a variant upstream of HLA-C alleles (Han et al., 2008, Thomas et al., 2009). These studies concluded that the -35C allele is a proxy for high HLA-C cell surface expression (Thomas et al., 2009). Though HLA-C alleles are not downregulated by HIV-1 Nef protein, the HLA-C expression pattern may have a significant role in HIV pathogenesis (Adnan et al., 2006, Makadzange et al., 2010). Individuals with high surface expression of HLA-C are associated with slow disease progression. HLA-B*27 and B*57 alleles are also known to be associated with the control of HIV replication especially when presenting immunodominant Gag epitopes (Streeck et al., 2007, Altfeld et al., 2006).

In acute HIV infection, there is a rapid and extensive destruction of CD4+ T cells populations that reside in the gut-associated lymphoid tissues (Mehandru et al., 2004). Other studies show that gut CD8+ T cells are more polyfunctional compared to peripheral

CD8+ cells and upon initiation of ART the rectal polyfunctional HIV Gag-specific CD8+ T cells were reduced (Critchfield et al., 2008, Brenchley and Douek, 2008). Evidence from animal models showed that SIV infection causes half or more of the gut CD4+ T cells depletion during primary infection. The CD4+ T cells loss results in the loss of mucosal integrity leading to malfunctioning of the gut (Li et al., 2005). All these events happen before adaptive immunity takes over, therefore early host immune responses are thought to be mediated by the innate immune system.

1.5. POLYFUNCTIONAL CD8+ T CELL RESPONSES IN HIV

CD8+ T cells kill viral infected cells by secreting cytokines (IFN-γ, TNF-α, IL-2), chemokines (MIP-1β), and expression of the degranulation marker (CD107a). CD8+ T cells that display all these functions are referred as to "polyfunctional" (Pantaleo and Koup, 2004, Betts et al., 2006, Harari et al., 2007). Previous studies indicated that in primary infection CD8+ T cells are polyfunctional but as the disease progresses their functions deteriorate resulting in high viral loads (Rehr et al., 2008, Streeck et al., 2008, Daucher et al., 2008). These studies reported that the loss of polyfunctional CD8+ T cells in chronic HIV infection is a consequence of the antigen load and antigen persistence and this may mean that viral burden impacts the natural functions of CD8+ T cells (Streeck et al., 2008). Likewise, in chronic HIV infection, HIV specific CD8+ T cells become monofunctional, frequently producing only IFN-γ or MIP-1β or CD107a (Betts et al., 2006, Zimmerli et al., 2005, Harari et al., 2007). HIV-1 specific CD8+ T cells progressively lose their functions during chronic infection and this is characterized by inability to produce particular cytokines such as IL-2 (Lichterfeld et al., 2004a). In most immunological studies, IFN-γ-production by CD8+ T cells was used to determine the magnitude of immune responses against the virus (Kiepiela et al., 2004).

The effectiveness of HIV-specific immune responses does not rely on the quantity of responses, but on the quality of CD8+ T cells (Daucher et al., 2008). HIV-specific CD8+ T cells play a vital role in the control of HIV replication and disease progression (Borrow et al., 1994, Koup, 1994, Schmitz et al., 1999, Altfeld and Allen, 2006). However,

differences exist in their antiviral effectiveness based on their HLA restriction, epitope specificity, functional epitope avidity and targeted viral protein (Kiepiela et al., 2007, Bihl et al., 2006, Yang et al., 2003). In particular, major histocompatibility complex (MHC) class I molecules have been shown to differ in their ability to mediate the control of HIV and SIV replication in humans and non-human primates respectively (Rolland et al., 2008, Kiepiela et al., 2004). For example, virus-specific Gag CD8+ T cell responses restricted by HLA-B*57, HLA-B*5801 and HLA-B*27 are associated with low viral loads or slow disease progression in HIV infection while Mamu-A*01 and Mamu-B*17 restricted responses are associated with control in SIV infection of rhesus macaques (Chung et al., 2007, Migueles et al., 2003, Loffredo et al., 2008, Maness et al., 2008).

In contrast to the beneficial outcomes associated with the protective MHC allele-restricted Gag CD8+ T-cell responses, Gag HLA-Cw*-restricted CD8+ T-cell responses were found to be associated with high viral loads (Kiepiela et al., 2007). Paradoxically, although HLA-Cw*-restricted CD8+ T lymphocytes appear to contribute little or even negatively to viral control *in vivo*, HLA-C is not down-regulated by HIV-1 Nef from the surface of infected cells to the same extent that HLA-A and HLA-B molecules (Cohen et al., 1999, Collins et al., 1998). Overall, the mechanisms underlying control of HIV by protective alleles such as HLA-B*57/5801, or the lack of control by HLA-Cw alleles remain unclear and this limited understanding has important implications for rational vaccine design.

HIV-specific CD8+ T cells may also display different differentiation status and activation profiles (Appay and Sauce, 2008b, Papagno et al., 2004). It has been suggested that these phenotypic differences are associated with divergent functional antiviral capacities of virus-specific T-cells (Almeida et al., 2009). Some studies have suggested that polyfunctional CD8+ T cells, able to secrete up to five different effector functions (IFN-γ, IL-2, TNF-α, MIP-1β and CD107a), have better antiviral activity (Betts et al., 2006, Precopio et al., 2007, Daucher et al., 2008). CD8+ T cell responses restricted by HLA-B*27 and HLA-B*57 alleles were reported to be polyfunctional when compared to CD8+ T cell responses restricted by HLA-A alleles within the same patients (Harari et al., 2007). However, recent data have suggested that the functional profile of CD8+ T cells is largely a consequence of the duration and level of antigen load, with prolonged continuous exposure to high levels of antigen resulting in exhausted CD8+ T cells characterized by a monofunctional effector profiles (Streeck et al., 2008, Rehr et al., 2008).

The aim of this study was to optimally define HLA-Cw* restricted CD8+ T cell responses in the Sinikithemba cohort in the Durban. The second aim was to investigate the polyfunctionality profiles of immunodominant HLA-B and HLA-Cw*-restricted CD8+ T cells in a cohort of HIV-1 clade C chronically infected individuals displaying both responses. This provided the unique opportunity to examine HLA-B and Cw* restricted responses in the context of matched viral loads and CD4 cell counts. The main focus was on the immunodominant HLA-B*57/5801 epitopes in Gag p24 and the immunodominant HLA-Cw*07 restricted epitope KY11 in Nef, in persons possessing both responses. We hypothesized that HLA-B CD8+ T cells will display a more

polyfunctional phenotype compared to HLA-Cw*-restricted CD8+ T cells. We also aimed to determine whether sequence variation within epitopes presented by these two alleles has a bearing on the magnitude and polyfunctionality of epitope-specific CD8+ T cell responses. The relationship between the frequency of polyfunctional HIV-1-specific CD8+ T cells and CD4+ T cell counts and viral loads was also investigated.

1.6. MAIN OBJECTIVES

1. To optimally define novel HLA-Cw* restricted HIV-1 specific CD8+ T cell epitopes in a cohort of individuals with chronic untreated HIV-1 clade C infection in Durban.
2. To assess the polyfunctionality of immunodominant HLA-Cw* and HLA-B*57/5801 restricted CD8+ T cells within the same study subjects and to investigate the impact sequence variation on T cell polyfunctionality in the same individuals.

Chapter 2. Material and Methods

2.1 BACKGROUND AND PRINCIPLES OF IMMUNOLOGICAL ASSAYS

2.1.1 ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS ASSAY

Peripheral blood mononuclear cells (PBMC) are blood cells which have round shaped nuclei (lymphocytes and monocytes). These cells play an important role in immune defence mechanisms against infection. They were separated from the whole blood samples using density centrifugation procedures. In the procedure, whole blood is layered over histopaque, a separation medium. After centrifugation, three layers are observed from top to bottom. The middle layer is the PBMC layer and this is carefully removed and washed to remove contaminants. Isolated PBMCs are used for different purposes: to assess the level of biological immune function, to quantify specific cell phenotype (T and B lymphocytes), and to determine the amount of specific drug within these cells and evaluate *in vitro*, the influence of a test drug on lymphocytes. PBMCs can be frozen using 10% DMSO in fetal calf serum and kept in liquid nitrogen for later use. However, without an effective and careful isolation, freezing and thawing of cells can detrimentally affect the immune monitoring of cells.

2.1.2 ENZYME-LINKED IMMUNOSPOT ASSAY

Enzyme-linked immunospot assay is adapted from the ELISA assay; it measures the concentration of cytokines that are released from CD8+ T cells stimulated with antigen. Enzyme-linked immunospot (ELISPOT) assay is widely used to measure the magnitude of responses of cytokine-producing cells when stimulated with specific HIV antigen (Fu et al., 2007, Maecker et al., 2001, Hickling, 1998). It is an effective assay for quantifying host immune responses, but it does not provide information about the quality of the immune response (Mwau et al., 2002, Chung et al., 2007). It provides information on the quantity of CD8+ T cells producing cytokines upon antigen stimulation (Fu et al., 2007). It is a very rapid, sensitive assay and allows multiple screening of multiple CD8+ T cell samples on a single day. The ELISPOT assay is widely used in evaluating immune responses in vaccine studies (Chung et al., 2007, Mwau et al., 2002); vaccines can elicit HIV-1 specific T cell immune responses which can be detected using ELISPOT, however some of this responses lack functional avidity to recognize HIV-1 infected cells (Bennetts et al., 2007, Arrode et al., 2007).

The ELISPOT assay results show the efficiency of a vaccine to induce an immune response; however, functional assays for vaccine development are also required (Hickling, 1998). The MHC-peptide tetramer staining assay is used to detect T cell receptor of a particular specificity irrespective of their functionality (Mwau et al., 2002, Goulder et al., 2001a, Hickling, 1998). In both intracellular cytokine staining (ICS) and

the ELISPOT assay, the whole protein, viral lysate or specific peptide may be used as antigen. In principle, the host antigen presenting cells (APC) process the antigen through the exogenous pathway of antigen presentation. Thereafter the peptide is presented on MHC- class II molecules and recognized by CD4+ T cells which proliferate and produce cytokines, similarly CD8+ T cell responses require antigen to be processed via the endogenous pathway of antigen presentation resulting in peptide presentation on MHC-class I molecules (Maecker et al., 2001).

In this assay, the 96-well plate is covered with PVDF membrane, and the bottom of each well is coated with primary antibodies that are specific to the protein of interest, for example cytokines, antibodies, surface marker, receptor. Peripheral blood mononuclear cells (PBMC) are cultured in an ELISPOT plate (Fig.3). Antigen or peptides are added into each well to stimulate the secretion of IFN-γ, which binds to the well bottom by forming a complex with antibodies (Mwau et al., 2002). The binding of antigen is very specific and therefore only molecules of interest stick to the bottom membrane. After incubating the cells and antigen overnight, the content of the well is discarded and the plate is washed with PBS thoroughly. Proteins remain bound at the bottom of the well. Biotinylated secondary antibody is added which binds to another epitope of the primary antibody protein and this is incubated for 90 minutes at most. The next step is to add enzyme that is coupled with streptavidin, which binds covalently with biotin, the marker of the secondary (detection) antibodies (Fig. 3). Multiple washing of the plate removes the excess of enzyme that remains unbound. The substrate is then added, which in the presence of enzyme changes into dye, BCIP/NBT is added (5-brom0-4-chloro-3-indolyl phosphate and nitroblue tetrezolium). The dye coated under catalytic influence of the

enzyme, sediments on the well bottom. After visible spots develop colour reaction can be stopped through washing and drying the plate. In the ELISPOT assay, it is assumed that one spot denotes one cell producing cytokine or protein of interest. The spots are counted using a specialized ELISPOT plate reader (Hickling, 1998). The number of spots produced by cells is expressed as spot forming units (SFU).

2.1.3. INTRACELLULAR CYTOKINE STAINING ASSAY

Intracellular cytokine staining (ICS) is a widely used technique in flow cytometry which measures the cytokine production upon antigen stimulation (Waldrop et al., 1997, Hickling, 1998). In this assay, T cells are stimulated with specific peptide in vitro for about one and half hour. Brefeldin A or monensin is added to the tube in order to block the transport of cytokine through the Golgi apparatus, which will prevent the secretion of protein products such as cytokines. Fixation and permeabilization of T cells is crucial because it allows cytokine specific antibodies to enter the cells. After fixation and permebialization the cells are stained for the presence of intracellular cytokines using directly conjugated anti-cytokine antibodies (Prussin and Metcalfe, 1995, Hickling, 1998). Intracellular cytokine staining has several advantages on measuring cytokine staining. Large number of CD8+ T cells can be analyzed in a very short time and multiple cytokines can be measure at the same time (polyfunctionality of CD8+ T cells measuring IFN-γ, IL-2, TNF-α, MIP-1β and CD107a). It can also be used to determine the phenotype of the cells that are producing cytokines. In addition, ICS has been used

successfully to characterize cytokine production by circulating CD4+ T cells in HIV positive individuals (Waldrop et al., 1997).

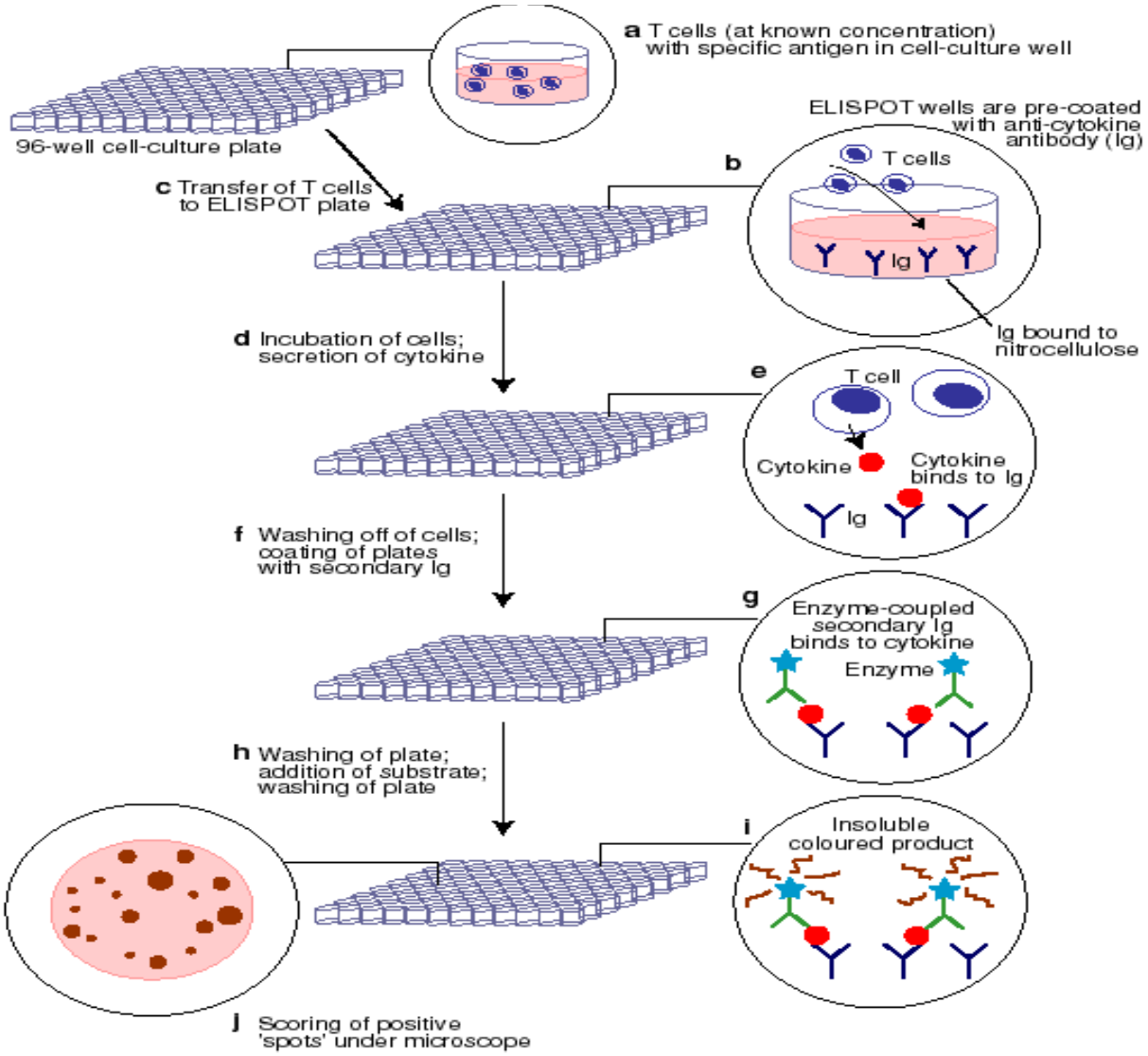

Figure 3. Schematic diagram showing the Enzyme-linked immunospot assay.

a) PBMCs with antigen in the cell culture, b) ELISPOT plate pre-coated with anti-cytokine antibody c) Cells are incubated with antigen on a pre-coated plate, d) Cells with antigens are incubated overnight for maximum secretion of cytokine, e) wash off the cells and coat the plates with secondary antibody and incubate for 1h 30 min, f) wash off the secondary antibody) add streptavidin and incubate for 45 min at room temperature in the dark, h)wash off the plate and add colour reagents to develop the spot NBT/BCIP and read the plates on the ELISPOT reader (Hickling, 1998).

2.1.4 FUNCTIONAL AVIDITY

CD8+ T lymphocytes possess the antibody-like T cell receptor (TCR) which binds antigen or foreign molecule, this binding initiates events that lead to the neutralization or eradication of foreign molecules (Margulies, 2001). TCR generates a repertoire of diversity in the thymus through somatic recombination. However, intrinsic affinity is commonly used to refer to the basic thermodynamic parameter, the equilibrium constant K_D that describes a single equilibrium binding reaction (Margulies, 2001, Bennetts et al., 2007). Functional avidity implies the ability of a particular T cell population to respond to the stimulation provided by tittered MHC-peptide complex (Yang et al., 2003, Bihl et al., 2006).

Functional avidity refers to the lowest peptide concentration that elicits half the maximal responses when stimulated with specific peptide (Bihl et al., 2006, Honeyborne et al., 2006). Avidity measurement depends on distal biological readout, and it may only indirectly reflect intrinsic affinity of the key components in the proximal events (Margulies, 2001). It may result from many interactions and steps, the relationship of intrinsic affinity of the particular TCR for its cognate peptide-MHC complex can at best be an approximation (Bennett et al, 2007). Immunological techniques have been developed to assess peptide functional avidity; these assays assess the secretion of IFN- γ when stimulated with the immunodominant peptide (Bihl et al., 2006). Peptide sensitivity is an important parameter in determining the efficacy of viral clearance by CD8 T cells. CD8 T cells with high responses at low concentration of peptide are termed as high

avidity and those that need high concentration are low avidity (Bennetts et al., 2007, Bihl et al., 2006). Functional avidity is a measure of the overall strength of the interaction between CTL and the target cell. Surface molecules of CTL are capable of contributing to the overall functional avidity of the cells. In addition, TCR affinity plays a role in determining the sensitivity of a CTL to peptide.

In addition, some studies have associated HLA-B* restricted CD8 T cells epitopes with high functional avidity as compared with HLA-A and HLA-C restricted epitopes (Bihl et al., 2006). Moreover, HLA-B alleles are associated with effective antiviral immunity compared to other HLA-A and C alleles (Kiepiela et al., 2007, Altfeld et al., 2003a). It has been reported that HIV-1 specific CD8 T cell in the early infection of HIV display high functional avidity compared to HIV-1 chronic infection (Lichterfeld et al., 2007). The changes in functional avidities in early and chronic HIV-1 infection was linked to a substantial switch in the clonotypic composition of epitope specific CD8 T cell resulting from the preferential loss of high avidity CD8 T cell (Lichterfeld et al., 2007). The loss of high avidity HIV-1 specific CD8+ T cell in chronic infection is due to the high level of viral replication which then impairs the functionality of CD8 T cell (Yang et al., 2003). Since HIV-1 specific CD8 T cells have high functional avidity in early infection, this might reflect higher overall avidity of the TCR/peptide MHC-1 interaction in early infection. This can be measured by using tetramer dissociation assay (Savage et al, 1999).

2.1.5 REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Polymerase chain reaction (PCR) is the assay that is used to amplify a single or few copies of DNA, in order to make more copies for different purposes. It provides a sensitive means of amplifying small quantities of DNA. It is widely used in molecular biology, immunology, genetics, diagnostics, clinical and forensic laboratories. In making up the PCR products from plasma RNA, SuperScript^{TM}III One-step RT-PCR system with Platinum® Taq High Fidelity was used. This is designed for sensitive, high-fidelity end-point detection and RNA analysis by RT-PCR. In this procedure both cDNA synthesis and PCR purification can be performed in a single tube using gene-specific primers, which can target RNAs and mRNA.

The one-step RT-PCR kits has the mixture of Superscript®III reverse transcriptase and Platinum^{TM} Taq DNA polymerase High Fidelity for enhanced RT-PCR yields and fidelity, and it can also detect longer templates. It can detect a range of RNA target from as small as 300bp to 10 kb and one can start from 1 pg to 1µg of total RNA. These two enzymes are engineered to reduce RNase H activity and provide increase thermal stability. The mixture of the two enzymes can synthesize cDNA at a temperature range from 45-60^{o}C, increasing specificity and high yields of cDNA and more full length product than other reverse transcriptases.

Platinum® Taq DNA polymerase high fidelity consists of recombinant Taq DNA polymerase, Pyrococcus species GB-D polymerase and Platinum® Taq antibodies. This may block the polymerase activity at ambient temperature. The denaturation of the antibodies results in polymerase activity restoration, during denaturation step in PCR at 94^{o}C which brings about a hot start, increasing the sensitivity and specificity and yield (Chou et al., 1992, Sharkey et al., 1994). This mixture of enzymes results in a six-fold increase in fidelity and allows amplification of simple and complex DNA templates. The 2x reaction mix has a propriety buffer system that has been optimized for reverse transcription and PCR and includes Mg^{2+} deoxyribonuclease (dNTPs) and stabilizers.

2.2. STUDY PATIENTS

The Sinikithemba cohort has 451 chronic HIV-1 infected individuals whose time of infection is unknown. The cohort, established in 2003 at McCord Hospital, Durban, South Africa consists of antiretroviral naïve patients (Kiepiela et al., 2007, Kiepiela et al., 2004). In this cohort, the HLA-C restricted CD8+ T cell responses were quantified using ELISPOT and intracellular cytokine staining assay (Fig. 4).

In this study population, Cw*0701 is in linkage disequilibrium with B*5801 (p=0.0239) and B*5703 (p=0.0097) and B*5702 (p=0.0034) as determined by the HLA linkage disequilibrium tool on the Los Alamos HIV database (http://www.hiv.lanl.gov/content/immunology/hla/hla_linkage.html).

In B*5702/5703-positive subjects, 64% co express Cw*07, and in B*5702/5703-positive subjects who do not express Cw*0701, 97% co express Cw*1801. In B*5801-positive subjects, 65% co express Cw*07 and in 88% of the remainder either Cw*0302 or Cw*0602 were co expressed with B*5801. Furthermore, nine HIV-1 infected patients were selected from the 78 patients from Sinikithemba cohort in order to study the CD8+ T cell polyfunctionality. The nine subjects studied for polyfunctionality assay were selected based on the possession of concurrent immunodominant HLA-B*57/5801- and HLA-Cw*07-restricted CD8+ T cell responses detected by gamma interferon (IFN-γ) ELISPOT, defined as a minimum magnitude of 500 spot forming cells per million (SFC/million) PBMC for each of these responses.

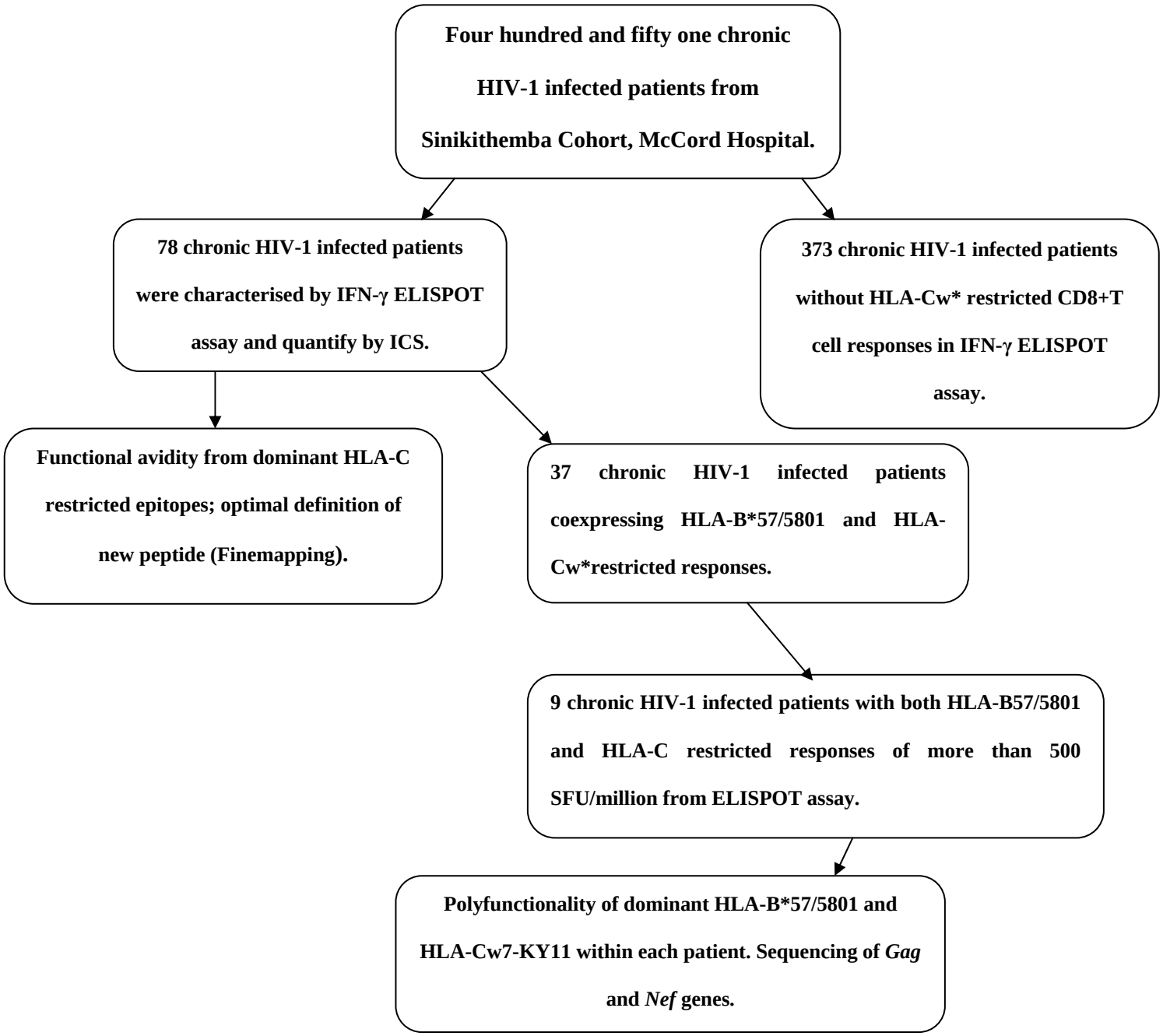

Figure 4. Schematic Diagram showing the selection of the study subjects.

The Sinikithemba cohort consists of 451 chronic HIV-1 infected individuals. All these patients were screened for immune responses using ELISPOT assay. A total of seventy eight patients with HLA-Cw* restricted CD8+ T cell responses were further assessed by intracellular cytokine staining. The remaining three hundred and seventy three patients were not studied because they did not have positive HLA-Cw* restricted responses on the ELISPOT assay. From the seventy eight patients who were studied, thirty seven possessed both HLA-B*57/5801 and HLA-Cw*7 restricted responses. From these thirty seven individuals, nine had immune responses ≥500 SFU/million PBMCs. These nine individuals were used for the polyfunctionality studies.

2.2.1 PROTOCOL OF PBMC SEPARATION

In lymphocytes separation, reagents must be sterile and kept inside the Class II Biological Safety Cabinet (BSC). Before placing objects in the Class II BSC, they were wiped down with 70% ethanol; 50 ml conical tubes were used in lymphocytes separation. The blood, PBS with antibiotics and histopaque were added in 1:1:1 ratio. The maximum amount of the blood that was separated in 50 ml conical was 45 ml. The tubes were labeled with the patient's ID and the date of sample collection. The histopaque media was added to the bottom of 50ml conical flask. The blood was pooled from Lithium heparin tubes (green top) or from EDTA tubes (purple top) to the 50 ml conical flask using 10 ml pipette with pipette aid. The blood was diluted with PBS supplemented with antibiotic (pen/strep). The amount of PBS used was equal to the amount of blood. The blood and PBS with antibiotics were mixed up with pipette aid using a 10 ml pipette. The empty blood tubes were discarded into the 20% Virkon or any other appropriate disinfectant. The blood and PBS were mixed, and then slowly layered on top of the histopaque in the labeled conical tubes taking care not to mix with histopaque. The layered blood was then centrifuged at room temperature for 30 minutes at 1,500 rpm with a slow start and no brakes. During spinning the empty 50 ml conical tubes were labeled with patient details. After spinning, the PBS/plasma was aspirated and the middle thin mononuclear cell layer was carefully removed and placed on the labeled conical tubes using 10 ml pipette, taking care to avoid aspiration of red blood cells. If there were some red blood cell contamination, a few drops of water were added to the PBMCs and allowed to stand for a few minutes to kill

blood cells. The cells were then washed twice with PBS and spun at 1,700 rpm for 10 minutes. The pellets were resuspended in 20 ml of R10 media (RPMI 1640 supplemented with 10% heat-inactivated FCs, 100U/ml penicillin, 1,7mM sodium glutamate, 5.5 ml HEPES buffer). PBMC were counted using Guava Cell Counter, the cells were made up to 1 million per ml concentration and were then ready to be used. If the cells were going to be used the following day, they were kept in T25 flask and maintained in the incubator at 5%$CO_{2;}$ 37 ^{o}C. Cells not for immediate use were frozen and were stored in liquid nitrogen until further use.

2.2.2. HLA TYPING

DNA for HLA typing was extracted using Puregene DNA isolation kit for blood (Gentra Systems, Minneapolis, Minn.) according to the manufacturer's instructions. HLA Class I typing was done by DNA PCR using sequence specific primers as previously described (Kiepiela et al., 2004, Ngumbela et al., 2008).

2.2.3. VIRAL LOADS AND CD4 T CELL COUNTS

Viral loads (VL) were determined from plasma using Roche Amplicor (version 1.5) and CD4+ T cell counts were enumerated from fresh blood by Tru-Count technology using a four-color FacsCalibur flow cytometer (Becton Dickinson) as previously described (Thobakgale et al., 2007, Ngumbela et al., 2008).

2.2.4. SYNTHETIC HIV-1 PEPTIDES

A panel of 410 overlapping peptides (18 amino acids long with 10-amino acid overlap) spanning the entire HIV-1 clade C consensus sequence were synthesized on an automated peptide synthesizer (MBS 396, Advanced ChemTech) and used in a matrix system in screening assays (Kiepiela et al., 2004, Goulder et al., 2001a). *Ex vivo* measurement of T cells for IFN-γ production was undertaken by ELISPOT assay as previously reported (Kiepiela et al., 2004, Ngumbela et al., 2008, Thobakgale et al., 2009). The antigen-specific T cell responses were considered positive if they were > 100 SFC above the unstimulated negative control wells.

2.2.5. ENZYME-LINKED IMMUNOSPOT ASSAY

Peptide titration and fine mapping of the HLA-C restricted epitopes were performed by IFN-γ ELISPOT assay. Before the addition of cells, 96 well polyvinylidene difluoride plate (Millipore) was coated with 0.5µg/µl of anti-IFN-γ antibody and incubated at least overnight at 4 ^{o}C. The plate was then washed 6x with PBS with 1% fetal calf serum (FCS). The protein in the FCS prevents non-specific binding of the anti-IFN-γ antibodies. After washing the plates, 50 µl of R10 media (RPMI 1640 supplemented with 10% heat inactivated FCS, 100U/ml penicillin, 1.7 mM sodium glutamate, 5.5 ml HEPES buffer) was added to each well of the ELISPOT plates. For peptide titration, the 2 µg/ml individual peptide was serial diluted from 10^{-4} to 10^{-9} Molar and 10 µl of peptide was added to each well and 10 µl of PHA (250 µg/ml) was added for positive wells. The cells

were diluted to give 50,000 cells per well and added to each well and incubated at 37 ^{o}C, 5% CO_2 overnight. On the following day, the plate was washed with PBS 6x. Biotinylated anti-IFN-γ Ab (0.5µl/ml) was added and the plates incubated for 90 minutes in the dark. The plates were washed 6x as above and incubated with streptavidin-alkaline phosphatase conjugate for 45 minutes. The plate was developed using BCIP and NBT diluted in TRIS buffer and the reaction stopped by washing with tap water when intense blue spots appear in the positive control wells. Spot forming cells (SFC) were counted using an automatic plate reader (ImmunoSpot Software); results were expressed as spot forming cells (SFC)/10^6 PBMCs.

2.2.6. DETERMINATION OF FUNCTIONAL AVIDITY USING PEPTIDE TITRATION ASSAY

This was determined by performing limiting dilution and determining the concentration of peptide required to induce half maximal response. In this assay, the peptide was added in serial dilutions ranging from 100µg/ml to 10pg/ml in an ELISPOT assay incubated with freshly isolated PBMCs. Standard protocol of ELISPOT assay (Fig.3) was followed except for the concentration of the peptide which was varied.

2.2.7. DEFINING OPTIMAL PEPTIDE BY FINEMAPPING

As previously described, 410 overlapping peptides, 18 amino acids in length and overlapping by 10 amino acids with adjacent peptide were synthesized (Honeyborne et al., 2006). In cases where overlapping peptides yielded a positive response, individual peptides (8-11 amino acids long) were designed and tested to confirm which specific optimal peptide elicited the response. Each peptide was diluted by means of serial dilution from high concentration to low concentration (10^{-1}M to 10^{-6} M). The truncated peptide with highest functional avidity is described as the optimal peptide.

2.2.8. GENERATION OF PEPTIDE-SPECIFIC T CELL LINE

Peptide-specific T cell lines were generated as previously described (Honeyborne et al., 2006, Goulder et al., 2001b). In this assay, PBMCs were incubated with 33µg/ml for about one hour. There were then re-suspended in R10/50 medium (RPMI 1640 (Sigma), 10% fetal calf serum (Sigma), 10 mM HEPES buffer with IL-2 (50U). There were then incubated for 1 week in culture at 37^{o}C and 5% CO_2 and medium was replaced every 3-4 days. The specificity of the T cells was tested using intracellular cytokine staining. Briefly, 100,000 T cells were incubated with 33 µg/ml peptide (TF10 or RM9), another tube with T cells only, and in the third tube the T cells were stimulated with non-specific peptide. Anti-CD28 and CD49d monoclonal antibodies (BD Bioscience) were added in each tube and incubated at 37^{o}C and 5% CO_2 for 30-60 minutes. Following the incubation, brefeldin A (10 µg/ml) was added, with further 6 hours incubation at 37^{o}C

and 5% CO_2. The tubes with the T cells were then taken out from the incubator and placed at 4^oC for further staining. The cells were washed and stained with surface antibody, anti-CD8 (PE) and anti-CD4 (FITC) (BD Bioscience).

2.2.9. B CELL TRANSFORMATION FOR HLA RESTRICTION

PBMCs were separated from the fresh blood using Histopaque; the cells were counted using Guava Via Cell Count. The cells were spun down at 1,700 rpm for 5 minutes, the supernatant was discarded and the cells were added on the 24 well plates. The cells were mixed with 0.5 ml filtered heat inactivated FCS, 0.5 ml EBV stock, 7.5 µl CSA (of a 20µg/ml stock) and the final concentration of cyclosporine A was 0.1 µg/ml (CSA inhibits T cells). The volume was made up to 1.5 ml with R20 (RPMI 1640 supplemented with 20% heat-inactivated FCS, 100U/ml penicillin, 1.7mM sodium glutamate, 5.5 ml HEPES buffer) . The mixture was added in each well of the 24 well culture plates, preferably using the middle well and in the outside well as sterile PBS. They plates were kept into the incubator for 7 days without disturbing them. The B cell lines take 3-6 weeks to transform, they were fed twice a week using R20 (RPMI 1640 supplemented with 20% heat-inactivated FCS, 100U/ml penicillin, 1.7 mM sodium glutamate, 5.5 ml HEPES buffer) until dense yellow colonies were observed on the plate. The cells were then transferred into T-25 flask with 6 ml of R20 (RPMI 1640 supplemented 20% heat-inactivated FCS, 100U/ml penicillin, 1.7mM sodium glutamate, 5.5 ml HEPES buffer) medium.

2.2.10. POLYFUNCTIONALITY ANALYSIS BY MULTICOLOR FLOW CYTOMETRY

Ex vivo measurement of CD8+ T cells for expression of IFN-γ, IL-2, MIP-1β, TNF-α and CD107a was assessed by multicolor flow analysis as previously described (Streeck et al., 2008). In brief, freshly thawed cryopreserved PBMCs were resuspended to 1-2 × 10^6 cells/ml in R10 media (RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 1.7mM sodium glutamate, 5.5 ml HEPES buffer) and rested for 2 hours at 37^{o}C and 5% CO_2. One million cells/ml were stimulated with optimal HLA-B*57/5801 or HLA-Cw*-restricted peptides that represented immunodominant (≥500 SFC on ELISPOT) responses in the study subjects (Fig. 7A) in the presence of anti-CD28 and anti-CD49 co-stimulatory antibodies. A negative control with PBMCs alone and a positive control containing PBMCs stimulated with *Staphylococcus* enterotoxin B (SEB) were included in the assays. Anti-CD107a-PE-Cy5 (BD Biosciences) antibody was added and incubated for 30 minutes at 37^{o}C, 5% CO_2, followed by addition of Brefeldin A (10 µg/ml, Sigma-Aldrich, St Louis, MO) and Monensin (2.5 µg/ml, Sigma-Aldrich) and incubated at 37^{o}C, 5% CO_2 for total of 6 hours. The cells were then washed with PBS (2% FCS), stained to differentiate between live/dead cells (violet viability dye, Invitrogen) and incubated for 30 minutes at 4^{o}C. Cells were then washed and stained with the following surface antibodies: anti-CD3-PE Cy5.5 (Caltag), CD4-APC, CD8-APC Cy7 (both from BD Biosciences) and incubated for 20 min in the dark at room temperature. Cells were again washed and fixed in 1 % paraformaldehyde (Fix Perm A, Caltag) for 20 min in the dark at room temperature and were then permeabilized (Fix

Perm B, Caltag) and stained intracellularly with the following antibodies: anti-IFN-γ PE Cy7, anti-TNF-α Alexa 700, anti-MIP-1β PE, anti-IL-2 FITC (purchased from BD Biosciences) before incubation for 20 min in the dark at room temperature. The cells were washed twice with PBS and resuspended in 200 µl of PBS before acquisition on LSRII flow cytometer (BD Bioscience).

2.2.11. SAMPLE ACQUISITION AND ANALYSIS

Between 200,000 and 1,000,000 events were collected per sample. Analysis was performed using DIVA and FlowJo 8.3.3 software (TreeStar, Ashland, OR). Initial gating was on the lymphocytes population, then forward scatter height (FSC-H) versus forward scatter area (FSC-A) to remove doublets. Subsequently, live CD3+ T cells gating, followed by identification of CD8+ T cells was done; then individual gates (set based on the negative control), for respective functions were made to identify positive responses. Boolean gating was performed to create a full array of possible combination of up to 32 response patterns. Positive responses were reported after background correction and the percentage of epitope-specific CD8+ T cell responses had to be at least two times higher than background for each tested marker. Analysis of multifunctional data was performed by PESTLE (version 1.6.2) and SPICE 5.0 (Mario Roederer, ImmunoTechnology Section, Vaccine Research Center, NIH, and Bethesda, MD).

2.3. RNA ISOLATION

RNA isolation was performed using QIAamp Viral RNA isolation kit (Qiagen). Five hundred and sixty (560µl) of Buffer AVL which contains carrier RNA was pipette into 1,5ml microcentrifuge tube. Plasma (140 µl) was added into the Buffer AVL-carrier RNA in the microcentrifuge tube and mixed by pulse-vortexing for 15 seconds. The mixture was incubated at room temperature (15-25^{o}C) for 10 minutes and centrifuged briefly to remove droplets from the lids. Ethanol (560 µl) 96%-100% was added into the sample and pulse vortexed for 15 s followed by centrifugation for 1 min. The solution from the above step was applied to the QIAamp Mini column without wetting the rim. The solution was centrifuged at 6000 x g (8000 rpm) for 1 min and the column was placed on a new centrifuge tube after discarding the filtrate. Buffer AW1 (500µl) was added into QIAamp Mini column and centrifuged at 6000 x g (8000 rpm) for one min. A new clean 2 ml collection tube was replaced after discarding the filtrate and 500µl of Buffer AW2 added and centrifuged at full speed (20,000 x g; 14,000 rpm for 3 min) and the step repeated. A new 2 ml collection tube was placed under QIAamp Mini column and centrifuged for 1 min. Finally, 1.5 ml microcentrifuge tube was placed under the column and 60 µl of water was added to elute the RNA from the column. It was incubated at room temperature for 1 min and centrifuge at 6000 x g (8000 rpm) for 1 min. This protocol was according to the manufacturer's instructions with minor amendments.

2.3.1. REVERSE TRANSCRIPTASE (RT) POLYMERASE CHAIN REACTION

0.2 ml nuclease free, thin walled PCR tube was placed in ice, while master mix was being prepared. For each PCR tube: 14.4 µl of DEPC water was added, 20µl of 2x Reaction mix, 0.8 µl of 10 µM gene-specific primers (Nef and Gag) for both 5' and 3' outer primers, 0.8 µl of Superscript ™ III RT/ Platinum® Taq High Fidelity Enzyme Mix and 5µl of RNA Template was added into the mixture. The mixture was gently mixed by centrifuging briefly in order to make sure that all ingredients settled at the bottom of the tube. The tubes were then placed in a preheated thermal cycler previously programmed with the following thermocycle conditions: cDNA denaturation and pre-denaturation was 55^{o}C for 30 min; 94^{o}C for 2 min, PCR amplification conditions; denaturation temperature 94^{o}C for 15 sec, annealing temperature 55^{o}C for 30 sec; extension step 68^{o}C for 2 min for 35 cycles.

The final extension temperature was 1 cycle of 68^{o}C for 5 minutes and finally kept at 4^{o}C. The second round PCR was performed using the product of the first PCR (cDNA). In the second round PCR, each PCR tube contains the following ingredients: 37 µl of DEPC water, 5 µl of 10x Taq buffer, 4 µl dNTPs, and 0.8 µl of 10 µM inner primers of gene-specific primers (*nef* and *gag*), 0.25 µl of Ex Taq and 3 µl of first round PCR product. The conditions of the second round PCR were pre-denaturation step (94^{o}C; 2 min), PCR amplification (94 ^{o}C; 15 sec, 56^{o}C;15 sec, 72^{o}C;1 min 30 sec) for 35 cycles.

The final extension was 1 cycle 72^{o}C; 7 min and then a hold at 4^{o}C until the product was characterized by electrophoresis on agarose gel.

The amplified DNA was characterized on 1% agarose gel electrophoresis; 0.5 g of agarose was weighed and dissolved in 1 x TBE buffer by heating. The mixture was cooled down and 2.5 µl ethidium bromide was added to the mixture and mixed thoroughly. It was then added on the gel cast with well formers or comb allowing for a few minutes for it to solidify. Tank buffer (1 xTBE (Tris/Boric acid/EDTA) was added on the tank and the contents of each DNA tube were loaded on each well. Each well was loaded with molecular weight marker to determine the size of the product. The gel was ran for 45 min at 100 V and 400 A.

2.3.2. GEL PURIFICATION

PCR product was purified using QiAquick gel extraction kit. In this procedure, DNA fragment was removed from the agarose gel with a clean sharp scalpel. Colorless, empty tubes were weighed and the excised DNA fragments in the tubes were also weighed to determine the weight of DNA, the weight of colourless tube was subtracted from the weight of the colorless tube with DNA. Three volumes of Buffer QG (from the kit) were added to one volume of gel (100 mg~100 µl). The gels together with the buffer were incubated at 50^{o}C for 10 min or until the gel slice had completely dissolved. In order to dissolve the gel slice, it was mixed with Buffer QG and centrifuged for every 2 to 3 min during incubation. After dissolving the gel slice, the color of the mixture was checked to

see whether it was similar to Buffer QG before dissolving the gel. One gel volume of isopropanol was added and mixed. The QIAquick spin column was prepared and the mixture was applied into the column and centrifuged for 1 min. The flow-through was thrown out and placed back in the collection tube. In order to remove all the traces of agarose 0.5ml of Buffer QG was added into the column and centrifuged for 1 min. For direct sequencing, 0.75 ml of Buffer PE was added into the column and allowed to stand for 2-3 minutes and centrifuged for 1 min. the flow through was discarded and continued with centrifugation for another 1 min. A new 1.5 ml microcentrifugation tube was placed into the QiAquick column and 50 µl of water was added to elute DNA from the column and let the column stand for 1 min, continue with centrifugation for the next minute. The purified DNA was then analyzed and characterized on agarose gel; one volume of loading dye was added to 5 volume of purified DNA. After analyzing the DNA from the gel, the DNA was quantified using low molecular weight marker and DNA concentration calculated for sequencing.

2.3.3. SEQUENCING OF *GAG* AND *NEF* GENES

HIV-1 RNA was extracted from 500µl of plasma (VL <5,000 HIV-1 RNA copies/ml) or 140µl plasma (VL >5,000 RNA copies) using QIAmp viral RNA extraction kit (Qiagen). RNA was reversed transcribed using one-step RT-PCR kit (Invitrogen) and gene specific primers under the following conditions: reverse transcription at 55 ^{o}C for 30 min, followed by amplification at 94 ^{o}C for 2 min, and 35 cycles of 94 ^{o}C for 15 sec, 55 ^{o}C for 30 sec, 68 ^{o}C for 2 min; and final extension at 68 ^{o}C for 5 min.

Gag PCR primers were 5'-CTAGCAGTGGCGCCCGAACA-3' and 5'GCAGTCTTTCATTTGGTGTCCTCC-3'. The same primers were used in the second round PCR reaction using the following conditions: 94 ^{o}C for 2 min, followed by 35 cycles of 94 ^{o}C for 15 sec, 58 ^{o}C for 30 sec, 72 ^{o}C for 1:30 min, with a final extension at 72 ^{o}C for 7 min. Nef PCR primers were 5'-TTCAGCTACCACCGATTGAGA-3' and 5'-TGAGGGTTGGCCACTCC-3'. The PCR conditions were similar to those for *gag* except for the second round PCR reaction, where the annealing temperature was set at 56^{o}C for 30 sec. Purified (Qiagen) PCR products were sequenced using Big Dye v3.1 Terminator sequencing kit (Applied Biosystems, Foster city, CA, USA) on a XL-3100 automatic DNA sequencer (Applied Biosystems). Nucleotide sequences were analyzed using Sequencer 4.8 software (Gene Codes Corporation, Ann Arbor, MI). Alignments of reference and newly generated *gag* and *nef* sequences were performed using ClustalX and edited by Bioedit Sequence Alignment editor.

2.3.4. STATISTICAL ANALYSIS

Mann-Whitney U test was used to compare the median magnitude of HLA-B versus HLA-Cw* responses. The same test was used to compare individual effector functions or polyfunctional responses between HLA-B*57/5801 and HLA-Cw responses in individuals. The Spearman rank correlation test was used to correlate individual and polyfunctional responses with viral loads and CD4 counts. Fisher's exact test was used to compare proportions of HLA-B and HLA-C epitopes with sequence variation.

Chapter 3. RESULTS

3.1 CHARACTERIZATION AND QUANTIFICATION OF HLA-CW* RESTRICTED EPITOPE

The Sinikithemba cohort was established at 2002 in McCord Hospital, Durban. It consists of 451 HIV positive patients followed up since 2002, of which the time of infection was not known. These patients were between 15 and 60 years, including both males and females. HLA-typing for these patients was previously performed using HLA-Class I gene-specific primers (Ngumbela et al., 2008, Kiepiela et al., 2004). Viral loads (Roche Amplicor) and CD4 counts (BD Facscalibur) of these patients were also determined. Viral loads were performed every six months while CD4 counts were performed at every clinic visit. The 78 patients analyzed in this study were from the Sinikithemba cohort, HLA-C restricted epitopes with maximum responses at least greater than 100 spot forming cells (SFC) from ELISPOT assay were selected from these patients and quantified by ICS. Using the standard ICS assay 78 patients with HLA-Cw* restricted CD8+ T cells responses were determined (Fig.5).

Cw*0701-KRQEILDLWVY (Nef) (n=30), Cw*0304-YVDRFFKTL (p24) (n=14), Cw*0801-TPQDLNTML (p24) (n=11), Cw*0801-GAFDLSFFL (p24) (n=6), Cw*18-FRDYVDRFF (p24) (n=8), Cw*0801-IVTDSQYAL (RT) (n=6) were the most targeted HLA-Cw* restricted epitopes and few patients had response to Cw*0801-TPGIRYQYNV (RT) (n=2), Cw*6- LTNNAKTIIVHL (gp120) (n=2) (Fig.5). Cw*0701-KY11 had high percentage of IFN-γ+ producing CD8+ cells when compared to other

HLA-Cw* restricted with the median percentage of 0.5%, followed by Cw*0304-YL9 (0.49%) and Cw*0801-IL9 (0.35%) while Cw*06-LL12 has the lowest median of percentage of CD8+ IFN-γ producing cells.

HLA-Cw*0701-KY11 epitope had high percentage of IFN-γ producing CD8+ T cell when compared to other HLA-Cw* restricted epitopes (Fig. 5). Previous studies has shown that HLA-Cw* restricted epitopes including Gag restricted epitopes are associated with high viral loads when compared with HLA-A and HLA-B while HLA-B*57 Gag-restricted epitopes are associated with low viral loads (Kiepiela et al., 2004, Kiepiela et al., 2007). For HLA-Cw* restricted CD8+ T cell responses with significantly high magnitudes of responses, their functional avidities were performed.

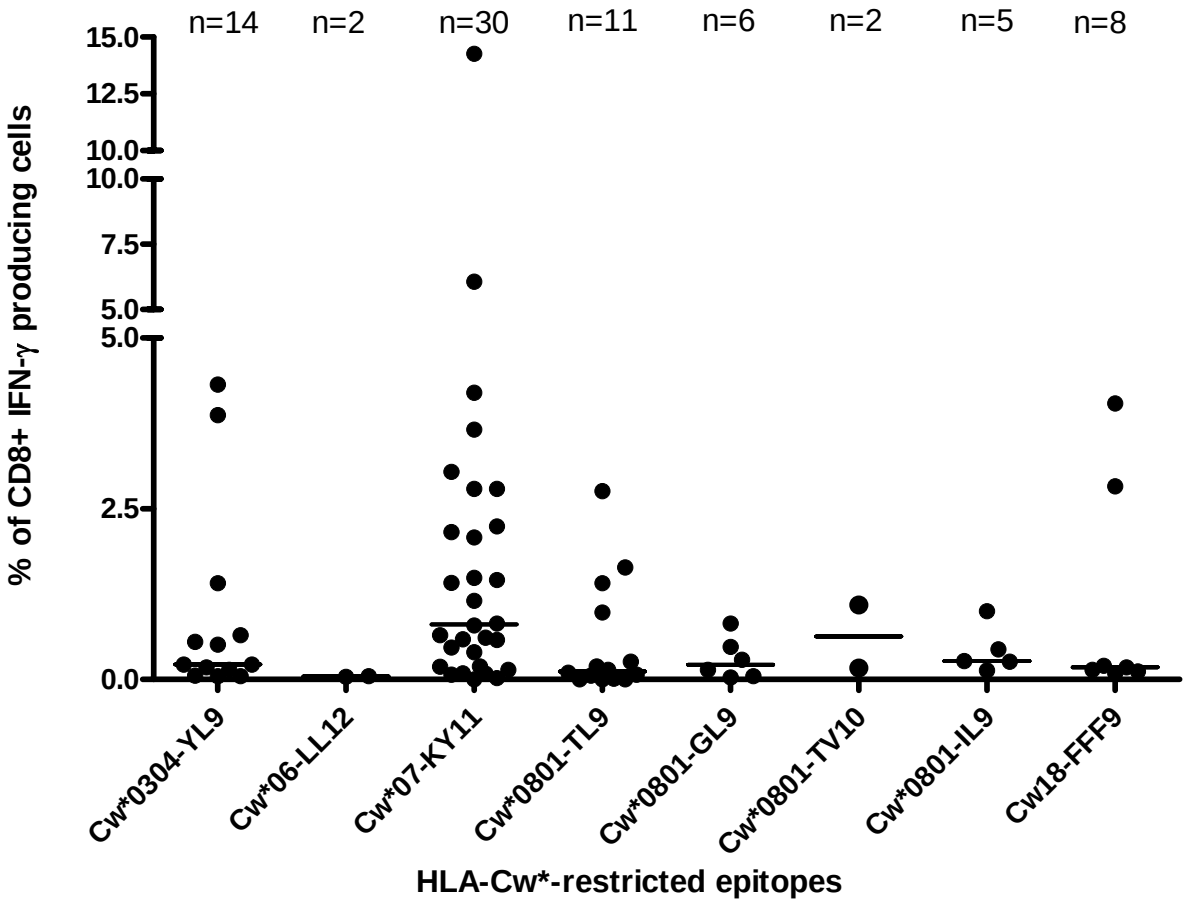

Figure 5. Characterization of HLA-Cw* restricted CD8+ T cell responses using intracellular cytokine staining (ICS). Y-axis shows the percentage of CD8+ T cells producing IFN-γ and x-axis shows the HLA-Cw* restricted epitopes.

3.2 DETERMINATION OF FUNCTIONAL AVIDITY USING PEPTIDE TITRATION

Functional avidity or TCR avidity to HLA/peptide complex is more important as a determinant of the magnitude of response than the peptide affinity to HLA molecules (Yang et al., 2003, Lichterfeld et al., 2007). There is a relationship between the functional avidity and CTL antiviral activity, and this was demonstrated by comparing CTL killing of cells infected with panels of epitope-variant viruses to the corresponding SD_{50} for variant epitopes, showing a sigmoid relationship between avidity and infected cell killing (Bennett et al., 2007). In this study, the HLA-Cw* restricted epitopes with high percentage of IFN-γ producing CD8+ T cells, and their functional avidities were performed. Peptide titration curve was generated using limiting dilution assessing the IFN-γ responses in ELISPOT when stimulated with low concentration.

From previous results (Fig. 5), Cw*0701-KY11 and Cw*0304-YL9 have high percentage of IFN-γ producing CD8+ T cells from intracellular cytokine staining, followed by Cw*0801-TL9 and Cw*18-FFF9. Here the HLA-Cw* restricted epitopes were serially diluted with R10, standard ELISPOT assay was performed assessing the IFN-γ production. HLA-Cw*0701-KY11 (Nef) and Cw*0801-TL9 (Nef) were shown to have high functional avidity compared to Cw*0304-YL9 (p24) and Cw*18 (p24) (Fig. 6).

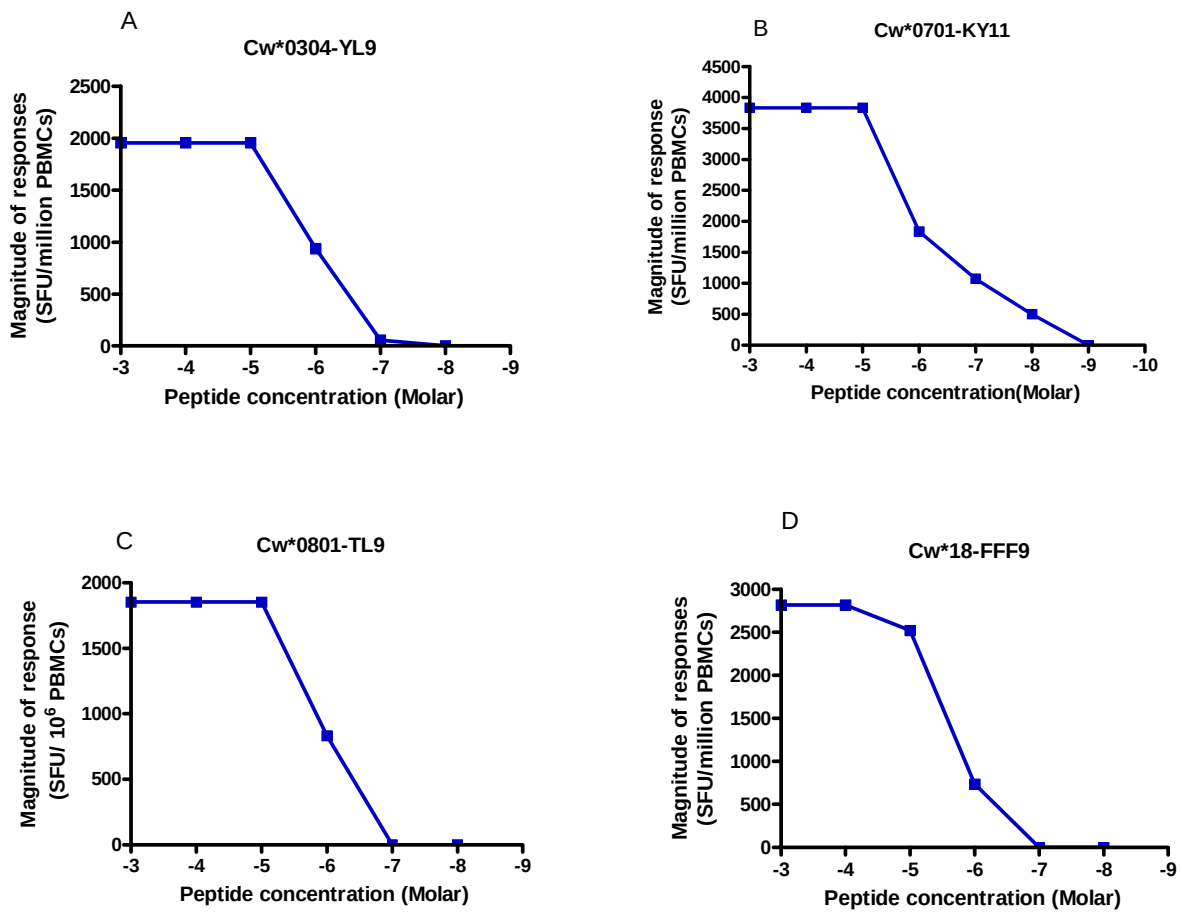

Figure 6. Determination of functional avidity of four dominant HLA-Cw* restricted CD8+ T cell responses. A. indicates the peptide titration curve of Cw*0304-YVDRFFKTL (YL9), B. Cw*0701-KRQEILDLWVY (KY11), C. Cw*0801-TPQDLNTML (TL9), D. Cw*18-FRDYVDRFF (FFF9). This peptide titration curve was the representative of each patient per peptide.

3.3 IDENTIFICATION AND CHARACTERIZATION OF HIV-1 SPECIFIC HLA-C RESTRICTED CD8+ T CELLS

Many studies have proved that HIV-1 specific CD8+ T cell play a crucial role in control of viral replication both in acute and chronic HIV-1 infection (Koup et al., 1994, Wong et al., 2010). The identification of new HIV-1 specific restricted epitopes is very important in development of vaccine especially those associated with the control of viral replication. HLA-B*27 and B*57- restricted HIV-1 specific CD8+ T cell epitopes are strongly associated with the control of viremia, however the role of HLA-Cw* restricted epitopes may be underestimated due its high linkage disequilibrium with other HLAs and there are few known HLA-Cw* restricted epitopes (Goulder et al., 1997a, Goulder et al., 2001a). In the present study, the characterization and identification of new HLA-Cw* restricted epitopes was performed as a requirement in understanding its role in HIV replication.

In the fine mapping assay, 18 amino acids long overlapping peptides were truncated into shorter peptides (8 to 11 amino acids long). Firstly, the screening of responses by ELISPOT assay was done for all the patients. After screening, the overlapping peptide was confirmed as the responses for that particular patient. Overlapping peptide number 180 (OLP#180) and OLP# 362 were determined as positive responses from these patients without knowing whether these responses are restricted by HLA-C or not. Each overlapping peptide consist of 18 amino acids sequence as depicted below and this long peptide was truncated into small peptides (between 8-11 amino acids long) to ascertain

the exact peptide sequence that constitutes an HLA class I peptide. We then performed HLA restriction assays using B cell lines that express particular HLA class I in order to confirm specific HLA-Cw* restriction.

The sequence of optimal peptide was KKKSVTVLDVGDAYFSV (overlapping peptide number 180), and the truncated peptides were TVLDVGDAYF (TF10), TVLDVGDAY (TY9), TVLDVGDAYFS (TS11), VLDVGDAYF (VF9) and VLDVGDAYFV (VV11). The other overlapping peptide that was truncated for fine mapping was IVQQQSNLLRAIEAQQHM (overlapping peptide number 362) and the short peptides were RAIEAQQHM (RM9), AIEAQQHM (AM8), RAIEAQQH (RH8), LRAIEAQQHM (LM 10) and RAIEAQQ (RQ7). Each of these peptides was serially diluted separately from low concentration to the highest concentration. PBMCs from the patients with immune response of these overlapping peptides (180 and 362) were stimulated with serial diluted peptide, using standard IFN-γ ELISPOT assay. From the titration curves, the results showed that the optimal peptide from overlapping peptide 180 was TF10 and optimal peptide from overlapping peptide 362 was RM9 (Fig 7).

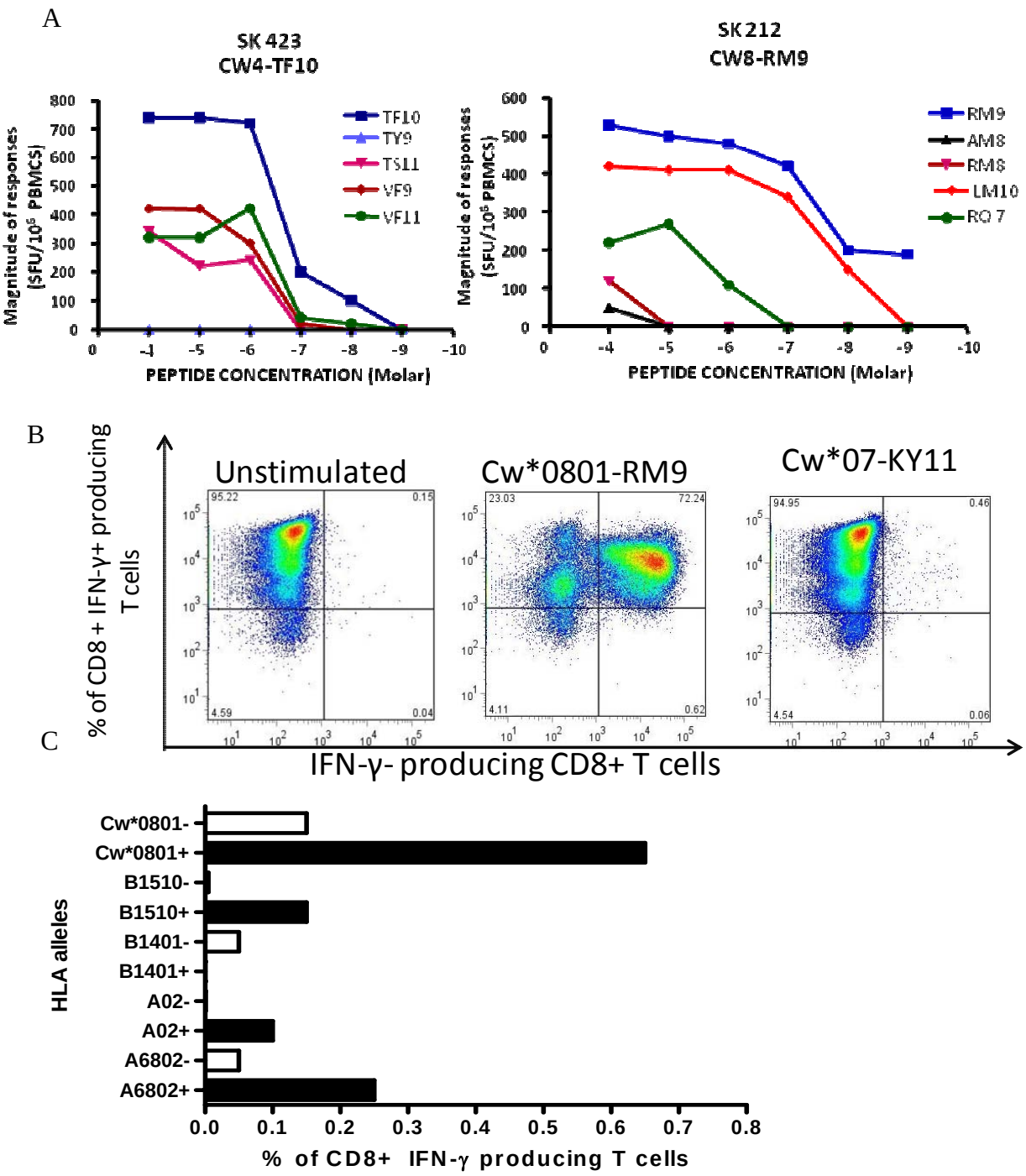

Figure 7. Optimal definition of two novel HLA-Cw*0401 (TVLDVGDAYF) and HLA-Cw*0801 (RAIEAQQHM) -restricted epitopes. A. Peptide titration curve showing the determination of optimal peptide B. Determination of the specificity of HLA-C restricted CD8+ T cell line using intracellular cytokine staining and HLA restriction C) HLA restriction of Cw0801-RM9 (patient's HLA A6802/02 B1401/1510 Cw*0801/0801) . The specificity of T cells and the HLA restriction was only performed for RM9 because TF10 B cell lines were insufficient.

To further prove that the peptides with high responses were optimal peptides, peptide-specific cell lines were generated as described in *Materials and methods*. The specificity of these optimal epitopes was determined by using standard intracellular cytokine staining. RM9 and TF10 epitopes have high specificity when compared to the cell line stimulated with other peptides (Fig. 7B). HLA restriction was performed in order to determine the restricting HLA of the peptide.

Previously, optimal epitopes were determined by either ELISPOT or intracellular cytokine staining assay (Honeyborne et al., 2006, Goulder et al., 2001a). However, the ELISPOT assay resulted in high background of spot forming cells (SFC); intracellular cytokine staining assay thus became the preferable assay for HLA restriction (Fig. 7C). In the HLA restriction assay, the HLA-matched B cell lines are generated from patients. The six mis-matching B cell lines and the lines with no HLA in common with patient's HLA type were chosen. The T cell line/PBMC's of the chosen patients were added into the tube with the B cell line and stimulated with optimal peptide. The standard intracellular cytokine staining assay was followed assessing the IFN-γ producing CD8+ T cells (Fig. 7C). In this assay, RM9 and TF10 peptide-specific cell line were incubated with B-cell line matched through several of the individual HLA-A, B, C expressed by donor. The results confirmed that RM9 is restricted by HLA-Cw*0801, while TF10 restricted by Cw*0401 consistent with data from other studies (Makadzange et al., 2010).

3.4 MAGNITUDE AND BREADTH OF HLA-B*57/5801 AND HLA-C RESTRICTED RESPONSES BY THE IFN-Γ ELISPOT ASSAY

In addition, 37 of 451 (8.2%) participants coexpressed HLA-B*57/5801 and HLA-Cw*07, 51 (11.3%) expressed HLA-B*57/5801 without HLA-Cw*07 and 81 (18) expressed HLA-Cw*07 without HLA-B*57/5801. The median age, CD4 cell counts and viral load for the 37 subjects who co expressed HLA-B*57/5801 and HLA-Cw*07 was 37 years, 490 cells/µl and 6,700 copies/ml respectively. Of these 37 individuals only the nine further studied here had concurrent immunodominant HLA-B*57/5801-and HLA-Cw*07-restricted IFN-γ ELISPOT responses defined as ≥500 SFC/ 10^6 (Table 1).

Table 1. Characteristics of study subjects.

Patient ID	Sex	Age (years)	CD4 count (cell/ml)	Viral load (copies/ml)	HLA type	HLA-B*57/5801 epitopes	HLA-C epitopes
SK 009	Male	32	291	47,000	A*2301/74 B*1503/5702 Cw*0202/0701	TSTLQEQIAW (p24)	KRQEILDLWVY(Nef)
SK 215	Female	35	202	34,800	A*6802/74 B*0702/5703 Cw*07/07	ISPRTLNAW (p24)	KRQEILDLWVY(Nef)
SK 236	Female	37	411	9,900	A*02/3002 B*0801/5801 Cw*07/07	ISPRTLNAW (p24)	KRQEILDLWVY(Nef)
SK 251	Female	58	271	2,530	A*02/3001 B*4201/5801 Cw*07/1701	QATQDVKNW (p24)	KRQEILDLWVY(Nef)
SK 318	Female	27	370	3,600	A*33/74 B*0702/5703 Cw*07/07	KAFSPEVIPMF (p24)	KRQEILDLWVY(Nef)
SK 358	Female	42	264	750,000	A*0202/2301 B*08/5701 Cw*07/07	ISPRTLNAW (p24)	KRQEILDLWVY(Nef)
SK 364	Female	38	305	11,500	A*02/3001 B*4201/5801 Cw*07/1701	QATQDVKNW (p24)	KRQEILDLWVY(Nef)
SK 379	Male	44	267	4,310	A*0205/0208 B*0702/5801 Cw*07/07	TSTLQEQIAW (p24)	KRQEILDLWVY(Nef)
SK 428	Female	45	214	272,000	A*0205/0208 B*1401/5801 Cw*07/08	TSTLQEQIAW (p24)	KRQEILDLWVY(Nef)
Median		38	271	11,500			

All subjects included in this study made HIV-specific CD8+ T cell responses to known HLA-B*57/5801 and HLA-Cw*07 epitopes as determined by IFN-γ ELISPOT (Fig 7A, Table 1). Only a few individuals within the Sinikithemba study cohort had responses $\geq 500/10^6$ SFCs for both HLA-B*57/5801 and HLA-Cw*07: these high magnitude responses were examined further in subsequent assays. The immunodominant HLA-B*57/5801-restricted responses were to the following four epitopes: TSTLQEQIAW (TW10), ISPRTLNAW (ISW9), QATQDVKNW (QW10) and KAFSPEVIPMF (KF11). In contrast, only one HLA-Cw* response was targeted by the study subjects: KRQEILDLWVY (KY11) restricted by HLA-Cw*07 (Fig.8A and B). The overall magnitude of the responses targeted by HLA-Cw*07 restricted epitopes was significantly higher than the magnitude of HLA-B*57/5801 restricted responses (p=0.0012, Mann-Whitney test; Fig 8B).

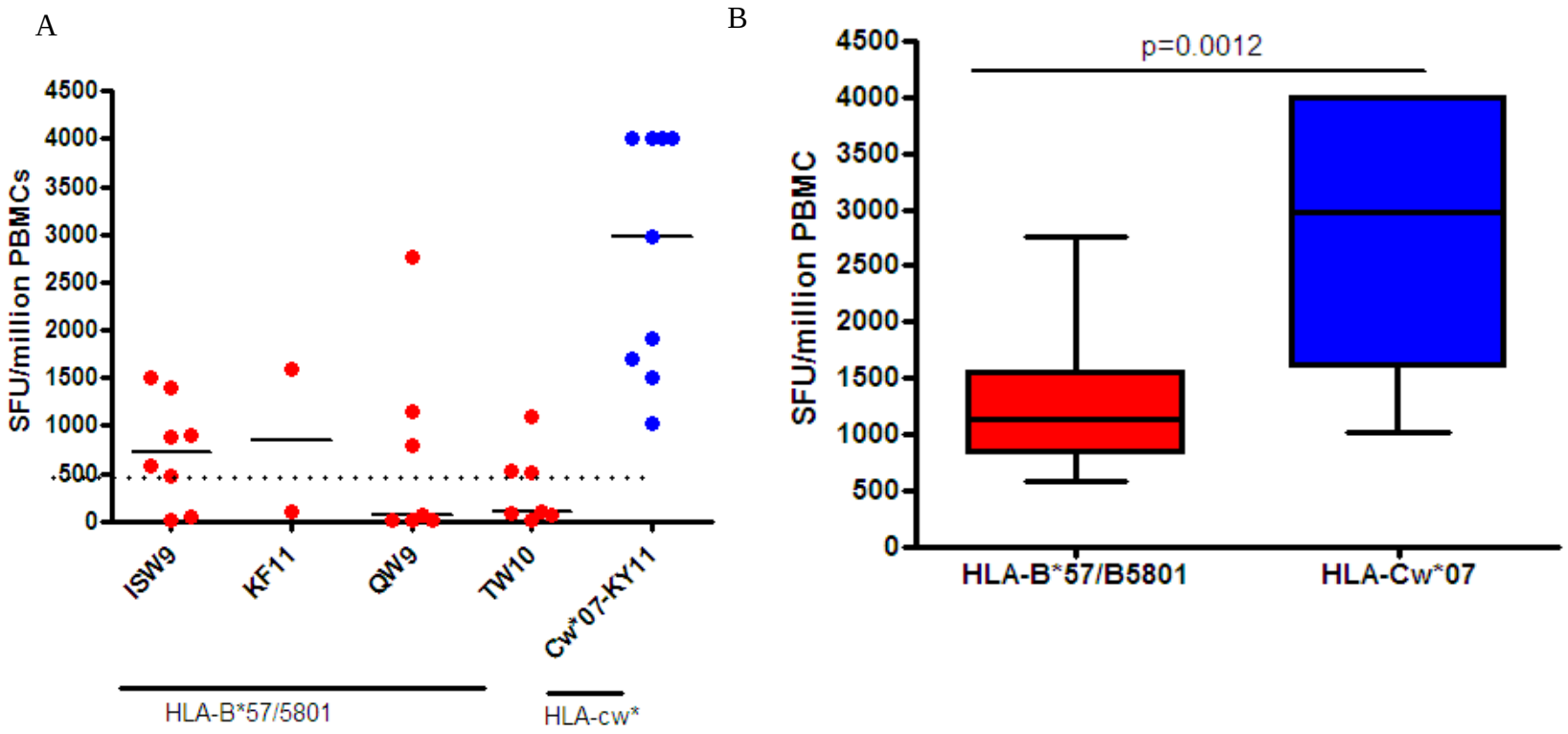

Figure 8. Measurement of HLA-B*57/5801 and HLA-Cw*07-restricted T cell responses

(A) Hierarchy of dominant epitopes presented by the study subjects expressing HLA-B*57/5801 and HLA-C alleles (n=9). Dotted line indicates responses above 500 SFCs from ELISPOT assay that were further tested in subsequent multicolor assays. **(B)** Combined total magnitude of T-cell responses presented by both HLA-B*57/B5801 and HLA-Cw*07 restricted epitopes in the study individuals (n=9).

3.5. FUNCTIONALITY PROFILES OF HLA-B*57/5801 AND HLA-C RESTRICTED HIV-1 SPECIFIC CD8+ T CELL EPITOPES

Previous studies of long term non-progressors have shown that HIV-1-specific CD8+ T cells restricted by protective alleles such as HLA-B*57 and HLA-B*27, may be more polyfunctional than CD8+ T cells restricted by other HLAs (Zimmerli et al., 2005, Harari et al., 2007, Champagne et al., 2001). To determine the functionality of HIV-specific CD8+ T cells restricted by alleles with different disease outcomes (HLA-B*57/5801 and HLA-Cw*07); we assessed the polyfunctionality of these responses in those who possessed them concurrently.

HIV-specific CD8+ T cells polyfunctionality was evaluated using multicolor flow cytometry by simultaneous measurement of five functions: IFN-γ, TNF-α, IL-2, MIP-1β, and CD107a as previously described in other studies (De Rosa et al., 2004, Betts et al., 2006, Streeck et al., 2008). On single function gating, IFN-γ expression was lower on HLA-B*57/5801 than on HLA-Cw*07 restricted HIV-1 specific CD8+ T cell epitopes ($p=0.06$; Mann-Whitney test) (Fig. 9A), consistent with ELISPOT data. A similar trend was observed for the other individual functions although the magnitude of other responses was lower than for IFN-γ. The vast majority of both HLA-B and HLA-Cw* restricted CD8+ T cells (75%) in this study cohort were monofunctional (Fig. 9B).

The most frequently expressed function was MIP-1β at 40% for HLA-B*57/5801 and 30% for HLA-Cw*07, with CD107a at 30% for HLA-B*57/5801 restricted CD8+ T cells and 28% for HLA-Cw*; and IFN-γ at 29 % for HLA-B*57/5801 versus 30% for HLA-Cw*.

Monofunctional IL-2 or TNF-α producing HIV-specific CD8+ T cells were very infrequent in this cohort of chronically infected patients and only a small proportion of HIV-specific CD8+ T cells expressed TNF-α responses confirming previous studies that this effector function is lost early in infection (Wherry et al., 2003, Lichterfeld et al., 2004b).

To further investigate potential differences in functional profiles upon stimulation with both HLA-B*57/5801- and HLA-Cw*-restricted peptides on all patients, we compared the frequencies of the different HLA-restricted CD8+ T cells expressing different functions detected in these individuals. The fraction of each function was determined as a percentage of the total CD8+ T cell response as previously described (Streeck et al., 2008). No significant differences were observed between HLA-B*57/5801- and HLA-Cw*-restricted responses with regard to five ($p=0.64$; Mann-Whitney test), four ($p=0.89$), three ($p=0.87$), two ($p=0.22$) and one function ($p=0.37$) (Fig. 9B). Taken together, these data suggest the differences in disease outcome observed for HLA-B*57/5801 and HLA-Cw* cannot be explained by the polyfunctionality of the HIV-specific CD8+ T cell responses during chronic infection. In addition, polyfunctionality of CD8+ T cell responses may not depend on the restricting HLA allele during the chronic phase of infection.

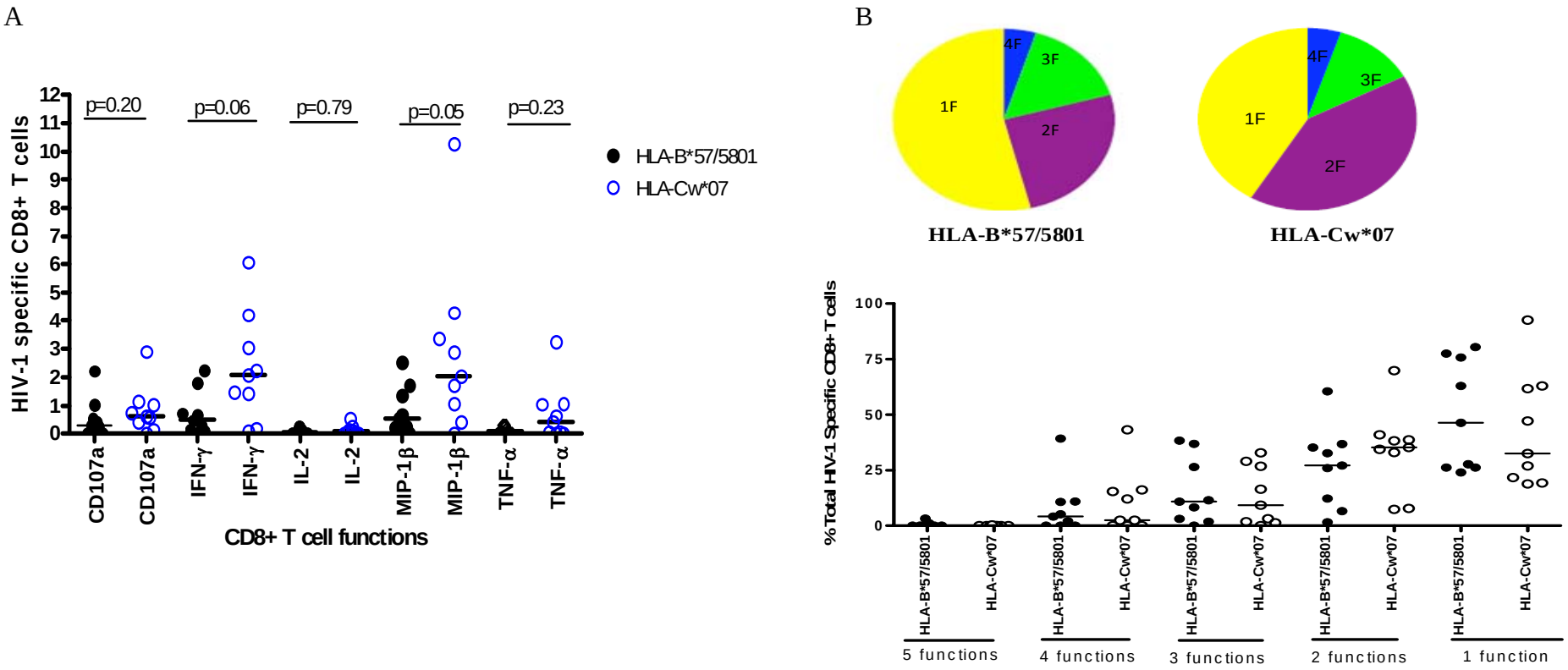

Figure 9. Polyfunctionality of HLA-B*57/5801 and HLA-Cw*07-restricted epitopes

(A) Magnitude of HLA-B*57/5801 and HLA-C restricted epitope responses using multicolor staining, single CD8+ T cell function responses are shown after background subtraction. **(B)** Comparison of the contribution of individual functions between HLA-B*57/B5801 (○) and HLA-C (●) restricted epitopes in the study subjects (n=9). The fractions of the response patterns are grouped and color-coded by the number of functions and summarized in pie chart form where each slice of the pie represents the fraction of the total epitope-specific response that consist of CD8+ T cells with the respective number of functions.

3.6. RELATIONSHIP BETWEEN THE POLYFUNCTIONALITY OF HLA-RESTRICTED HIV-1 SPECIFIC CD8+ T CELLS, CD4 COUNTS AND VIRAL LOADS

Previous studies have suggested that HLA-B-restricted HIV-1-specific CD8+ T cells are more polyfunctional than HLA-A and HLA-Cw* (Harari et al., 2007, Zimmerli et al., 2005) and that the proportion of the HIV-1-specific CD8+ T cell with the highest functionality inversely correlate with viral loads in non-progressive disease (Betts et al., 2006). The proportion of HIV-1-specific CD8+ T cell responses were plotted against CD4 counts and viral load, for all the functions (Fig. 10A and B). Since no differences were noted in polyfunctionality profiles of HLA-B*57/5801 and Cw*07-restricted epitopes, the total percentages across different (1-5) functions were added together to determine whether or not there was a relationship between the polyfunctional CD8+ T cells, viral load and CD4 counts. There was a significant positive correlation between 3 or more functions and CD4 counts ($p=0.006$; $r=0.85$, Spearman test) (Fig 9A). In contrast, there was a negative correlation between monofunctional cells and CD4 counts ($p=0.05$; $r=-0.79$). However, no correlation was noted between the polyfunctional or monofunctional CD8+ T cells and viral loads (Fig. 10B).

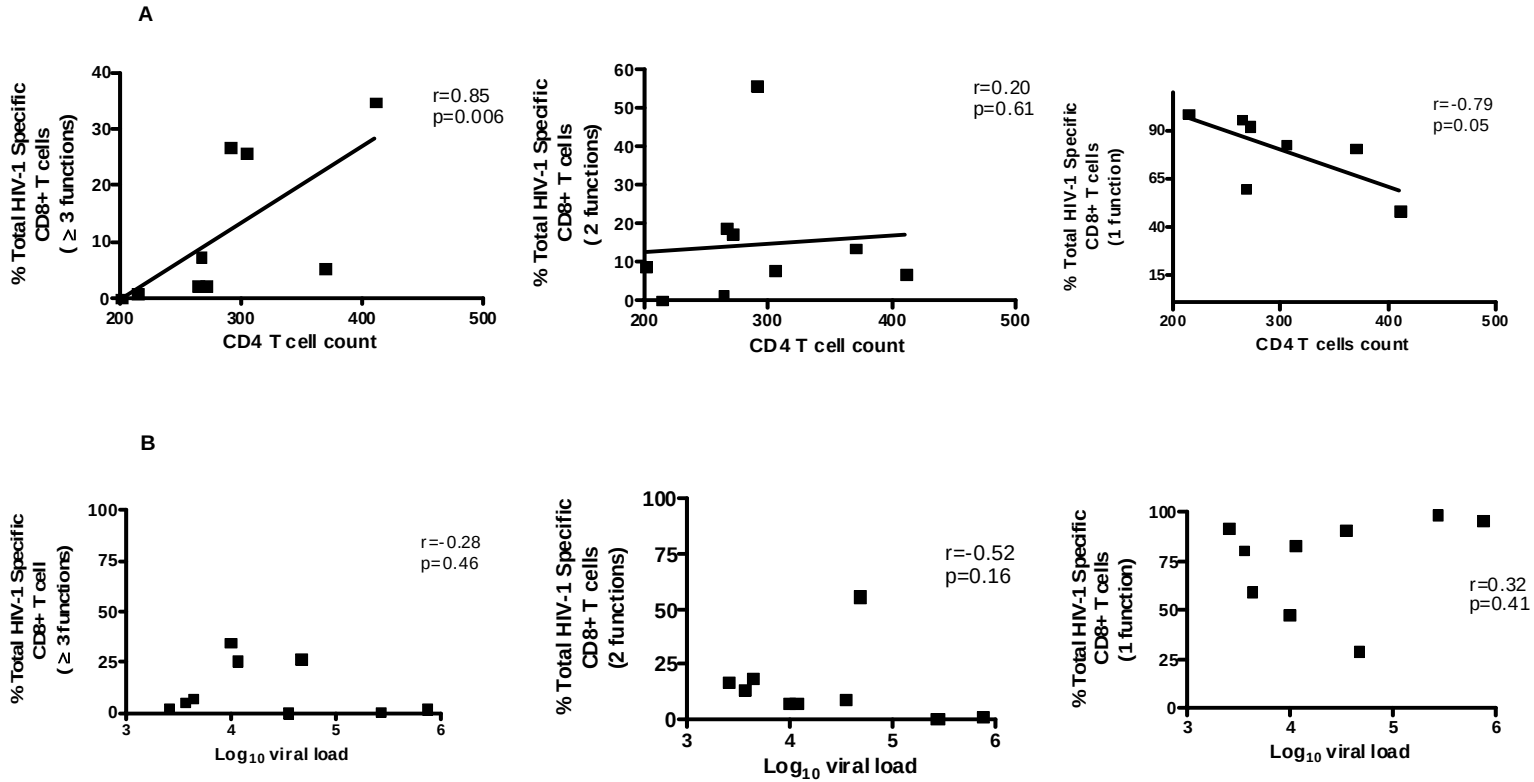

Figure 10. Relationship between mono-functional and poly-functional CD8+ T cell responses

(A) CD4+ T cell counts and **(B)** viral loads.

3.7 SEQUENCE VARIATION WITHIN HLA-B*57/5801 AND HLA-C RESTRICTED EPITOPES AND ITS IMPACT ON CD8+ T CELL POLYFUNCTIONALITY

Table 2 Sequence variation in CD8+T cell epitopes presented by HLA-B*57/B5801 and HLA-Cw*07 alleles.

			HLA-B*57/5801	**epitopes**	**HLA-C epitopes**
Patient ID	ISW9	KF11	TW10	QW9	CW*7-KY11
	ISPRTLNAW	KAFSPEVLPMF	TSTLQEQIAW	QATQDVKNW	KRQEILDLWVY
SK009	L---------	----------	--S-------	---------	----------
SK215	L---------	-N---------	--N-------	---------	---D-------
SK236	L---------	----------	--N-------	---------	----------
SK251	L---------	-G---------	--N-------	---------	----------
SK318	---------	----------	----------	---------	----------
SK358	L---------	----------	--N-------	---------	----------
SK364	---------	----------	----S-----	---------	----------
SK379	---------	----------	----S-----	---------	----------
SK428	L---------	----------	--N-------	---------	----------

Highlighted -epitopes tested in ICS

Unhighlighted- epitopes not tested in ICS

Sequence variation and the accumulation of mutations over course of infection are known to affect epitope recognition, specificity of the T-cell receptor binding and recognition by HLA (Nixon et al., 1988, Goulder et al., 1997b). Next, we evaluated whether the low magnitude of responses noted in HLA-B*57/5801 compared to high HLA-Cw*07-restricted CD8+ T cell responses was due to the sequence variation in the CD8+ T cell epitopes. *Gag* and *nef* genes were sequenced in all nine patients as the epitopes studied here were located in these viral proteins. HLA-B*57/5801-restricted HIV-1-specific CD8+ T cell epitopes had more sequence variation (6/9) when compared to HLA-Cw*07-restricted epitopes (1/9), ($p=0.05$; Fisher's exact test), despite the fact that the HLA-C-restricted response examined is in the highly variable Nef protein. The most frequent sequence changes were noted in the TSTLQEQIAW (TW10) and ISPRTLNAW (ISW9) Gag epitopes restricted by HLA-B*57/5801, and a single sequence variation was noted in the Nef Cw*0701-restricted epitope KRQEILDLWVY (KY11) restricted by HLA-Cw*07 (Table 2). These data suggest that HLA-B*57/5801-restricted epitopes have high sequence variation compared to HLA-Cw*07-restricted epitopes and this may in part account for the low magnitude of HLA-B*57/5801- compared to HLA-Cw*07-restricted responses.

We also investigated whether or not sequence variation impacted on CD8+ T-cell polyfunctionality. CD8+ T cell polyfunctionality was assessed by comparing HLA-B and HLA-Cw*-restricted HIV-1-specific CD8+ T cell epitopes with or without the

sequence variation against the percentage of the total HIV-1-specific CD8+ T cell response (Fig. 11). No differences were noted between epitopes with or without sequence variation with regard to 3 or more functions, 2 functions and monofunctional T-cells. However, there was a high frequency of monofunctional HIV-1-specific CD8+ T cells when compared to other functions e.g. monofunctional HLA-B*57/5801 epitopes with or without sequence variation were higher than HLA-B*57/5801 epitopes with or without sequence variation expressing 3 or more functions ($p=0.004$, $p=0.10$ respectively, Mann-Whitney test).Consequently, whereas the decrease in the frequency of the HLA-B*57/5801- restricted responses compared to HLA-Cw*07-restricted CD8+ T-cell responses may have been due to sequence variation, the data implies that sequence variation alone may not affect the polyfunctionality of HLA-B*57/5801 or HLA-Cw*07 restricted HIV-1-specific CD8+ cells.

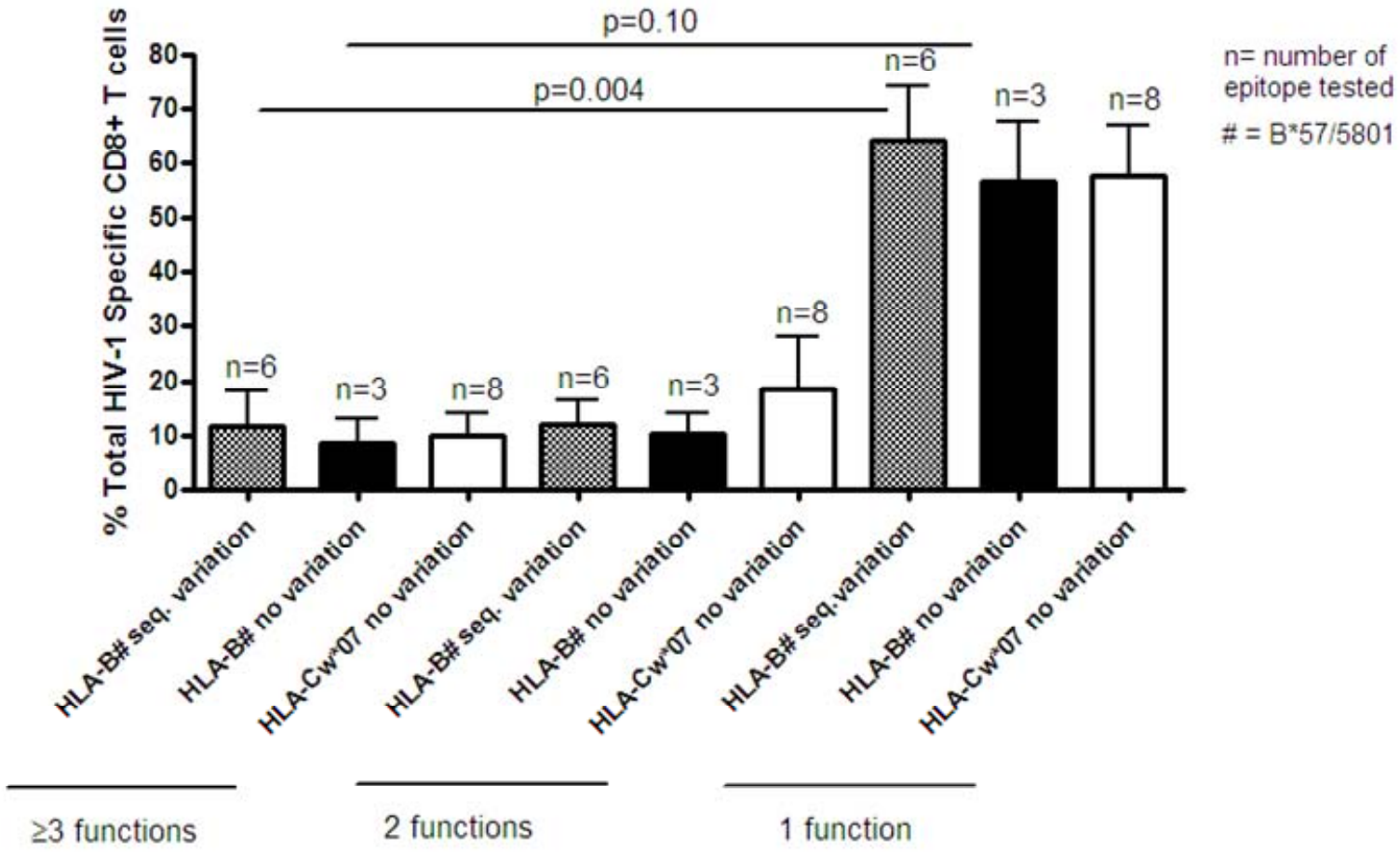

Figure 11. Evaluation of the relationship between epitope sequence variation and polyfunctionality of HIV-specific CD8+ T cell responses.

HLA- B*57/5801 and HLA-Cw*07 restricted epitope responses with and without sequence variation were plotted against the percentage of total HIV-1 specific CD8+ T cells with 3 or more functions, bi-functional and monofunctional T cell responses generated by the study individuals (n=9)

Chapter 4. DISCUSSION AND CONCLUSION

There is clear evidence that CD8+ T cells act on the virus infected cells by executing a variety of diverse functions which result in the control of viral replication (Lichterfeld et al., 2004a, Betts et al., 2006, Zimmerli et al., 2005). However, the mechanism of how the CD8+ T cell can mediate this killing effect is still unclear. It is likely that phenotypic differences may impact in the ability of CD8+ T cells to mediate cytolysis and secrete suppressive factors (Migueles and Connors, 2002, Migueles et al., 2002). There was no association between the breadth or the magnitude of HIV-1 specific CD8+ T cells when quantifying responses by ELISPOT assay and intracellular cytokine staining (Addo et al., 2003, Kiepiela et al., 2004). The ability of CTL to kill virally infected cells is dependent on the HLA restriction, epitope specificity and functional affinity and avidity (Yang et al., 2003, Kiepiela et al., 2004).

Most studies have indicated that the control of HIV replication is driven by the restricting HLA. HLA-B restricted epitopes are mostly associated with the control of HIV replication, especially HLA-B*57 and HLA-B*27 (Kiepiela et al., 2007, Altfeld et al., 2003a). There are few studies that have investigated the role of HLA-Cw* restricted epitopes in the control of HIV replication, however, there are generally few epitopes restricted by HLA-Cw* (Makadzange et al., 2010, Adnan et al., 2006). HLA-Cw* restricted epitopes were previously underestimated due to poor serological techniques and close linkage disequilibrium between certain HLA-B and HLA-Cw alleles, and some

HLA-Cw* restricted epitopes were assigned to the linked B molecules (Goulder et al., 1997a). Most HLA-A and B restricted epitopes have been characterized and studied, whereas very few HLA-Cw* restricted epitopes are known at present. In this study, HLA-Cw* restricted epitopes were characterized and quantified using ELISPOT and ICS assay. Cw*07-KY11 had the highest magnitude of responses in the present study and the Cw*07 allele has strong linkage disequilibrium with HLA- B. As reported before the magnitude of responses does not correlate with the viral control (Addo et al., 2003). In this study, two new HLA-Cw* restricted epitopes Cw*0401-TF10 and Cw*0801-RM9 were optimally defined using the fine-mapping method. These new epitopes may contribute to the study of HLA-Cw* restricted CD8+ T cell responses in association with (or lack of) viral control.

ELISPOT and intracellular cytokine staining (ICS) assays are widely used in vaccine development, measuring the immune responses induced by infection or candidate vaccine (Betts et al., 2005). In this study, these assays were used to measure the cytokine production by CD8+ T cells when stimulated with either HLA-Cw*-or HLA-B- restricted epitopes. The ELISPOT assay is used to determine the secretion of cytokines especially the IFN-γ and IL-2. In the chronic disease stage of HIV, CD8+ T cells secrete more IFN-γ as compared to other cytokines while in controlling subjects, CD8+ T cells produce other cytokines particularly IL-2. Due to the fact that the study subjects were chronically infected, IFN-γ production was assessed, while other cytokines were assessed in a subset of patients in the polyfunctionality assays. Recently, a whole genome wide association study found that a polymorphism in HLA-C may have an important role in viral control

(Fellay et al., 2007). HIV-1 Nef downregulates HLA-A and B and not HLA-Cw, and this is thought to reduce the recognition of HIV-infected cells by CTL. However, total HLA downregulation on the cell surface may result in elimination of virus infected cells by natural killer (NK) cells. The selective down regulation (of HLA-A and HLA-B but not HLA-C) results in the recognition of HLA-Cw* by inhibitory NK receptors thus sparing the infected cells from NK cell mediated elimination (Collins et al., 1998, Adnan et al., 2006). However, HLA-Cw* surface expression is generally low but it is also known that HLA-Cw* restricted responses are generated during chronic infection.

High levels of ELISPOT responses in chronic HIV infected patients reflect viral persistence and viral infection. The production of interferon gamma (IFN-γ) by CD8+ T cell does not define the mechanism of how the CD8+ T cell control the viral replication but more parameters may be a better correlate (Addo et al., 2003, Chung et al., 2007). The overall frequency of CD8+ T cell is not the sole determinant of immune-mediated protection in HIV infection (Betts et al., 2001, Addo et al., 2007). Quantification of the magnitude and the breadth of the CD8+ T cell responses cannot explain the impact of T cell in viral replication (Addo et al., 2003).

HLA-Cw*07-KY11 has high functional avidity compared to other defined HLA-Cw* restricted epitopes. This suggests that Cw*07-KY11 (Nef) may bind strongly to the T cell receptor which induces strong immune responses. HIV-1 Nef selectively downregulates HLA-A and HLA-B but not C (Adnan et al., 2006, Cohen et al., 1999, Collins et al.,

1998). Functional avidity simply refers to the half the maximal concentration required to induce the maximum responses and it is a measure of how strongly a peptide binds to the T cell receptor (Yang et al., 2003). CD8+ T cells are important in protective immunity in HIV-1 infection, however the factors that determine the effectiveness of antiviral activity of these cells still unknown. Antigen functional avidity and epitope specificity are proposed to influence the antiviral activity (Yang et al., 2003).

The relationship between the high functional avidity of Cw*07-KY11and viral loads was not investigated in this study, this will require additional studies. Cw*0304-YL9 (p24) and Cw*18-FFF9 (p24) have low functional avidity even though they are restricted in the Gag region which is associated with control. Other studies show that using cytokine secretion and tetramer decay assays, in chronic infection, CTL functional avidity is lower than in early infection in the presence of high viral replication (Lichterfeld et al., 2006, Lichterfeld et al., 2007). In contrast, high functional avidity and high magnitude of responses does not mean high functional antiviral activity of that specific CD8+ T cell.

In addition, two HLA-Cw* restricted epitopes were optimally defined using IFN-γ ELISPOT titrating the truncated peptide. These were HLA-Cw*0401-TF10 and Cw*0801-RM9. Cw*0401-TF10 is located in the RT region of the HIV proteome while Cw*0801-RM9 is located in gp120. These new epitopes added new information in studies of HIV-HLA interaction- specifically furthering the study of HLA-Cw* restricted epitopes and correlation with viral control. Other studies has shown that HLA-Cw* restricted CTL are functionally and phenotypically the same with HLA-A and HLA-B

restricted CTL (Makadzange et al., 2010). HIV-1 infected cells presenting HLA-C viral epitope are susceptible to lysis by HLA-Cw* restricted CTL. It is believed that a functional assay may provide information that will determine the relationship between the high functional avidity and the functionality of responses.

A recent study has reported that antigen sensitivity determined the polyfunctionality of CD8+ T cells and HIV suppressive activity and a Gag-restricted cell line has high functional avidity compared to Env-specific cell line, but the high avidity did not correlate with the killing activity (Almeida et al., 2009). Therefore the high functional avidity does not imply that the epitope has high functional activity in killing the viral infected cells. Further functional assays like HIV suppression/inhibition assays may require to be performed to determine the functionality of this high functional avidity.

Additionally, the mechanisms underlying better control of HIV-1 by certain HLA alleles are still not well understood. Understanding these mechanisms could facilitate the rational design of an effective HIV-1 vaccine. Therefore, the functional characteristics of CD8+ T cells responses restricted by either HLA-B*57/5801 or HLA-Cw*07 alleles within the same individuals in order to better understand the differences in disease outcome mediated by these two alleles was investigated. IFN-γ producing CD8+ T cell responses restricted by HLA-Cw*07 were significantly higher than responses mediated by HLA-B*57/5801, both on ELISPOT and intracellular cytokine staining (Fig. 8B and Fig. 8A).The targeted responses in the nine study subjects were representative of other HLA-B*57/5801-possesing persons in the larger cohort. However, the nine study

participants were in a relatively advanced stage of infection, as evidenced by the low median CD4+ T cell count of 288 cells/µl, and it is possible that this disease progression status may have influenced the magnitude of HLA-B*57/5801-restricted responses relative to HLA-C responses. On the other hand, the more obvious explanation of the high magnitude of HLA-C-restricted responses is the higher conservation of these epitopes compared to the escaped HLA-B*57/5801-restricted epitopes (Table 2).

We hypothesized here that protective HLA-B restricted CD8+ T cells have a more polyfunctional profile compared to the non-protective HLA-C-restricted responses as has been described in some studies (De Rosa et al., 2004, Betts et al., 2006). In contrast to these earlier studies, we found no significant differences in polyfunctional CD8+ T cell responses mediated by the two alleles within patients with both responses (Fig. 8B). Whereas in the other studies differences were analyzed between patients, here we studied responses occurring concurrently within a patient thus eliminating confounding by disease status, environmental and genetic factors. We observed a positive correlation between polyfunctionality and CD4+ T cell count, but no correlation with viral load suggesting that disease progression but not viral antigen load *per se* is associated with loss of CD8+ T cell polyfunctionality (Fig. 9A). We also observed an inverse correlation between the proportion of monofunctional CD8+ T cells and CD4+ T cell counts, which suggests that immune dysfunction as seen in late chronic HIV-1 infection is characterized by increasing proportion of exhausted monofunctional cells.

It was recently observed in a longitudinal study of recent HIV-1 infection that CD8+ T cells directed against conserved epitopes lost their polyfunctionality whereas escaping-epitope targeting CTLs appeared to maintain their original polyfunctional profile (Streeck et al., 2008). We investigated here whether there were sequence variation differences in HLA-B*57/5801 versus HLA-Cw*07-restricted epitopes and whether such differences resulted in divergent CD8+ T-cell polyfunctionality profiles HLA-B*57/5801-restricted epitopes were more variable, perhaps suggesting increased immune selection pressure on these epitopes, however, there were no differences observed in polyfunctionality between these and immunodominant HLA-Cw*07 restricted CD8+ T cells.

These results imply that sequence variation in targeted epitopes may have no impact on the level of polyfunctionality of CD8+ T cells during chronic HIV-1 infection, although as shown previously, sequence variation within a targeted epitope can impact on polyfunctionality over time (Streeck et al., 2008).

The strength of this study is that CD8+ T cells were analyzed concurrently within study subjects with both responses of interest, thus eliminating across subject confounders. However, limitations include a small sample size and relatively advanced phase of infection of study subjects. It should be noted that the reason we analyzed such a small number of study participants is that most of the individuals who concurrently expressed HLA-B*57/5801 and Cw*07 in our cohort did not have high magnitude of response to epitopes restricted by these alleles, thus making it technically impossible to analyze these

responses in multicolor flow cytometry for those subjects with low magnitude responses. We have therefore focused on immunodominant responses (≥ 500 spot forming cells/10^6 PBMCs) as explained under *Materials and Methods*. Further studies are needed to compare polyfunctionality between dominant and subdominant epitopes restricted by these two alleles although the technical hurdles outlined above will need to be addressed before these studies can be done. CD8+ T-cell antiviral function was not measured directly and are thus unable to conclude whether polyfunctionality had any association with antiviral functional capacity.

In summary, in late chronic HIV-1 infection, immunodominant HLA-B*57/5801 restricted IFN-γ CD8+ T cell responses were of lower magnitude compared to immunodominant HLA-Cw*07-restricted responses in patients with both responses possibly due to differences in sequence variation in targeted epitopes. No significant evidence of polyfunctionality differences between HLA-B*57/5801 versus HLA-C-restricted CD8+ T cells. Polyfunctionality of CD8+ T cells correlated positively with CD4 T cell counts, suggesting that polyfunctionality is lost as disease progresses. There was no impact of sequence variation within targeted epitopes on the polyfunctionality of restricted CD8+ T cells. Larger longitudinal studies are needed to better elucidate the mechanisms that underlie protective versus non-protective HLA-mediated CD8+ T cell responses.

Chapter 5. APPENDIX

5.1 IMMUNODOMINANT HIV-1-SPECIFIC HLA-B- AND HLA-C-RESTRICTED CD8+ T-CELLS DO NOT DIFFER IN POLYFUNCTIONALITY

This manuscript was published in *Virology*

Title of the Article:

Immunodominant HIV-1-specific HLA-B- and HLA-C-restricted CD8+ T cells do not differ in polyfunctionality.

Authors (sequence as in the articles)

Nompumelelo Mkhwanazi, Christina F. Thobakgale , Mary van der Stok , Shabashini Reddy , Zenele Mncube , Fundisiwe Chonco, Bruce D. Walker, Marcus Altfeld , Philip J.R. Goulder , Thumbi Ndung'u

Journal

Virology, 2010 doi: 10.1016/j.virol.2010.06.002

Please state your contribution to the article under the following heading.

1. Formulation of the thesis

   I was actively involved in developing the hypothesis of this study

2. Study design

   The Sinikithemba cohort was developed by Prof Philip Goulder, Bruce Walker, and Prof Thumbi Ndung'u. However, I was actively involved in designing this specific study, I put together a proposal including the literature review, specific aims and data analysis.

3. Work involved in the study.

   I performed all the immunology and virology experiments involved in this study including ELISPOT, flow cytometry work (polyfunctionality assays) and viral sequencing.

4. Data analysis

   I was involved fully in analyzing the data generated from ELISPOT and polyfunctionality data and sequencing.

5. Write up

   I was the first (primary) author of this article.

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

Signature: Date 06 August 2010

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