UNIVERSITY OF KWAZULU-NATAL

Analysis of Viral Inhibitory Activity of Cytotoxic T Lymphocytes Targeting Identical Epitopes Restricted by Different Class I HLA alleles from the same HLA Supertype

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Research Output

The journey undertaken in completing this degree has allowed me to attend some prestigious conferences:

1. The Canada-Sub-Saharan Africa HIV/AIDS Networks of Investigators, 19-20th June, 2014. Durban, South Africa.

2. Southern African Immunology Workshop and 6th Infectious Diseases in Africa Symposium (IUIS-FAIS), 20-23rd October, 2015. Cape Town, South Africa.

Oral and Poster Presentations

Ogunshola F, Mewala N, Wright J, Shen H, Ismail N, Walker B, Ndung'u T, Ndhlovu Z. 2015. Comparative Analysis of the Inhibitory Activity of Cytotoxic T Lymphocytes Targeting Identical TL9 Epitopes Presented by HLA-B*81:01 and B*42:01 Alleles (IUIS-FAIS, Cape Town, South Africa).

Award

I received an award as the 2nd best poster presenter in the IUIS-FAIS symposium and I was given a travel award to attend the 6th South Africa Immunology Society Meeting, 2016.

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Summary

Human leukocyte antigen (HLA) polymorphism and the genetic diversity of human immunodeficiency virus (HIV) are the major obstacles for designing an effective HIV Cytotoxic T Lymphocytes (CTLs) based vaccine. Interestingly, recent studies have demonstrated that multiple class I alleles can recognize common epitopes "supertopes" due to the homology of amino acids within the major binding pockets of the peptide binding cleft. The implications of this for vaccine design is that a vaccine containing a small number of highly promiscuous supertopes can confer protection against a wide range of HIV variants. This notion makes supertopes immunogen design an attractive option. However, it is not clear whether supertopes presented in the context of different class I HLA alleles would induce functional equivalent CTL responses.

In this study, we investigated the inhibitory activity of CTLs targeting identical epitopes presented by class I HLA alleles from the same superfamily. The viral inhibitory activity was measured using a newly developed CEM-GFP reporter T-cell line (GXR-cell) as target cell. We first compared the inhibitory activity of CTLs from 8 subjects targeting TPQDLNTML (Gag p24 residue 180-188-TL9) epitope presented by HLA-B*81:01 or B*42:01 alleles. We then assessed the inhibitory activity of the 8 subjects' CTLs when presented with *in-vivo* occurring mutant (Q182S)-TL9 epitope by HLA-B*81:01 or B*42:01 alleles. Furthermore, we compared the inhibitory activity of CTLs from 4 subjects targeting ISPRTLNAW (Gag p24 residue 147-155-IW9) epitope presented by HLA-B*57:03 or B*58:01 alleles.

Comparative analysis of the inhibitory activity of the 8 subjects' CTLs showed no statistical significant difference when TL9 epitope was presented by HLA-B*81:01 or B*42:01 alleles (1:1; p-value = 0.8785, paired t test), even at low target to effector ratio (1:8; p-value = 0.4418). No statistical significant difference was observed in the inhibitory activity of the 8 subjects' CTLs when mutant (Q182S)-TL9 epitope was presented by HLA-B*81:01 or B*42:01 alleles (1:1; p-value = 0.8042), same result was observed at low target to effector ratio (1:8; p-value = 0.9396).

Comparative analysis of the inhibitory activity of the 4 subjects' CTLs targeting identical IW9 epitopes presented by HLA-B*57:03 or B*58:01 alleles showed a trend towards significance at target to effector ratio 1:1 (1:1; p-value = 0.0924), but at low target to effector ratio, no significance difference was observed (1:8; p-value = 0.1496).

In conclusion, we have demonstrated that there is no observable significant difference in the inhibitory activity of CTLs targeting wildtype TL9 or mutant (Q182S)-TL9 epitopes

presented in the context of HLA-B*81:01 or B*42:01 alleles. Thus, TL9 epitope could be immunogenic for individuals expressing HLA-B*81:01 or B*42:01 alleles. We have also shown that the inhibitory activity of CTLs targeting identical IW9 epitopes presented by HLA-B*57:03 or B*58:01 alleles is comparable. Indicating that IW9 epitope could be included in immunogen design for individuals expressing HLA-B*57:03 or B*58:01 alleles. These findings are relevant for HIV vaccine approach that seeks to identify immunogenic supertopes that can be cross-presented in a broadly cross-reactive T cell based vaccine design.

Abbreviations

AIDS	Acquired immune deficiency syndrome
APC	Antigen Presenting Cell
CA	Capsid
CCR5	Chemokine Receptor 5
CD4+ T cells	Human T cells expressing CD4+ antigen
CD8+ T cells	Human T cells expressing CD8+ antigen
cDNA	Complementary deoxyribonucleic acid
CDR	Complementary determining region
CTL	Cytotoxic T lymphocyte
CXCR4	CXC chemokine receptor 4
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunosorbent spot assay
Env	Envelope
FACS	Fluorescent activated cell sorting
FCS	Fetal Calf Serum
Gag	Group-specific antigen
GALT	Gut associated tissue
GIT	Gastrointestinal tract
GXR	CEM-GFP GXR25
HAART	Highly active antiretroviral treatment
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
ICS	Intracellular cytokine staining
INF_Y	Interferon gamma
MA	Matrix
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
Nef	Negative regulatory factor
NC	Nucleocapsid (p7)
P24	p24 Capsid protein

PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
Pol	Polymerase
Rev	Regulator of virion expression protein
RNA	Ribonucleic acid
SIV	Simian immunodeficiency virus
SSO	Sequence specific oligonucleotide
Tat	Transactivator of transcription
TCR	T cell receptor
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U

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CHAPTER ONE: Literature Review

1.1 Introduction

The isolation of human immunodeficiency virus (HIV) in 1983 has led to more than three decades of intense research on its structure and pathogenesis. Most of the work has been motivated by the need to develop new approaches to test, treat and prevent HIV infections (Barre-Sinoussi et al., 2013). Highly active antiretroviral treatment (HAART) has proven to be effective in controlling disease progression and transmission by reducing the viral load (Tanser et al., 2013, Bor et al., 2013). However, HAART is faced with several limitations such as; the development and transmission of drug resistant viruses, occurrence of drug toxicity, cost implication of obtaining the drugs and non-adherence of patients to therapy (Pontali, 2005, Tozzi, 2010, Falutz, 2011).

The development of a safe and effective HIV vaccine remains the best hope to control HIV epidemic (Verma et al., 2015). Like other vaccines, a preventive HIV vaccine could help save millions of lives. Induction of broadly neutralizing antibodies and HIV-specific Cytotoxic T lymphocytes (CTLs) are the two immune effectors that HIV vaccine research has focused on. Although broadly neutralising antibodies are the best correlate of protection for many viral vaccines (Plotkin, 2010, Amanna and Slifka, 2011), eliciting neutralizing antibodies against HIV has been difficult due to rapid mutation rates of HIV (Amanna and Slifka, 2011). Hence, inducing functional CTLs response provides a very viable option.

Data have shown the importance of the CTLs response in controlling HIV infection (Jin et al., 1999, Schmitz et al., 1999). CTLs can recognize and kill HIV-infected cells by specific recognition of viral epitopes presented by class I HLA molecules (Yang et al., 1997). However, huge genetic diversity of the class I HLA molecules and the considerable genetic variation of HIV are the major obstacles for designing effective HIV CTL-based vaccine.

In order to develop HIV CTL-based vaccine, it would be important to define effective CTLs responses directed against HIV epitopes presented on the surface of infected cells (Yang et al., 1997, Sacha et al., 2007, Bennett et al., 2008). The benchmark towards this goal is to identify beneficial CTLs responses and to exclude ineffective or even potentially harmful ones.

1.2 Global Overview of HIV/AIDS Epidemic

Human immunodeficiency virus (HIV) is the aetiological agent of acquired immune deficiency syndrome (AIDS) and is one of the most devastating pathogens worldwide. Following its isolation in 1983, HIV has evolved to become a threat to global health (De Cock et al., 2012). Since the discovery of HIV, approximately 25 million AIDS related deaths have been recorded. In 2013, an estimate of 35 million people were living with HIV globally. In the same year, approximately 2.3 million new infections occurred, a number that is still high despite it being 33% decline from the 3.4 million infections that occurred in 2001 (UNAIDS, 2013).

More than two-thirds of HIV infections, roughly 24.7 million persons, were in Sub-Saharan Africa which also accounted for close to 80% of women and 90% of children living with HIV (De Cock et al., 2012). South and south-east Asia accounted for approximately 4 million HIV infected persons, and the Americas, including the Caribbean, accounted for 3 million, (Figure 1). Despite the decline in the new infections, South Africa, in the Sub-Saharan Africa region, still bears the highest burden of HIV epidemic globally (UNAIDS, 2013).

Figure 1: Global View of the Estimated Number of People Living with HIV in 2013. The HIV pandemic affects all regions of the world, but Sub-Saharan Africa is the most affected region with approximately 24.7 million HIV infected individuals (UNAIDS, 2013).

1.2.1 HIV/AIDS Epidemic in South Africa

South Africa is the most affected country in the Sub-Saharan region by HIV pandemic (Shisana and Onoya, 2014). In 2012, the overall prevalence of HIV in South Africa was estimated at

17.9 % in 15 to 49 year old people, and an estimated 6.1 million people were living with HIV/AIDS (UNAIDS, 2013). Provincial data on HIV prevalence is shown in Figure 2. The results show substantial variation of HIV prevalence by province. KwaZulu-Natal continue to lead South Africa in HIV prevalence (16.9%), followed by Mpumalanga (14.1%), Free State (14.0%) and North West (13.3%). The Western Cape had the lowest HIV prevalence (5%) followed by the Northern Cape (7.4%) and Limpopo (9.2%). In most provinces, HIV prevalence in women; aged 15 years and above, remains significantly higher than in men (Shisana and Onoya, 2014).

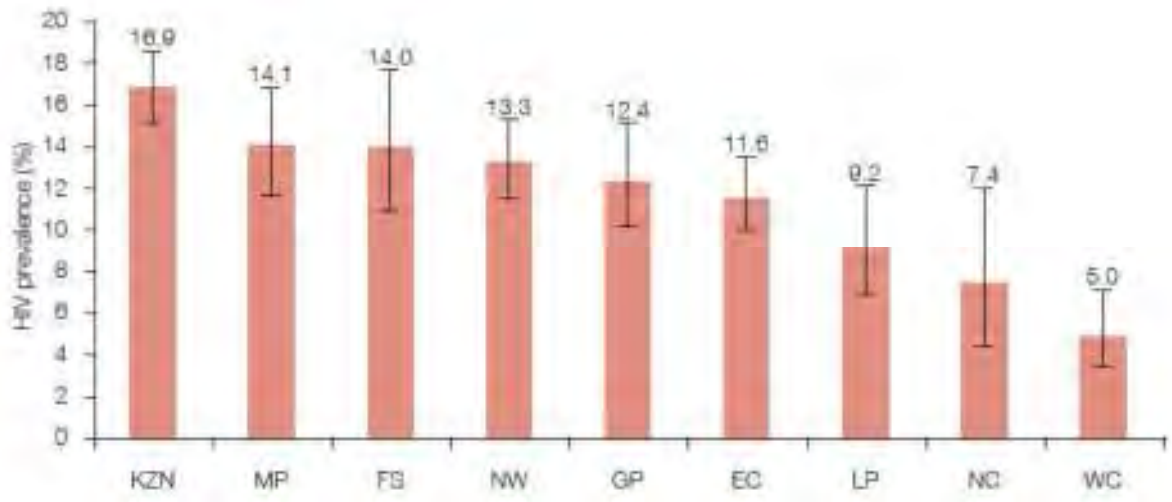

Figure 2: Overall HIV Prevalence by Province in South Africa. KwaZulu-Natal is the most affected province while the Western Cape is the least affected province. Key: **KZN**-KwaZulu-Natal, **MP**-Mpumalanga, **FS**-Free State, **NW**-North West, **GP**-Gauteng, **EC**-Eastern Cape, **LP**-Limpopo, **NC**-Northern Cape, **WC**-Western Cape (Shisana and Onoya, 2014).

1.3 Molecular Biology of HIV

1.3.1 Structure of HIV Virion

HIV is a retrovirus belonging to the *Retroviridae* family and *Lentivirus* genus with a genome of approximately 10 kilobase (kb) in size (Ratner et al., 1985). Like other viruses, HIV can only survive and replicate upon infection of a host cell expressing its corresponding receptor (Smith and Daniel, 2006). Outside the host cell, HIV exists as a spherical particle called 'virion' with a diameter of about one 10,000th of a millimetre (Figure 3).

HIV possess a lipid bilayer membrane that surrounds the viral Envelope glycoprotein and some cellular proteins (Sierra et al., 2005). The Envelope glycoprotein is heterodimer of the gp120 which is attached to the gp41 (transmembrane glycoprotein).

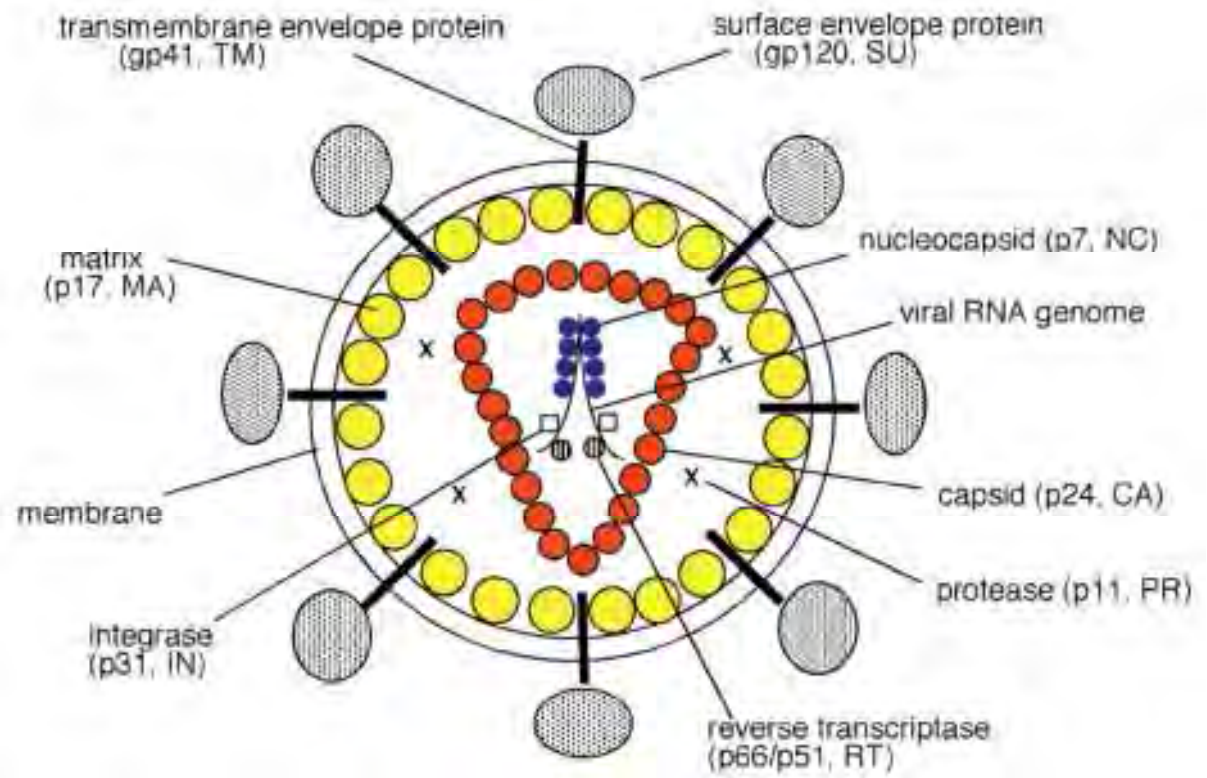

Figure 3: Schematic Representation of HIV Virion. Inside the virion is the conical capsid. The capsid contains the enzymes reverse transcriptase, integrase and protease, and the viral genome. Two copies of single-(+) stranded RNA molecules, which are complexed by the nucleocapsid protein p7. The capsid is surrounded by lipid membrane. The viral glycoprotein gp41 and host cell surface proteins are integrated into the viral membrane, (Freed, 1998).

HIV encodes 9 proteins; 3 major structural proteins (Gag, Pol and Env) are required for the formation of new virus particles, accessory proteins (Vif, Vpr, Vpu, and Nef), and regulatory proteins (Tat and Rev) are essential for regulatory function and virion assembly (Frankel and Young, 1998).

The Gag gene encodes a polyprotein that is cleaved by viral protease to mature Gag proteins (p17, p24, p7 and p6). The matrix (p17), which is surrounded by the capsid (p24), ensures the integrity of the virion particle. The matrix is important for the incorporation of viral surface glycoproteins into virion and early post entry events (Dorfman et al., 1994, Freed,

1998). The capsid forms the cone shaped shell of the mature virion that encloses the viral genomic RNA (Gelderblom et al., 1987), and plays a crucial role in virus assembly and maturation (Freed, 1998). The nucleocapsid, found in the core tightly associated with the viral RNA, is required for the encapsidation of the viral RNA, membrane binding, preintegration and transcription (Accola et al., 1998, Freed, 2001). The single stranded RNA and the enzymes; reverse transcriptase (RT), protease (PR), ribonuclease and integrase (IN), which are important for the virion development are tightly bound to the protein p7. The protein p6, located at the C-terminal of Gag is essential for the release of assembled virion from the cell surface (Paxton et al., 1993, Kondo and Gottlinger, 1996).

The pol gene encodes a polyprotein, which is then cleaved by viral protease to produce viral enzymes reverse transcriptase, protease and integrase, (Figure 4). Reverse transcriptase catalyses the conversion of single stranded RNA into double stranded DNA, while integrase catalyses the insertion of linear double stranded viral DNA into the host cell chromosome (Hill et al., 2005). Protease cleaves the Gag and the Gag-Pol polyproteins and allows conformational changes within the particle to produce mature infectious viruses (Frankel and Young, 1998).

Synthesis of the Env gp160 takes place in the endoplasmic reticulum and then transported through the secretory pathway to the plasma membrane (Murakami, 2008). The Env protein is heavily glycosylated and cleaved by cellular protease into surface subunit proteins; gp120 and transmembrane gp41 (Chan and Kim, 1998, Freed, 2001). The other 6 genes encode the proteins that antagonise host defence and enhance pathogenicity (Freed, 2001). These genes are known as vif (viral infectivity), vpu (viral protein U), vpr (viral protein R), nef (negative regulator protein), tat (transactivator protein) and rev (regulator of expression of viral protein), (Figure 4).

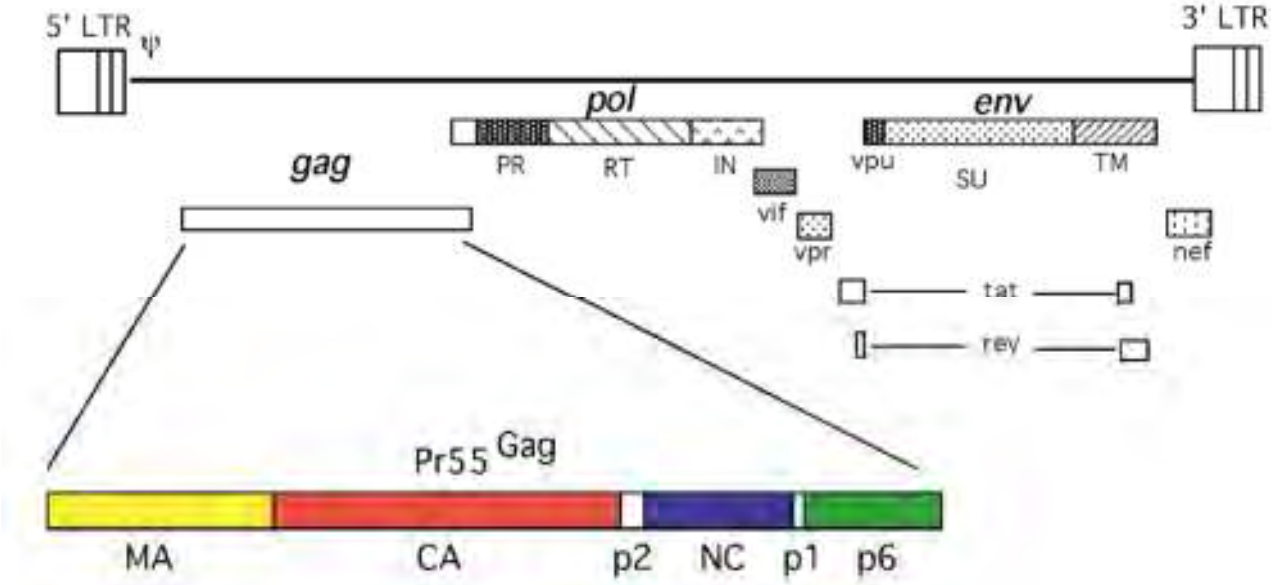

Figure 4: Schematic Representation of HIV Genome. The diagram indicates the regions coding for HIV proteins (Freed, 2001).

1.3.2 HIV Replication

The life cycle of HIV can be divided into early and late phase. The early phase involves: binding and entry, uncoating, reverse transcription and provirus integration. The late phase includes: virus protein synthesis, budding and maturation (Fanales-Belasio et al., 2010).

In the early phase, HIV particles bind specifically to CD4 expressing cells through the interaction between surface subunit Env protein (gp120) and the co-receptors; such as CCR5 and CXCR4 (Sierra et al., 2005, Bartolo et al., 2009). This interaction triggers a conformational change in gp120 which uncovers the transmembrane Env protein (gp41) binding site. After binding to the co-receptor, the virus and cellular membrane fuse thereby allowing for uncoating and release of the viral core into the host cell (Freed, 2001, Gomez and Hope, 2005). The single stranded viral RNA is then reverse transcribed by the enzyme reverse transcriptase to double stranded DNA which is then transported to the nucleus of the host cell (Pomerantz and Horn, 2003). Following transportation to the nucleus, the viral DNA is then integrated with the host cell genome by the enzyme integrase.

the late phase, viral mRNA transcripts are synthesized and transported out of the nucleus for translation into proteins such as; gag-pol, env and nef (Sierra et al., 2005). These early proteins are assembled near the cell membrane and once the virion bud-off the cell, they

are cleaved by the enzyme protease to produce independent enzymes as well as structural proteins of a mature viral particle (de Oliveira et al., 2003). (Figure 5).

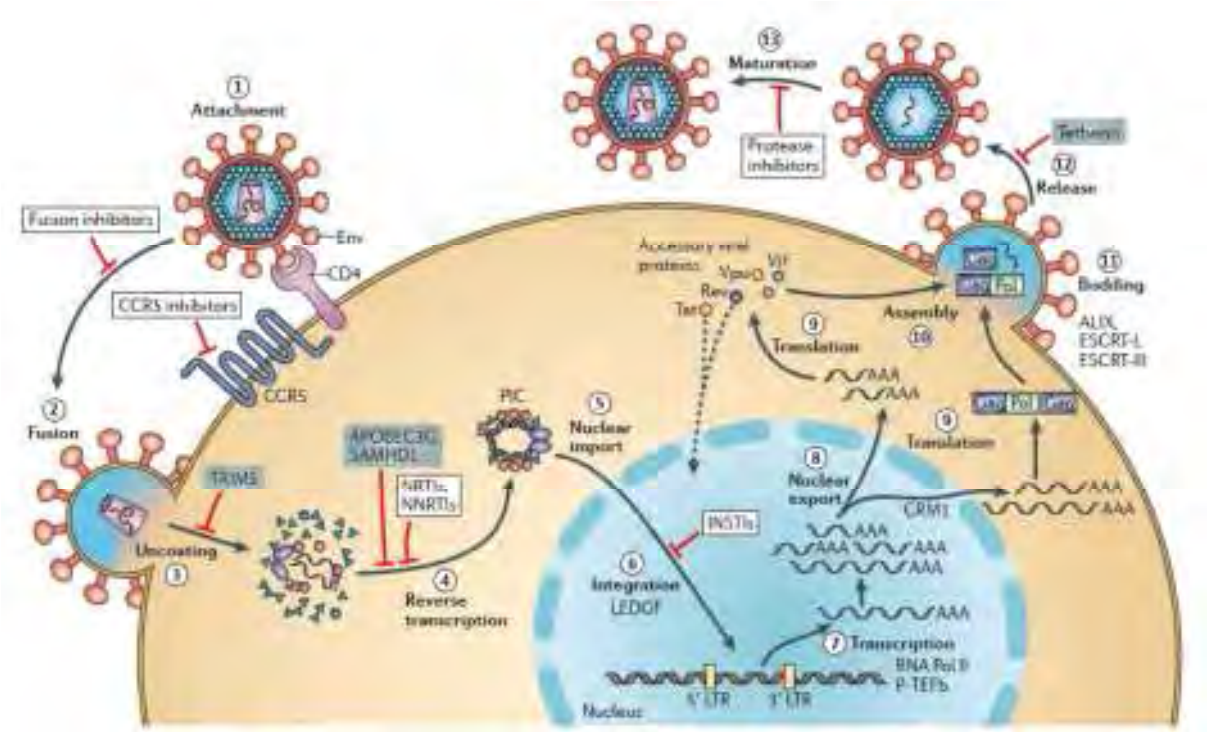

Figure 5: Schematic Overview of HIV Replication Cycle. The replication cycle can be divided into early and late phase; the early phase is described with points 1-6, while the late phase is described with points 7-13, (Engelman and Cherepanov, 2012).

1.4 HIV Pathogenesis

Exposure to HIV is primarily through the mucosal route; either gastrointestinal or reproductive tracts, which is thought to result in initial local replication of the virus within target cells of the mucosal tissue (Douek et al., 2003), followed by systematic spread with considerable dissemination in the gut-associated lymphoid tissue (GALT) (Yuki et al., 2007). The establishment of HIV infection is dependent on the target cells expression of CD4 and a chemokine receptor (CCR5 or CXCR4), (Doms, 2001). The course of HIV infection can be categorized into 3 phases as illustrated in (Figure 6); (1) the acute phase, (2) the asymptomatic phase and (3) the AIDS phase.

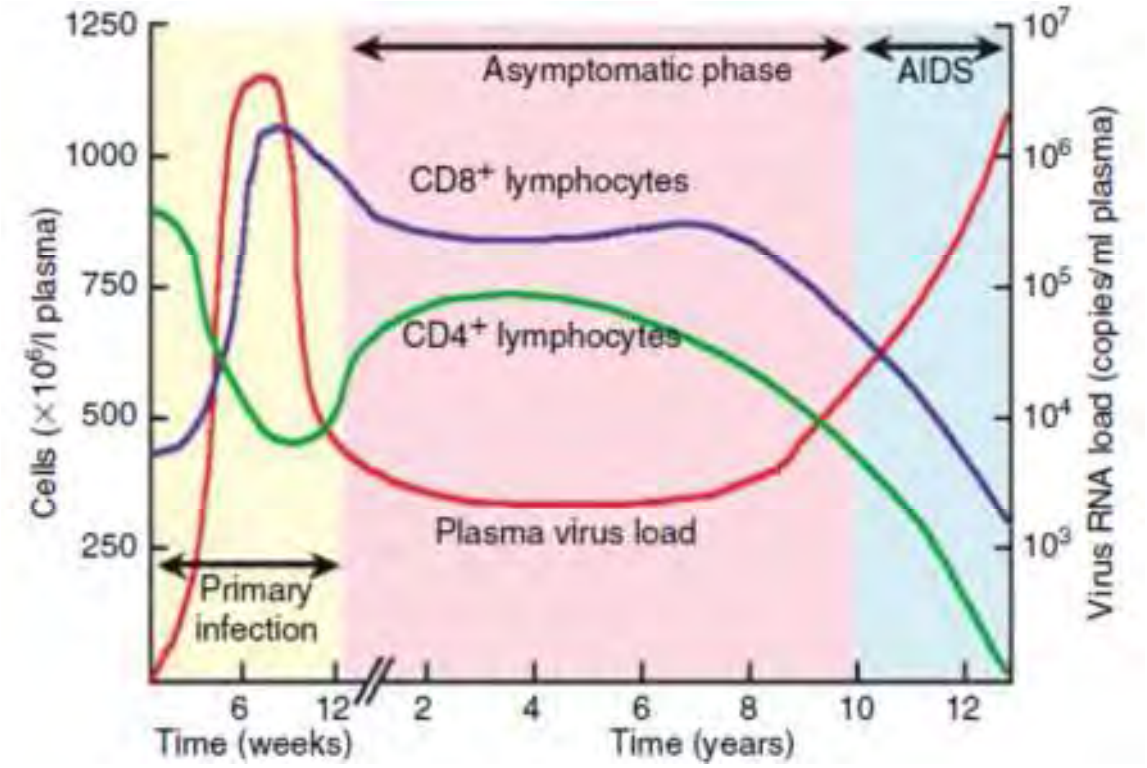

Figure 6: HIV Pathogenesis. Representation of a typical course of HIV infection showing CD8+ T and CD4+ T-cells dynamics and viral load changes over-time of infection (Munier and Kelleher, 2007).

1.4.1 The Acute Phase

Following transmission of HIV, approximately 10 days is required for viral RNA to be detectable in the plasma (McMichael et al., 2010). During this acute phase, diagnosis of acute HIV infection is often missed because the symptoms are common to many other infections (Altfeld and Walker, 2001). HIV replicates rapidly during the acute phase of infection with viral titres reaching a peak, usually more than a million RNA copies per ml of blood. CD4+ T-cells numbers are significantly lowered at the time of peak viremia (McMichael et al., 2010). Subsequently, the emergence of CD8+ T-cell responses coincides with a decrease in viremia (Mogensen et al., 2010), suggesting that the suppression of viral replication is largely mediated by CD8+ T-cell (McMichael et al., 2010).

A few studies have measured HIV-specific CD8+ T-cell responses during early HIV infection (Borrow et al., 1994, Koup et al., 1994, Pantaleo et al., 1994, Wilson et al., 2000, Ndhlovu et al., 2015). The CD8+ T-cell responses peaks at about 1-2 weeks later as the viremia declines (McMichael et al., 2010). Following the peak in the CD8+ T-cell responses, the virus

sequence start to change dramatically (McMichael et al., 2010). As viremia decline to the viral set point, rapid selection of mutations occurs at discrete sites in the virus genome (Bernardin et al., 2005, Salazar-Gonzalez et al., 2009). Further studies on the early events during the acute phase of HIV infection may be critical in determining the course of diseases progression.

1.4.2 Asymptomatic Phase

The asymptomatic phase of HIV infection is where the viral load reaches a viral set-point (McMichael et al., 2010). During this phase, there is a gradual decline in the circulating CD4+ T-cells and loss of immune function. The decline in the CD4+ T-cells is not only caused by direct infection but also as a result of chronic immune activation and inflammation (Appay and Sauce, 2008). Furthermore, the immune system becomes progressively exhausted and there is a decline in its ability to regenerate the depleted CD4+ T-cells (Ndhlovu et al., 2015). The steady replication of HIV results in progressive exhaustion of CD8+ T-cells, thus impairing the ability of the immune system to control the virus (Appay and Sauce, 2008, McMichael et al., 2010).

1.4.3 The AIDS Phase

In the absence of antiretroviral treatment, HIV establishes a chronic, progressive infection of human immune system that invariably, over the course of the years, leads to its destruction and severe immunodeficiency (Douek et al., 2003). The AIDS phase is defined by the rapid decline in the CD4 cell count with an increase in viral load. At this phase, the host becomes highly susceptible to opportunistic infections (e.g. tuberculosis, pneumococcal infections, oral candidiasis) and certain cancer (e.g. Kaposi's sarcoma) (Paranjape, 2005). The average time from infection to AIDS is 8-10 years, but this may vary considerably due to host and viral factors (Derdeyn and Silvestri, 2005, Chatterjee, 2010). At this phase of advanced disease, the immune system is overwhelmed and death ensues as a result of the opportunistic infections. The loss of HIV-specific immunity might play a role in the steady acceleration of viral replication that is observed in late disease (Mueller et al., 2001, Kostense et al., 2002).

1.5 CYTOTOXIC T LYMPHOCYTES (CTLs)

Cytotoxic T lymphocytes (CTLs), also referred to as CD8+ T-cells, are the host's major defence mechanism against invading intracellular pathogens (Lehmann-Grube et al., 1985). CTLs are thought to control HIV replication and help to maintain clinical stability in infected individuals (Borrow et al., 1994, Koup et al., 1994). The intimate involvement of CTLs in the control of HIV will be discussed in the following sub-sections.

1.5.1 History of HIV-Specific CTL

HIV-specific CTL was first isolated in 1987 by Walker *et al*., (1987) and Plata *et al*., (1987). The high frequencies of HIV-specific CTLs in HIV infected individuals was detected by measuring the ability of freshly isolated Peripheral blood mononuclear cells (PBMCs) to lyse autologous B cells infected with recombinant vaccinia-HIV vector (Walker et al., 1987) or by peptide pulse targets (Nixon et al., 1988). Subsequent studies using other approaches such as detection of interferon γ ELISPOT (Dalod et al., 1999), intracellular cytokines (Maecker et al., 2001) and peptide major histocompatibility complex (MHC) tetramer assay (Altman et al., 1996), confirmed and quantified CTLs robustness in HIV infected individuals. Rapid expansion and the ability of single CTL to destroy more than one target cell, while sparing "innocent" bystanders, make CTLs very efficient Ag-specific effector cells (Broere et al., 2011).

1.5.2 CTLs Differentiation

The development of CTLs is a multistep process with several built-in check points to ensure appropriate differentiation. Progenitor T-cells migrate to the thymus from the bone marrow; where they are produced. At this phase, the cells only express CD44 (adhesion molecule) and CD25 (α chain of the IL-2 receptor). Subsequently, they differentiate to express CD3 and CD8 molecules in the thymus. The differentiated cells become committed to TCR lineage rearrangement. Cells bearing the T cell receptors that have high affinity for binding self-peptides presented on Major histocompatibility complex (MHC I) are deleted, while those that do not recognise self-peptides are positively selected and exit the thymus to the periphery as naïve CTLs (Mosmann and Coffman, 1989).

1.5.3 Activation of Naïve CTL

Activation of naïve CTL requires specific recognition of cognate peptide presented by antigen presenting cell (APC), and a costimulatory signal, (Figure 7), (Abbas et al., 1996). In the case of HIV, naïve CTL usually encounter HIV antigenic peptide in a secondary lymphoid organ. Upon priming with HIV antigen, naïve CTL undergo activation, proliferation, clonal expansion and differentiation into effector T cells, most of which are short lived (Abbas et al., 1996).

The effect of HIV-specific CTL is seen in the vigorous destruction of infected cells presenting the cognate peptides. Most of these HIV-specific CTLs die by apoptosis after eliciting their effector function. A few of the effector T cells mature into memory T cells, which can respond faster and more effectively upon re-encountering their cognate antigen (Roderick Nairn., 2007).

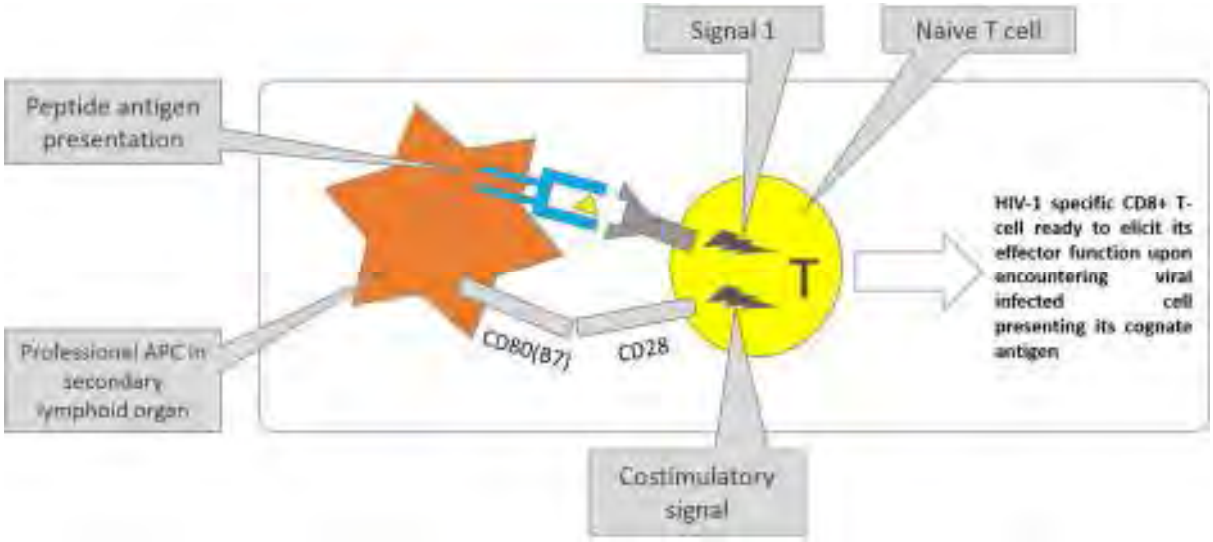

Figure 7: Activation of Naïve CTL. The APC (brown) presents antigenic peptide to the naïve CTL (yellow). Recognition of antigenic peptide coupled with the costimulatory signal activates the naïve CTL and differentiate it to effector T-cell.

1.5.4 Mechanism of Action of HIV-specific CTL

An HIV-specific CTL recognizes its cognate peptide fragment of 8 to 12 amino acids in length presented by the infected cell. This peptide fragment is generated intracellularly through protein degradation. The fragmented peptide is loaded into the class I human leukocyte antigen (HLA) molecule and transported to the cell surface for presentation to HIV-specific CTL (Paranjape, 2005). HIV-specific CTL receptor (TCR) recognizes the complex of class I HLA

molecule and viral peptide (Figure 8), and this recognition triggers the CTL to lyse the infected cell by releasing effector molecules such as perforins and granzymes. These effector molecules are capable of direct killing of the infected cells or inducing apoptosis in them (Gandhi and Walker, 2002). Thus, CTL responses in an individual may depend on the TCR repertoire that recognise the restricted peptide, expressed class I HLA allele and the peptide presented.

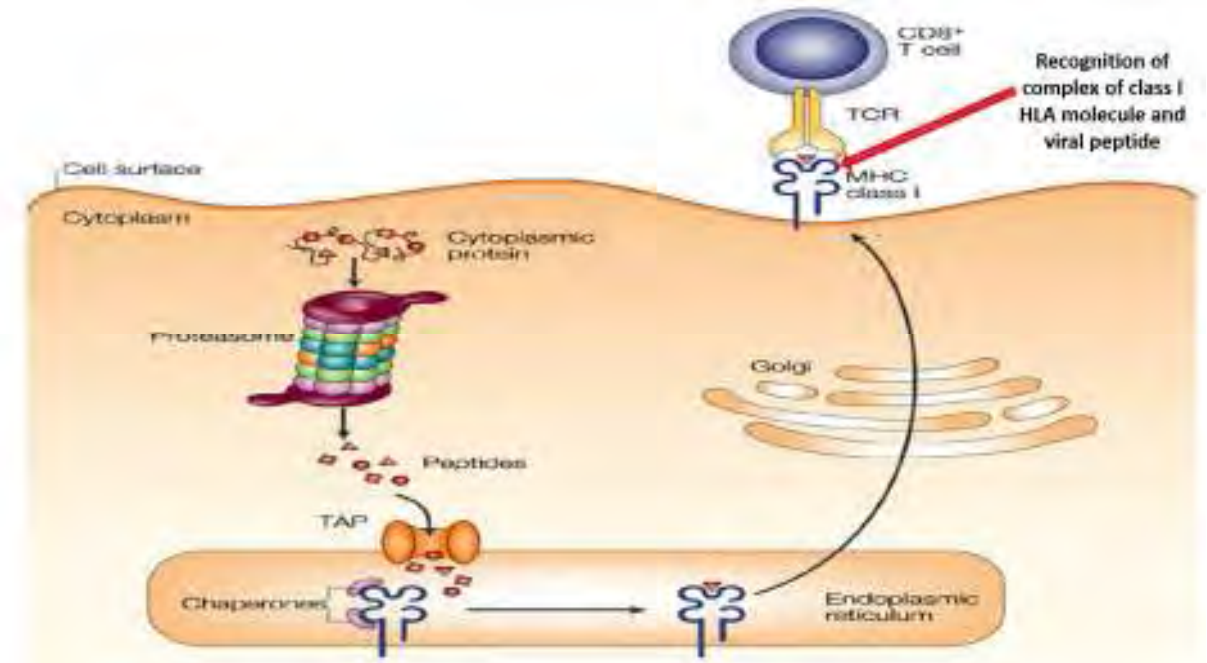

Figure 8: Schematic Representation of HIV-Specific CTL Mechanism of action. The infected cell process the viral peptide and present to CTL via class I HLA molecule. CTL recognizes the presented peptide and releases effector molecules on the infected cell, thereby resulting in the killing of the infected cell (Yewdell et al., 2003, Jixin Zhong, 2011).

1.5.5 CTL Receptors

The CTL receptors (TCRs) are surface heterodimers consisting of disulphide-linked α- and β-chains. Each TCR chain is composed of variable and constant Ig-like domains, followed by a transmembrane domain and a short cytoplasmic tail (Davis and van der Merwe, 1996). CTL recognizes peptides presented by the infected cell through the α/β binding site of the TCR (Turner et al., 2006). The binding of the peptide fragment takes place through the three loop region on each of α and β chains and the complementarity determining regions (CDRs)**.** The CDR is composed of three domains; CDR1, CDR2 and CDR3. The CDR3 is mainly involved in the interactions with the peptide fragment, while CDR2 interacts with the heavy chain of

class I HLA molecule (Figure 9). In most TCRs, CDR1 has limited interaction with the peptide fragment and the class I HLA molecule (Rudolph et al., 2006). Studies have suggested that engineered TCRs might provide a means of generating HIV-specific polyfunctional T-cell responses and can engage epitope variants presented by the infected cells (Price et al., 2004, Varela-Rohena et al., 2008, Chen et al., 2012). Hence, engagement of TCRs could be modulating virus inhibitory capacity and recognition of naturally occurring HIV peptides.

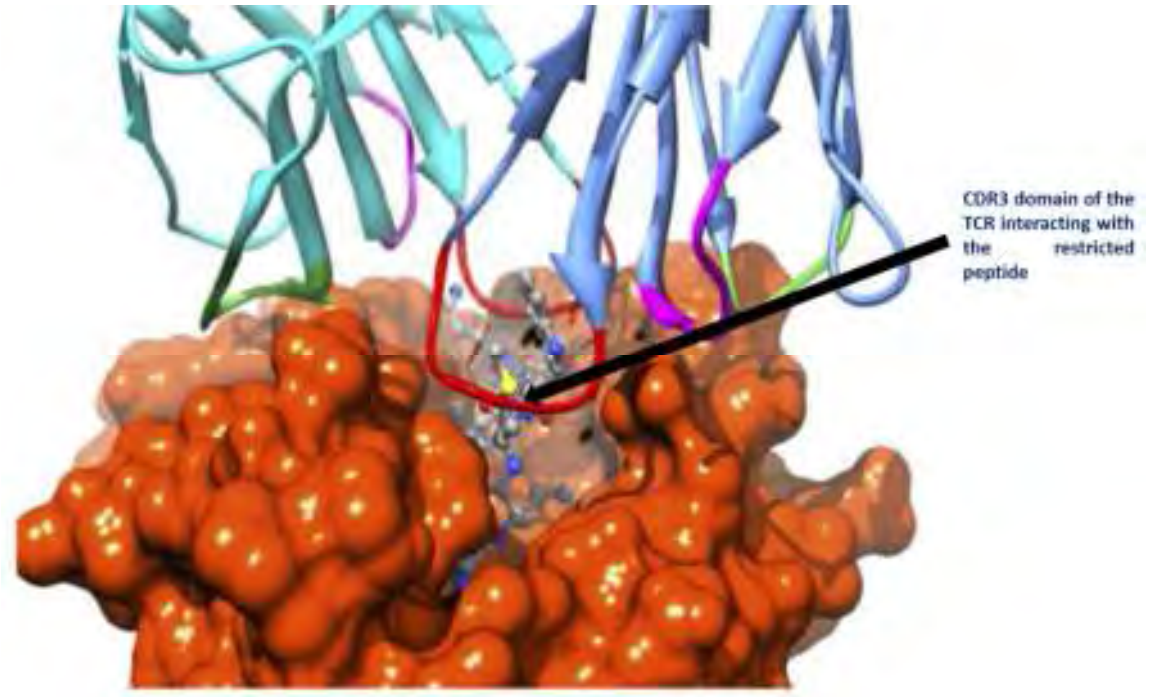

Figure 9: Position of the TCR CDRs Over the Class I HLA Allele/Peptide Surface. CDR1, CDR2 and CDR3 are colored in magenta, green and red respectively (Zoete et al., 2013). CDR3 is mainly involved in the interraction with the peptide preseneted by the class I HLA molecule.

1.5.6 The Role of HIV-specific CTL in HIV Infection

There is a substantial evidence on the significant role that HIV-specific CTLs play in combating viral replication during the acute infection phase (Pantaleo et al., 1994). The massive expansion of HIV-specific CTLs few days after exposure to HIV coincide with the decline in plasma viremia (Koup et al., 1994, Ndhlovu et al., 2015). HIV-specific CTLs are thought to be responsible for the initial control of viral replication with viral control from both blood and lymph nodes (Borrow et al., 1997, Ndhlovu et al., 2015). In addition, *in-vivo* studies on the control of viremia in macaques during primary simian immunodeficiency virus (SIV) infection shows that the depletion of CTLs abrogates their ability to control primary viremia

(Schmitz et al., 1999, Jin et al., 1999, Schmitz et al., 2005). *In-vitro* assay has also shown that the CTLs taken from HIV infected individuals are able to inhibit viral replication in autologous CD4+ T-cells (Yang et al., 1996). However, the factor influencing the effectiveness of the HIV-specific CTL responses in controlling viral replication and in subsequently establishing different viral set points in individuals remains elusive.

The relationship between the earliest CTL responses, viral set point and disease progression remains controversial (Walker and McMichael, 2012). Studies have suggested that the initial CTL responses are low in magnitude and narrowly directed towards viral proteins (Dalod et al., 1999, Altfeld et al., 2001, Cao et al., 2003a, Radebe et al., 2011). The responses may be localized at the inductive sites within the lymphoid tissue (Altfeld et al., 2002), and are regarded as the most effective in controlling viral replication as they have the greatest antiviral activity (Cao et al., 2003a). Although most of these detectable responses persist even in the chronic phase of infection (Koibuchi et al., 2005), most of them are less effective in further reducing the viral load. Thus, there is a need to understand the causes of ineffectiveness of the persistent responses at the chronic phase.

1.5.7 HIV-Specific CTL Responses during the Chronic Phase of Infection

Despite the presence of HIV-specific CTLs in chronic phase, majority of HIV infected patients still lose viral control (Addo et al., 2003, Draenert et al., 2004b, Frahm et al., 2004). Reported data from various studies on the immune correlate of HIV-specific CTL with viral load at chronic phase of infection have not been consistent (Ogg et al., 1998, Betts et al., 1999, Shankar et al., 2000, Betts et al., 2001, Migueles and Connors, 2001). On the other hand, specificity of responses during the chronic phase of infection repeatedly suggests that Gag targeting is associated with lower viral load (Edwards et al., 2002, Zuniga et al., 2006, Kiepiela et al., 2007, Geldmacher et al., 2007, Ndhlovu et al., 2015), while Env-specific response is associated with higher viral load (Ngumbela et al., 2008). These findings suggest that responses targeting Gag epitopes could be effective in reducing the rate of HIV disease progression.

1.5.8 Gag-Specific CTL Responses are Superior

While the overall breadth and magnitude of the CTL responses does not correlate with the rate of disease progression (Addo et al., 2003), studies have established an association between

preferential Gag-specific CTL responses and favourable clinical outcomes among other HIV proteins. Zuniga and colleagues (2006) showed that the contribution of Gag p24-CTL responses is positively associated with low viral load and high CD4+ T-cell counts. This was further confirmed with a large population study infected with clade C virus (Kiepiela et al., 2007), showing a correlation between the number of CTLs responses to the conserved Gag-proteins and viral load. In this same study, Env-proteins show a correlation between CTL responses and higher viral load. Pereyra et al., (2008) reported that HIV controllers (HIV infected patients without antiretroviral therapy for at least one year and plasma HIV-1 RNA is < 50 copies/ml by ultrasensitive PCR), preferentially target Gag over other viral proteins. Other studies also reported the superior *in-vitro* suppression of viral replication in autologous CD4+ T-cells using inhibition assays as mediated by Gag-specific rather than Nef or Env-specific CTL responses (Saez-Cirion et al., 2009, Julg et al., 2010, Ndhlovu et al., 2012). Taken together, these data suggest that among viral proteins, Gag-proteins are important for CTL mediating viral control.

1.5.9 CTL Selection Pressure and Viral Escape

The impact of CTL selection pressure is readily observed by sequencing autologous virus, which undergoes rapid immune-driven evolution *in-vivo* following acute infection (Walker and McMichael, 2012). Genome amplification techniques have shown clear evidence of CTL escape within 25-32 days of infection (Goonetilleke et al., 2009). Ultra-deep sequencing has also revealed that the virus explores multiple pathways to escape CTL pressure (Fischer et al., 2010). The pathways to immune escape appear limited, as shown by studies of genetically identical twins infected by the same virus strain, in whom the earliest targeted epitopes and escape variants were similar (Draenert et al., 2006).

Escape from CTL responses can occur through multiple mechanisms; mutation within CTL-epitopes can clearly impact processing and subsequent recognition (Draenert et al., 2004b, Le Gall et al., 2007, Tenzer et al., 2009). Mutations in the TCR contact residues have a variable impact on CTL recognition, and the ability to cross-recognize variants appears to be influenced by thymic selection (Kosmrlj et al., 2010). HLA alleles associated with better outcome following HIV infection are associated with presentation of fewer self-peptides in the thymus, resulting in a repertoire in which more clones survive negative selection, and are more likely to be cross-reactive to variants that arise (Kosmrlj et al., 2010, Chen et al., 2012). Taken

together, these studies give insight to the association between HLA alleles and HIV infection outcome.

1.6 Human Leukocyte Antigen (HLA)

The major histocompatibility complex (MHC) coding region, known as HLA in human and located on the short arm of chromosome 6, is the most polymorphic of the entire human genome (Goulder and Watkins, 2008, Goulder and Walker, 2012). Functional MHC molecules are made of a heavy (α) chain and a β2-microglobulin chain genes encoding class I loci (Sidney et al., 2008). Peptide binding by MHC class I molecule is accomplished by interaction of the peptide amino acid side chains with discrete pockets within the peptide-binding groove of the MHC molecule formed by the α1 and α2 domain of the heavy chain, (Figure 10), (Sidney et al., 2008). The main binding energy of peptide to the class I HLA molecule is provided by the interaction of residues in position 2 and the C-terminus of the peptide with the B and F binding pockets respectively, (Figure 11), (Madden, 1995).

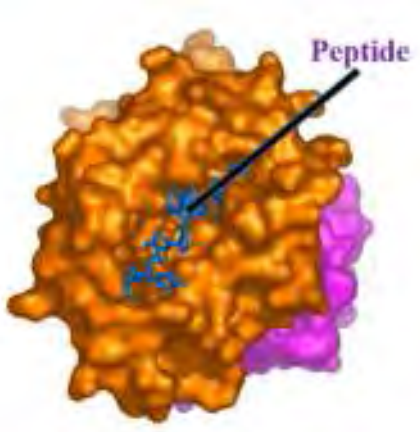

Figure 10: Schematic Representation of HLA Molecule Housing a Peptide. The peptide (blue) is the amino acid sequence sitting in the peptide binding pocket of HLA molecule (Khan et al., 2000).

Of the three MHC class I loci in humans (HLA-A, HLA-B and HLA-C), HLA-B is the most polymorphic, with 817 different HLA-B molecules described, compared to 486 distinct HLA-A and 263 distinct HLA-C molecules (Mungall et al., 2003). The polymorphism of HLA molecules influences the peptide-binding repertoire. However, multiple class I HLA alleles can bind identical peptides due to the similarity in their peptide binding motifs. HLA alleles sharing similar peptide binding motifs have therefore been referred to as HLA supertype, (Figure 12),

(Sette and Sidney, 1999). Polymorphism of class I HLA molecules have been shown to contribute to differences in disease outcome (Goulder and Watkins, 2008).

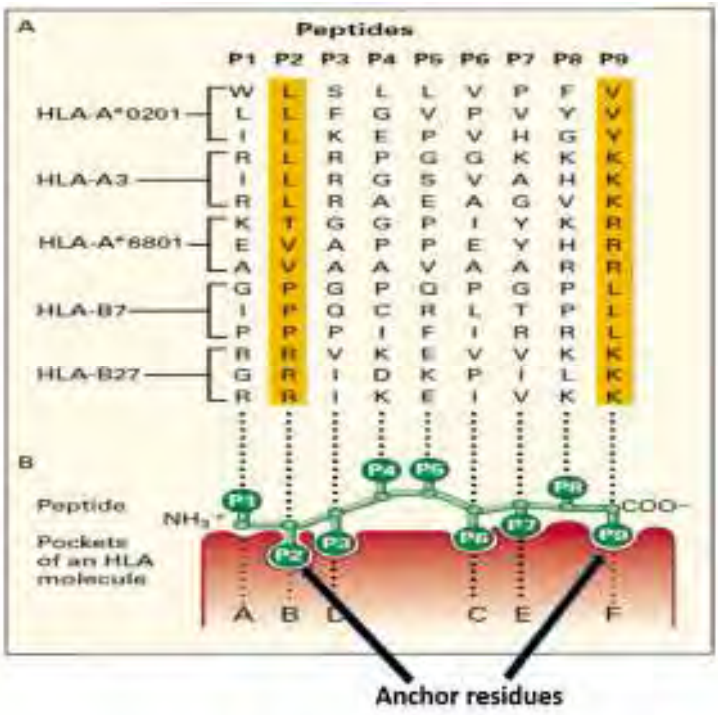

Figure 11: Interactions between HLA Molecules and Peptides. Panel A illustrate examples of peptide motifs. The listed amino acids as well as many others have been found to complex with the respective HLA class I molecules. The anchor residues are highlighted in yellow. Panel B is a longitudinal section through the peptide-binding groove of pocket A to F. Pocket B and F bind the peptide residue P2 and P9 respectively. Some of the peptide residues pointing into the HLA molecule have a greater influence over binding while the residues that point outward affect the T-cell receptor recognition (Klein and Sato, 2000).

1.6.1 Class I HLA Supertype

The peptide repertoire of a specific molecule is to a large extent determined by the molecular structure accommodating the main anchor positions of the presented peptide (Sidney et al., 2008). Multiple class I HLA alleles can recognize common peptides due to the homology of amino acids within the anchor positions of the presented peptides (Sette and Sidney, 1998). Examples; A2 supertype share specificity for peptides with aliphatic hydrophobic residues in position 2 and at the C-terminus. A3 supertype recognize peptides with small or aliphatic residues in position 2 and basic residues at the C terminus (Sidney et al., 2008).

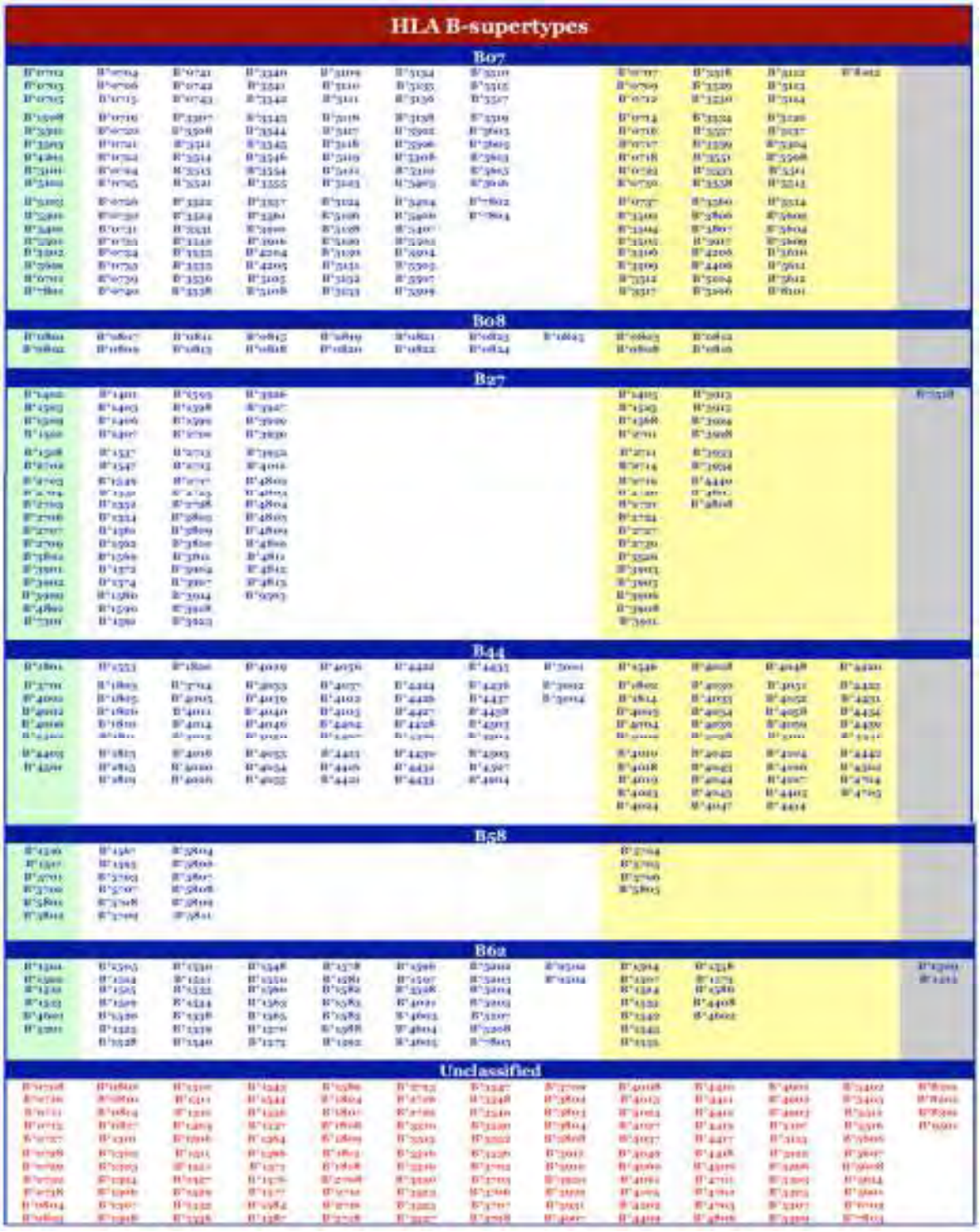

Figure 12: Supertype Classification of HLA-B Alleles. The alleles associated with each HLA-B supertype, multiple supertype, or those that are unclassified, are shown. Under each supertype, alleles are grouped by colour on the basis of the stringency of selection: experimentally established motif (i.e., reference panel) light green: exact match(es) in the B and F pockets, white: one exact and one key residue pocket match, yellow: key residue match(es) at B and F pockets. Alleles with no match at one or both pockets are listed with red font (Sidney et al., 2008).

Overlapping peptide with distinct binding pocket have been demonstrated among class I HLA-B supertype (Sidney et al., 1995, Sidney et al., 1996b). A study illustrated that proline is preferably selected at position 2 (anchor-motif) by HLA-B7 supertype (Leslie et al., 2006a). Another study showed that HLA-B*27 binds peptides with arginine at position 2 and HLA-B*57 binds peptides with a tryptophan at the C-terminus (Marsh et al., 1999). There are nine HLA supertypes that have been well described based on their structural similarities and the overlapping peptide-binding motifs (Sette and Sidney, 1999). Each of these supertypes allows coverage of about 35-55% of the general population irrespective of ethnicity (Sidney et al., 1996a), 83-89% coverage could be achieved when 3 of the supertypes are combined (Sette and Sidney, 1998). These analysis suggest that an effective, non-ethnically biased HIV CTL based vaccine design is feasible.

1.6.2 The B57 Supertype

The most reproducible class I HLA association with low HIV viremia and prolonged AIDS-free survival has been observed with HLA-B*57 and related HLA alleles (Kaslow et al., 1996, Keet et al., 1999, Hendel et al., 1999, Trachtenberg et al., 2003). Nearly all HLA-B*57 alleles are characterized by unique valine at position 97, which contribute to the formation of the C-pocket of the class I antigen-binding cleft (Lardy et al., 1998). HLA-B*58 also belongs to the B57 supertype both by structural and functional similarities. Other alleles within HLA-B*57 supertypes are; B*57:01, B*57:02, B*58:01, B*58:02, B*15:16, and B*15:17 (Figure 12) (Sette and Sidney, 1999). These alleles share a preference for antigen supermotifs with small aliphatic residues in position 2 and aromatic or hydrophobic residues at the C-terminus of their peptide ligands (Sette and Sidney, 1999). The B57 supertype alleles have a combined phenotypic frequency of some 10-25% in Caucasoid and African populations (Sette and Sidney, 1999). In addition, HLA-B*57:01 in Caucasoids and HLA-B*57:03 in Africans demonstrate broad functional cross-reactivity to both common and rare variants of a dominant p24 epitope (Gillespie et al., 2002). These studies further suggest that broad based vaccine that can cover more than one ethnic group and region is feasible.

1.6.3 The B07 Supertype

Human leukocyte antigen-B*42:01, B*81:01, B*39:10, B*35:01, B*07:02, fall into the HLA-B07 supertype, (Figure 13), (Sidney et al., 2008). Alleles in this supertype harbour a proline in the B pocket and an aromatic, aliphatic and hydrophobic residue in the F pocket (Sidney et al., 2008). The B07 supertype in Caucasoids and African Americans population infected with HIV subtype B is associated with high viremia, relatively poor responses and fast progression to AIDS (Pereyra et al., 2010). In contrast, in African populations infected with HIV subtype C, it is associated with low viremia (Matthews et al., 2008, Leslie et al., 2010). A study by Leslie and colleagues in 2006 that sought to address the role of HLA-B07 supertype in the immune control of clade C HIV, using a large cohort of clade C infected patients of Zulu/Xhosa ethnic origin from KwaZulu-Natal, South Africa, showed the promiscuity of epitopes restricted by the alleles within this supertype (Leslie et al., 2006a). However, the assay used in this study has been shown not to correlate with the responses of CTL to the restricted epitopes *in-vivo* (Bennett et al., 2008). Therefore, there is need for the development of functional assay that can inform on the direct correlate of CTL responses *in-vivo* when presented with promiscuous epitope restricted by alleles within the same supertype.

1.6.4 Immunodominant Epitopes Restricted by Alleles within B57 Supertype

Strong HIV-specific CTLs responses targeting multiple epitopes in the conserved p24 region of Gag protein are likely to contribute to viral control (Scherer et al., 2004, Altfeld et al., 2006, Kiepiela et al., 2007). Alleles within B57 supertype restrict multiple HIV peptides with predominant responses by HIV-specific CTL against Gag (Goulder et al., 1996, Klein et al., 1998). A study in South African HIV infected population showed that targeting epitopes within the Gag protein, especially those restricted by B57 could increase the expected time to AIDS from 2-3 years after infection to >8-10 year (Kiepiela et al., 2007). In the study, HLA-B*57:03 and HLA-B*58:01 were shown to be the most prevalent alleles within the B57 supertype. HLA-B*57:03 showed a dominant response to KAFSPEVIPMF (Gag residue 162-172), while HLA-B*58:01 restricted immunodominant response is against TSTLQEQIAW (Gag residue 240-249). ISPRTLNAW (Gag residue 147-155) is also a well characterised p24 Gag immunodominant epitope restricted by most alleles within B57 supertype (Goulder et al., 1996). Although HLA-B57 is comprised of "protective" alleles, not all HIV infected patients expressing these alleles exhibit slow HIV disease progression (Chopera et al., 2011). The

reason for the differences observed within HLA-B57 alleles could be the apparent small differences (micropolymorphisms) existing between these alleles (Kloverpris et al., 2012b).

Mutations at specific positions of HIV-Gag peptides occur frequently and are unique to HLA-B57 and -B58 positively individuals that are infected with HIV clade B and C (Leslie et al., 2004). The gradual accumulation of mutations within the restricted immunodominant HIV-Gag peptides can result into functional loss of T-cell recognition (Yang et al., 2003). This indicates that positive selection for these mutations is being driven by CTL specific for the restricted epitope. However, the extent to which HIV-specific CTL can recognise and kill infected targeted cells restricting wildtype and variant epitopes is unknown.

1.6.5 Immunodominant Epitopes Restricted by B07 Supertype

The most common B07 supertype class I HLA alleles in the Zulu/Xhosa population of South Africa are HLA-B42:01, B81:01, and B07:02, which occur at phenotypic frequencies of 18.6, 9.6 and 8.2%, respectively (Leslie et al., 2006a). These alleles are closely related, with no amino acid differences in the residues forming their B and F primary binding pockets. Clade C p24-TL9 (TPQDLNTML, Gag residue 180-186) is restricted by majority of the class I HLA alleles within B07 supertype. Leslie et al., (2006a) showed that there is a significant greater variation in HIV-specific CTL response to TL9-Gag in individuals expressing each of the alleles within B07 supertype, with HLA-B81:01 having a higher CTL response than other alleles. Although TL9 is a dominant epitope restricted by HLA-B81:01, B42:01 and B39:01, there were striking differences in the frequency of polymorphism between these alleles, which could invariably have profound impact on T-cell immunity (Gao et al., 2001a, Geldmacher et al., 2009, Goulder and Walker, 2012, Kloverpris et al., 2012a, Kloverpris et al., 2012b) The impact of these differences observed within class I HLA molecules restricting identical epitopes on CTL was investigated in our study.

1.7 Class I HLA Molecules and Disease Outcome

Class I HLA alleles bind peptide fragments derived from the intracellular pathogens and present them to CTLs (Figure 8), thereby initiating cytotoxic T-cell responses. The fragmented peptides are loaded into the peptide-binding groove of the HLA molecules and transported to the cell surface for presentation to CTLs (Sette and Sidney, 1999). Class I HLA alleles have

been identified consistently to have significant impact on the rate of disease progression (Kaslow et al., 1996, Hendel et al., 1999, Migueles et al., 2000, Lazaryan et al., 2006).

Class I HLA molecules and the peptides they present are significantly important (Goulder and Watkins, 2008), single amino-acid differences between closely related class I HLA alleles may have crucial consequences in the antiviral efficacy of CTL (Archbold et al., 2009, Kloverpris et al., 2012b); examples include the association of HLA-B*35:02 and HLA-B*35:03 with rapid disease progression and a lack of any such association for HLA-B*35:01, which differs from HLA-B*35:02 and HLA-B*35:03 by only three and one amino acid respectively (Steinle et al., 1995, Gao et al., 2001a). These small changes would be expected to affect the binding of the C-terminal amino acid of the F pocket of the HLA molecule (Smith et al., 1996).

Study on HIV infection showed a strong association between HLA-B*58:01 and low viral load, whereas HLA-B*58:02, which differs from HLA-B*58:01 by three amino acids, was associated with high viral load (Table 1), (Kiepiela et al., 2004a). Another study of four closely related HLA alleles within the B7 supertype (B*07:01, B*42:01, B*39:02, B*81:01) demonstrated that, even when identical peptides are presented by these closely related alleles, different selection pressure on the virus could be observed (Leslie et al., 2006a). Hence, the existence of class I HLA polymorphism could be the reason for the differences in disease outcome observed within class 1 HLA alleles restricting identical epitopes and this could have implications on the development of effective CTL based vaccine.

Table 1: Some of the Class I HLA-B Alleles, HIV Infection Outcome and their Restricted Epitopes (Goulder and Watkins, 2008).

MHC class I allele	Progression	Viral setpoint	Epitope restricted	HIV protein
HLA-B*13:02	Slow	Low	RQANFLGKI	Gag 429-437
HLA-B*18:01	Rapid	High	RQANFLGKI	Gag 429-437
HLA-B*27:05	Slow	Low	KRWIILGLNK	Gag 263-272
HLA-B*35:02 HLA-B*35:03	Rapid	High	KRWIILGLNK	Gag 263-272
HLA-B*51:01	Slow	Low	TAFTIPSI	RT 128-135
HLA-B*57:01 HLA-B*57:02 HLA-B*57:03	Slow	Low	TSTLQEQIAW KAFSPEVIPMF IVLPEKDSW	Gag 240-249 Gag 162-172 Gag 147-155
HLA-B*58:01	Slow	Low	TSTLQEQIAW ISPRTLNAW	Gag 147-155 Gag 147-155
HLA-B*58:02	Rapid	High	QTRVLAIERYL	Gp41 577-587
HLA-B*81:01	Slow	Low	QTRVLAIERYL TPQDLNTML	Gp41 577-587 Gag 180-188
HLA-B*42:01	Rapid	High	TPQDLNTML	Gag 180-188

1.8 Measurement of HIV-specific CTL responses

Despite the strong evidence of the important role CTL plays in the control of HIV infection, current assays to quantify T cell immunity have failed to correlate HIV-specific CTL responses with protection from infection or control of viral replication (Addo et al., 2003, Frahm et al., 2004). The rapid recognition and subsequent elimination of HIV infected cells before production of new viruses represent critical effector functions of HIV-specific CTL that are not reflected in the current assays (D'Souza and Altfeld). The merits and demerits of several assays currently employed to quantify HIV-specific CTL would be discussed briefly below:

1.8.1 Chromium Release Assay

Chromium (^{51}Cr) release assay (CRA) is the earliest method to detect HIV-specific CTLs (Sun et al., 2003). It is performed either on fresh cells by measuring CTL activity (Walker et al., 1987, Plata et al., 1987), or on CTL lines by evaluating memory CTL reactivity (Nixon et al., 1988). In CRA, class I MHC matched and mis-matched target cells expressing HIV antigen are labelled with radioactive ^{51}Cr and incubated with HIV-specific CTL for 4-6 hours. CTL killing is measured indirectly by detecting ^{51}Cr released by dying target cells into the supernatant (Shacklett, 2002).

Although CRA is useful in the identification of optimal epitopes recognized by CTL, it requires large numbers of PBMC, uses radioactive material, and is time consuming. More importantly, it may introduce a bias in CTL analysis since the cells which easily expand *in-vitro* are mainly memory T-cells, while the CTLs which have proliferated *in-vivo* for many cycles are more prone to apoptosis and do not readily proliferate *in-vitro* (McMichael and O'Callaghan, 1998).

1.8.2 ELISPOT

Enzyme-linked immunospot (ELISPOT) IFNγ assay; which is an adaptation of ELISA, is used to enumerate the function of CTL by quantifying the amount of IFNγ secreted when stimulated with peptide (Czerkinsky et al., 1988). Upon stimulation of CTL with exogenous peptide, CTL specific for the peptide secrets IFNγ and the secreted cytokine is captured on membrane coated by anti-cytokine monoclonal antibody (Streeck et al., 2009). Biotinylated polyclonal antibody specific for the secreted cytokine and alkaline-phosphatase conjugated with streptavidin are used to identify the blue-black colour formed at the spot of cytokine localization. The secreted IFNγ is viewed using a computerized camera that quantify cytokine secreted by CTL in response to antigen-specific stimulation (Nylander and Kalies, 1999).

Despite the usefulness of ELISPOT to quantify CTL magnitude in response to stimulation with HIV peptide, ELISPOT assay usually relies on IFNγ as a functional readout and may therefore miss antigen-specific cells that do not produce IFNγ. In addition, ELISPOT has also been shown not to correlate with *in-vivo* virus control (Pantaleo and Koup, 2004, Valentine et al., 2008, Gray et al., 2009).

1.8.3 Tetramer Binding Assay

Tetramer binding assay provides an elegant, sensitive means of quantifying antigen specific-CTL frequency in a population of T-cells and is used in characterising immunodominant epitopes (Altman et al., 1996, Ogg et al., 1998). Tetramer binding assay include coupling of tetrameric complexes of MHC-class I with CTL population. The tetrameric complexes directly bind the T cell receptors of CTL specific for the tetrameric molecule and the tetramer specific CTL can be quantified using flow cytometry (Ogg et al., 1998). However, responses of CTL to well-characterized immunodomionant epitopes by tetramer binding assay overlooks the potential complexity of these responses (Shacklett, 2002), such as: capacity to secret cytokines and their ability to proliferate in response to stimulation with HIV peptides.

1.8.4 Intracellular Cytokines Assay

Antigen-specific stimulation, followed by inhibition of cytokines secretion and measurement of accumulated intracellular cytokines by flow cytometry is referred to as intracellular cytokines staining (ICS) (Shacklett, 2002). The produced cytokines are stained with their corresponding antibodies, followed by quantification using flow cytometry. ICS provides important insight into the dynamics of CTLs upon encountering their cognate antigens. ICS is sensitive and allow screening for responses against multiple CTLs' epitopes in a single assay and require small number of PBMC.

However, studies have shown that cytokines production by CTLs do not correlate with *in-vivo* control of HIV replication (Pantaleo et al., 1994, Yang et al., 1996, Valentine et al., 2008, Gray et al., 2009), and did not predict the lack of efficacy in the vaccine trials (Buchbinder et al., 2008, McElrath et al., 2008). Therefore, as peptide based assays do not approximate the ability of CTL to suppress the replication of the virus *in-vivo,* novel assays that will represent the direct picture of CTL suppressive capacity of HIV replication *in-vivo* is needed.

1.8.5 Viral Inhibition Assay (VIA)

The viral inhibition assay (VIA) earlier demonstrated by Bennett *et al*. (2008), has been shown to be a suitable assay that reflects *in-vivo* CTL function. The VIA incorporates steps in viral entry, antigen processing and presentation and relies on the recognition of endogenously

generated viral peptides within targets cells. The assay readout is by quantifying HIV-p24 released into the media through enzyme linked immunosorbent assay (ELISA). Although the ELISA-VIA can be used to quantify the antiviral activity of CTL against different clades of viruses, the assay is faced with some challenges such as: it is labour intensive, it requires the separation of CD4+ T-cells and CTLs and measurement of p24 over time, the cell-killing ability can vary with manipulation and culture of lymphocyte populations, *in-vitro* HIV replication can vary greatly and is dependent on input virus strain and cellular activation. (D'Souza and Altfeld).

1.8.6 The Use of CEM-GFP Reporter T Cell Lines (GXR cell lines) as Target Cells in Viral Inhibition Assay

The CEM-GFP reporter T cell line (GXR-cell line) was used as target cells to obtain the precise physiological correlate of the antiviral activity of HIV-specific CTL. The GXR-cell line was first described by Gervaix *et al*. (1997), and was further modified to be susceptible to both CXCR4 and CCR5 co-receptors by Brockman *et al*. (2006). The cell lines express green florescent protein in a tat-dependent manner upon HIV infection. In addition, it has been genetically engineered to over express specific class I HLA molecule.

The GXR-VIA has advantages over the conventional ELISA-VIA such as: it is free of autologous virus, incubation period is reduced to 3 days instead of 7 days, and it also minimizes variations introduced by long term culture. Different GXR-cell lines were used as target cells in GXR-VIA to address the objectives of our study.

1.9 Study Rationale

Studies on HIV infection strongly suggest that HIV-specific CTL and the restricting class I HLA alleles play a critical role in immune control of HIV. CTLs recognize virus infected cells through antigenic viral peptide fragments of 8 to 12 amino acids in length, that are presented by class I HLA molecules. Class I HLA alleles bind specific virus derived peptide fragments. Interestingly, more recent studies have demonstrated that closely related class I alleles can present identical epitopes due to the homology of amino acids within the major binding pockets of the peptide binding cleft. However, the antiviral efficacy of HIV-specific CTL when identical epitopes are presented by closely related HLA alleles is unknown.

Few studies have shown that class I HLA alleles can restrict promiscuous mutant epitopes, no study has shown the functional consequence of HIV-specific CTL when presented with mutant epitope commonly restricted by closely related class I HLA alleles.

1.10 Aim of the Study

The aim of the study is to conduct a systematic analysis of the viral inhibitory activity of HIV-specific CTL targeting identical epitopes restricted by different closely related class I HLA alleles from the same supertype.

1.10.1 Specific Objectives

1. Validation of modified GXR viral inhibition assay with the standard viral inhibition assay using autologous activated CD4+ T-cells as target cells.

2. To conduct a comparative analysis of viral inhibition activity of CTL which recognise identical epitopes restricted by different class I HLA alleles from the same supertype.

3. Investigate whether class I HLA alleles belonging to the same supertype can efficiently present *in-vivo* occurring commonly recognized CTL variants epitope.

CHAPTER TWO: Methodology

2.1 Study Ethics Consideration

This study was approved by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal and all study subjects gave written informed consent for immunology, genetics and virological studies.

2.2 Study Subjects

Subjects with chronic and acute untreated HIV-1 subtype C infection from Zulu/Xhosa ethnic origin were recruited from Sinikithemba (SK) clinic at McCord Hospital and Prince Mshiyeni (AS) Hospital, Durban, South Africa. Frozen peripheral blood mononuclear cells (PBMCs) samples from the subjects stored at vapour phase of liquid nitrogen were used to conduct this study.

2.3 Inclusion Criteria

The participants included in this study were patients with known HLA type; B7 supertype (B*81:01, B*42:01 alleles) and B57 supertype (B*57:03, B*58:01 alleles).

2.4 HLA Typing

HLA typing was performed on patients' DNA samples at the Mary N. Carrington's Laboratory, Centre for Cancer research, National Cancer Institute, Fredrick, USA, as previously described (Kiepiela et al., 2004b). Briefly, DNA samples were first typed to an oligo-allelic level using Dynal RELITM reverse Sequence Specific Oligonucleotide (SSO) kits for the HLA-A, HLA-B and HLA-C loci (Dynal Biotech). Refining the genotype to the allele level was performed using the Dynal Biotech Sequence-Specific priming (SSP) kits in conjunction with the previous SSO type. Where alleles were still not defined to the allele level, the sequence specific primer mixes were used. All HLA class I alleles in the IMGT allele release 24.0 were considered in the typing.

2.5 Separation, Counting and Cryo-preservation of PBMCs

The blood was diluted with PBS (Whitehead scientific) in a 1:1 dilution. This was then layered very slowly over Histopaque 1077 using a Drummond pipette aid in a 1:1 dilution in a 50 ml tube. The tube was held at a 45º angle and the diluted blood was allowed to run down the side of the tube until the 40 ml mark was reached. The blood floated on top of the Histopaque. The tube was then centrifuged (Eppendorf 5810 AG) at 1,500 rpm for 30 minutes at room temperature. A pipette was used to aspirate off the opaque interface containing the mononuclear cells. The mononuclear cells were transferred to a second 50 ml tube. The tube was filled to 40 ml with PBS. The tube was centrifuged (Eppendorf 5810 AG) at 1,500 rpm for 10 minutes at room temperature. The supernatant was poured off and the lymphocytes pellet was gently resuspended. Twenty ml of warm R10 medium was then added to the tube and the sample was incubated at 37 $^{\circ}$C in a CO_2 incubator until it was ready to be counted.

An aliquot of cell suspension was prepared for counting. Twenty µl cells was added to 180 µl Guava counting solution (Merck, Germany) in a micro-centrifuge tube to produce a 1:10 dilution. The mixture was incubated for 8 minutes in the dark before being acquired on the Guava counter (Merck) according to the manufacturer's instructions.

Incubated PBMCs were centrifuged (Eppendorf 5810 AG) at 1,500 rpm for 10 minutes at 4 ºC and the supernatant was discarded. The cell pellet was gently re-suspended using a pipette tip and all clumps were resolved. The tube was then transferred to an ice box containing crushed ice. Ten million cells were thoroughly mixed with 500 µl of fetal calf serum (FCS) (BioCom Technology, USA), freezing media (500 µl of FCS with 20% dimethyl sulfoxide (DMSO) (Merck, Germany)) were further added to the mixture in a drop-wise manner. The cells in freezing solution were then transferred to the cyovial and the cryovial was transferred into special containers called Mr. Frosty's (Nalgene). The freezing containers were then placed into a -80 ºC freezer. The Mr Frosty's contain iso-propanol and cool down the cells at a rate of 1 ºC per minutes which is considered optimal for the cells quality. The vials were transferred from the Mr. Frosty's to the vapour phase of liquid nitrogen freezer for long term storage after 24 hours.

2.6 Thawing of the Cryo-preserved Cells

Cells were rapidly thawed at 37 ºC in the water bath until only a silver of ice remained. The outside of the cryo-tube was swabbed with 70% ethanol before the cells were transferred to the 15 ml tube containing 9 ml of warm R10. The tube was centrifuged (Eppendorf 5810 AG) at 1,800 rpm for 6 minutes. The supernatant was discarded, ensuring that maximum amount of the media was removed because of the freezing solution that contained dimethyl sulfoxide (DMSO) (Merck) which is toxic to the cells. The pellet was then resuspended with 20 µl of DNAse (Roche, USA) to disintegrate clumpy cells, 10 ml of R10 was further added and centrifuge at 1,800 rpm for 6 minutes. The cells were then resuspended in R10 medium, rested for 2 hours, thereafter counted manually. Cells were counted manually by adding 10 µl of the suspended cells to 90 µl of trypan blue. Ten µl of the mixture were pipetted on the haemocytometer (HYCOR Biomedical, USA), and counted using Olympus CH20 microscope (Olympus Optical, Japan). The count was multiplied by the dilution factor and by 10^4 and reported as the number of cells per l ml of media.

2.7 Viral Inhibition Assay (VIA)

2.7.1 Principle

Target cell is infected with HIV. An enzyme proteasome in the infected cell helps in the intracellular degradation of the HIV peptides. Transport associated protein (TAP) transfer the degraded peptides into the endoplasmic reticulum; where class I HLA molecule binds to the cognate peptide and transport it to the cell surface for presentation to CTL. T-cell receptor of the CTL recognizes the presented foreign antigenic peptide and trigger the release of effector molecules in the CTL. The effector molecules are directly release on the infected target cell, leading to the killing of the target cell.

2.7.2 Procedure

The various components of VIA are: generation of HIV stocks, Preparation of target and effector cells. In this section, components of ELISA-VIA and the GXR-VIA would be described.

2.7.2.1 Generation of HIV Stock

Integrated plasmid with all the HIV genomic regions and the GXR cells were obtained from Dr. Jacklyn Mann, HPP, Durban, South Africa. The plasmid was used to transfect GXR cells. Four million GXR cells were electroporated (Genepulser II Capitance, Biorad) using 5 µg of the NL4-3 plasmid at 250 Volts, and 950 µF for 10 seconds. The electroporated cells were transferred to T25 flask containing 1 million GXR cells suspended in 9 ml of R10 and 4 µl of polybrene (Sigma, USA). The polybrene was added to soften the GXR cells walls for easy penetration of the viral particles into the cells. The cells were cultured for 8 days by removing and adding 2 ml of fresh media. The percentage infectivity was monitored using LSRII (BD, Germany). At approximately 30% infection, the virus were harvested and cryo-preserved.

2.7.2.2 Harvesting and Cryo-preservation of the Generated Virus Stocks

The infected cultured containing the generated virus was transferred to 50 ml tube and centrifuged (Eppendorf 5810 AG) at 1,800 rpm, 4 ºC for 6 minutes. The virus-containing supernatant was aspirated, aliquoted in labelled cryovials and stored at -80 ºC until use.

2.7.2.3 Generation of Recombinant Virus Stock

Patient derived first round PCR product harbouring Q182S mutation within the Gag region was obtained from Dr. Michelle Gordon, HPP, Durban, South Africa. Second round PCR was carried out by Miss Avashna Singh as previously described by (Wright et al., 2010).

The subtype B pNL4-3Δgag-protease backbone stock, containing a BstE II restriction enzyme site in place of the viral gag-protease coding region, was obtained from Dr. Jacklyn Mann, HPP, Durban, South Africa. To amplify pNL4-3Δgag-protease stock, Luria-Bertani (LB) broth (Sigma, St Louis, USA) containing 100 µg/ml ampicillin was inoculated with 17.5 µl STBL3 stock containing pNL4-3Δgag-protease per 100 ml of LB broth and incubated overnight at 37 ºC in a shaking incubator (INFORS HT, Bottmingen, Switzerland). The plasmid was then purified using the plasmid Maxi kit (Qiagen), quantified using a Nano-drop spectrophotometer (Thermo Scientific, Delaware, USA) and stored in a -80 ºC ultrafreezer (Snijders Scientific, Holland) until use.

GXR cells were co-transfected with the mutant amplified patient's gag-protease and the pNL4-3Δgag-protease backbone. Prior to the co-transfection, pNL4-3Δgag-protease (10 µg per

sample) was digested with BstE II enzyme (Promega, Madison, USA) for 2 hours at 60 ºC in a water-bath to linearize the plasmid at the site of gag-protease deletion and allow for insertion of mutant amplified patients derived gag-protease by recombination. Recombination of gag-protease with pNL4-3Δgag-protease was made possible by the use of long primers exactly complementary to NL4-3 on either side of gag-protease to generate gag-protease PCR product.

During the BstE II digestion of pNL4-3Δgag-protease, the GXR cells were counted and the required number of cells was removed, pelleted and resuspended in fresh R10 medium. Four million cells per samples were suspended in 300 µl of R10 and transferred into 4 mm cuvettes (Biorad, USA). Ten µg of BstE II-digested pNL4-3Δgag-protease and 90 µl of patient's gag-protease PCR product was transferred into the cuvettes containing the GXR cells and electroporated at 250 V and 950 µF (Genepulser II Capitance, Biorad). As a negative control, GXR cells were electroporated with pNL4-3Δgag-protease only. Electroporation allows for the temporary disruption of cell membranes, allowing uptake of molecules and is a techniques used to transfect cells with DNA (Spencer, 1991). Following 1-hour incubation at room temperature to allow for cell recovery, electroporated GXR cells were gently transferred to T25 flasks containing 1 million GXR cells suspended in 9 ml of pre-warmed R10 and 4 µl (1 µg/µl) of polybrene and incubated at 37 ºC and 5% CO_2. Five days later, 5 ml R10 medium was added to each flask followed by further incubation.

Electroporated GXR cells were incubated for 12 days before virus production was monitored to allow time for recombination of gag-protease amplicons with the NL4-3Δgag-protease plasmid, followed by viral transcription, translation, particle production and spread in the culture. The percentage of the infected cells was monitored using LSRII (BD). At approximately 30% infection, the virus were harvested and cryo-preserved as described above.

2.7.2.4 Sequencing and Titration of the Virus Stocks

To confirm the presence of mutation under investigation in the harvested virus stock, the generated recombinant stock was sequenced. Virus stock was titrated to determine stock's concentration. One million GXR cells were infected with 400 µl of each of the stock for 8 hours. Thereafter, the infected cells were washed and 100, 000 cells per well were plated in duplicate in a 96-well plate and incubated for 3 days. An aliquot of 100, 000 uninfected GXR cells per well were also plated in duplicate alongside the infected cells as negative control. On day 3, incubated GXR cells were fixed and assessed the infectivity levels using LSRII (BD). Infectivity levels was used to calculate the desired multiplicity of infection (MOI) as follows:

$$\text{Volume of virus to be used per 1 million cells} = \frac{\text{Desired Multiplicity of Infection}}{\text{Titre value} * 400}$$

2.7.2.5 Generation of CD4+ T Cells and CTLs

2.7.2.5.1 Principle

The CTLs were magnetically labelled with CD8 microBeads (Miltenyi Biotec). The suspended cells were loaded onto a calibrated MACS Column, which was placed in the magnetic field of a MACS separator. The magnetically labelled CTLs were retained within the column. The unlabelled cells (majorly CD4+ T cells) ran through; CD4+ T cells fraction was thus depleted of CTLs. After removing the column from the magnetic field, the magnetically retained CTLs were eluted as the positive selected cell fraction.

2.7.2.5.2 Procedure

2.7.2.5.2.1 Preparation of Reagents for CD4+ T Cells and CTLs Isolation

Bovine Saline Albumin (BSA) (Whitehead Scientific) solution was used to prepare MACs buffer, which was used in the depletion experiment. To prepare BSA solution, 10g of BSA concentrate was added to 100 ml of distilled water and placed in the refrigerator (4 ºC) for 24 hours to dissolve. MACs buffer was prepared by adding 49 ml of PBS (Whitehead Scientific) to 1 ml of BSA solution and placed on ice. R10/50 was also prepared by adding 25 µl of interleukine-2 (IL-2) to 50 ml of R10 and kept on ice until used.

2.7.2.5.2.2 Separation of CD4+ T Cells and CTLs

LS column (MACS Miltenyi Biotec GmbH, Germany) and CD8 MicroBeads human (MACS Miltenyi Biotec GmbH, Germany) was used to isolate CTLs and CD4+ T cells from PBMCs. The PBMCs were counted to know the volume of CD8 microBeads that would be needed for the isolation step. Cells were centrifuged at 1,800 rpm (Eppendorf 5810 AG) for 6 minutes at 4 ºC and the supernatant was aspirated. The cells were resuspended in MACs buffer and wash. The supernatant was aspirated completely and 20 µl of CD8 MicroBeads was used per 10^7 cells. The cells were mixed with the CD8 MicroBeads and incubated for 15 minutes at 4 ºC. After incubation, MACs buffer was added to make up 10 ml per 10^7 and the cells were

centrifuging at 1,800 rpm for 6 minutes at 4 ºC. Supernatant was aspirated and the cells were resuspended in 500 µl of MACs buffer.

The MS column was placed in the magnetic field and column was calibrated using 1 ml of MACs buffer. Thereafter, the suspended cells were added to the column. The unlabelled cells fraction; which contained majorly the CD4+ T-cells were collected in a new 15 ml tube and 3 ml of MACs buffer was further added to the column. The total effluent was passed through the column twice to ensure maximum separation of CTLs from the CD4+ T-cells. The column was removed from the magnetic field and placed on a new 15 ml tube. Five ml of MACs buffer was dispensed into the column and plunged to release all the bounded CTLs.

2.7.2.5.2.3 Purity of CD4+ T Cells and CTLs

Purity of the CD4+ T cells and CTLs was determined by performing cell surface staining on the isolated fractions. Cells surface staining was carried out on the aliquoted PBMCs (before depletion), depleted CTLs and the enriched CTLs.

Cells were placed in labelled FACs tubes and washed by centrifugation at 1,800 rpm (Eppendorf 5810 AG) for 6 minutes. The supernatants were aspirated and cells were suspended in 40 µl of 2% FCS/PBS, 0.1 µl of dead cells aqua-dye and incubated at room temperature in the dark for 10 minutes. After incubation, 200 µl of 2% FCS/PBS was added to each FACs tube containing the cells and centrifuged at 1,800 rpm for 6 minutes. The supernatant was aspirated and 34 µl of 2% FCS/PBS and 2 µl each of CD3 (Alexa fluor 647), CD4 (Alexa fluor 700), and CD8 (CD8-PE) –antibodies (BD, Pharmingen) were added to each of the tubes and further incubated for 20 minutes. Thereafter, 200 µl of 2% FCS/PBS was added to each of the FACs tube to wash the cells. The supernatants were aspirated and each tube containing the stained cells were resuspended in 250 µl of PBS and were ran on the LSR II.

2.7.2.6 ELISA-VIA

Enzyme linked-immunosorbent viral inhibition assay (ELISA- VIA) is an inhibitory assay that determine the inhibitory capacity of CTLs by the quantification of p24 antigen through ELISA. The target CD4+ T cells were activated by culturing in R10/50 media and 0.1 µl (0.5µg/ml) of CD3 CD8-bispecific monoclonal antibody per 1 million cells for 3 days. The CTLs were rested in R10 for 3 days at 37 ºC.

On day 3, activated CD4+ T cells were counted, an aliquot of CD4+ T cells was set aside as negative control for the assay. The rest of the activated CD4+ T cells were infected at a multiplicity of infection (MOI) of 0.1 with the generated wild type NL4-3 virus for 4 hours at 37 ºC. After 4 hours infection, cells were washed twice, counted and resuspended in appropriate amount of R10/50 to make up 1 million cells per 1 ml of media. Uninfected, infected (positive control) and infected (for bulk culturing) target cells were plated in triplicate at 10^5 cells per well in a 96-well plate. Resting CTLs were washed, counted and resuspended in appropriate amount of R10/50 media to make 1 million cells per 1 ml of R10/50 media.

To assess inhibition, CTLs were added to the plated infected CD4+ T cells at a ratios of 1:1, 1:2 and 1:4. At day 3, 5 and 7, the co-cultures were fed by removing and adding 90 µl of R10/50. HIV in the removed supernatant were inactivated by the addition of 10 µl of triton (PerkinElmer, Boston, MA) to 90 µl of the supernatant containing virus. The harvested supernatants were cryopreserved at -80 ºC for p24 quantification using PerkinElmer ELISA-kit (PerkinElmer, Boston, MA).

2.7.2.6.1 PerkinElmer ELISA

2.7.2.6.2 Principle

Harvested supernatants were transferred to microplate wells which were coated with a highly specific mouse monoclonal antibody for HIV-p24. The immobilized monoclonal antibody captured the free HIV-p24 that have been released by the infected cells. The captured antigen was complexed with biotinylated polyclonal antibody to HIV-p24, followed by a streptavidin-HRP (horseradish peroxidase) conjugate. The resulting complex was detected by incubation with ortho-phenylenediamine-HCl (OPD) which produces a yellow colour that was directly proportional to the amount of HIV-p24 captured. The absorbance of each coated plate was determined using a microplate reader (Promega, USA), and calibrated against the absorbance of an HIV-p24 antigen standard curve.

2.7.2.6.2.1 Procedure

The inhibitory activity of CTLs was assessed by quantification of HIV-p24 production in the harvested supernatants on day 3, 5 and 7. Serial dilutions of the harvested supernatants were made to reduce the concentration of HIV-p24 down to 1-400 pg/ml. The positive control was also diluted according to the manufacturer's instruction. The positive control was dispensed

accordingly into the wells coated with HIV-p24 antigen. Diluted samples were also loaded into the 96-well plate and incubated for 2 hours at 37 ºC. Thereafter, 200 µl of the washing buffer provided by the kit manufacturer was dispensed into each of the well, washed 6 times and blotted. Detector antibody was measured based on the number of the tested wells, 200 µl was added to each well and incubated for 1 hour at 37 ºC. Washing step was repeated. Diluted streptavidin-HRP was added at 100 µl per well and incubated in the dark for 30 minutes at 37 ºC. Washing step was repeated. Ortho-phenylenediamine-HCl substrate was added at 100 µl per well and incubated at 37 ºC for 20 minutes. Thereafter, stopping solution was added at 100 µl per well and the plate was read using a microplate reader (Promega, USA).

CTLs mediated inhibition was first expressed as the $\log_{10}$ reduction in the quantification of HIV-p24 of day 7 infected target cells without CTLs minus infected target cells with CTLs. The $\log_{10}$ inhibition values were converted to percentage inhibition values using the formula:

$$\% \text{ inhibition} = \frac{[100^* (\text{Day 7 p24 } \log_{10} \text{ values without CTLs} - \text{Day 7 p24 } \log_{10} \text{ values with CTLs})]}{(\text{Day 7 p24 } \log_{10} \text{ values without CTLs})}$$

2.7.2.7 GXR-VIA

A CEM-GFP reporter T-cells (GXR cells) that were over expressing class I HLA were used as target cells in the GXR-VIA. The cell lines were genetically modified to express green fluorescence protein (GFP) in a Tat-dependent manner upon HIV infection. In GXR-VIA, Infected target GXR-cell line were co-cultured with CTLs. Reduction in the cells expressing GFP measured by flow cytometry was used as a read-out for the inhibitory capacity of CTLs.

The GXR-cell lines over expressing class-I-HLA molecules were developed by Dr. Mark Brockman (Simon Fraser University, Burnaby, Canada), using leukaemia cells. The endogenous HLA molecules of these cells were downregulated, though still present at low levels. Genes of Class I HLA of interest were used to transduce the cell line using Puromycin (3 µg/ml). The cells expressing high HLA molecule of interested were selected using 100 µg/ml hygromycin and stained clones using CCR5 antibody clone (Pharmingen). Cells were later sorted using pan-HLA antibody (clone W6/32). Sorted GXR cell line were cultured in R10 media. At log growth phase, cells were harvested and 10 million cells were frozen down per vial.

The frozen GXR cell line were thawed using the protocol described in section 2.6, and suspended in R10 media for 5 days before use. Cultured GXR cell line were counted and

appropriate amount needed for the assay was infected at MOI of 0.5. An aliquot of GXR cell line were set aside as negative control. Infected GXR cell line were incubated for 24 hours at 37 ºC. After 24 hours incubation, infected and uninfected GXR cell line were washed twice with R10 medium and 100, 000 cells were plated per well in duplicate in a 96-well plate.

The isolated CTLs were counted and co-cultured with the infected GXR cell line expressing class I HLA under investigation at different target to effector ratios as showed in table 2. The setup plate was incubated for 3 days at 37 ºC.

On day 3, cells population were harvested by spinning down the plate at 2,000 rpm (Eppendorf 5810 AG) for 6 minutes. The pallets were fixed using 300 µl of paraformaldehyde and transferred into cluster tubes for flow cytometry interrogation.

One hundred thousand events were acquired for each experimental setup condition. Cells were hierarchically gated on GXR cell line with the cultured CTLs, infected cells expressing GFP. It was expected that if the CTLs were inhibiting, they should recognise and kill the infected target cells presenting their cognate antigenic peptide, thereby reducing the population of cells in the GFP expressing gate. The formula used to determine the inhibitory capacity of CTLs was:

$$\% \text{ inhibition} = 100*[1-(\% \text{ of GFP with CTLs} / \% \text{ of GFP without CTLs})]$$

Table 2: GXR VIA Setup. The table shows the experimental setup of uninfected, infected GXR cells and the CTLs in GXR-VIA.

Target : Effector	1:1	1:2	1:4	1:8
Target cell line	100,000	100,000	100,000	100,000
TL9-specific CTL	100,000	50,000	25,000	12,500

2.8 Validation of GXR-VIA

To validate GXR-VIA, CTLs from 8-HIV infected subjects were concurrently assessed using GXR-VIA and ELISA-VIA. The attribute of the GXR-VIA was evaluated by correlating the CTLs' inhibitory values obtained using GXR-VIA with the ELISA-VIA.

2.9 Comparison of the Inhibitory Activity of CTL Targeting Identical Epitopes Restricted by Different Class I HLA Molecules within the Same Supertype

2.9.1 Expansion of HIV-Specific CTLs

Cytotoxic T lymphocytes specific for the restricted identical epitopes under investigation were expanded from the whole PBMCs. Thawed PBMCs were rested for 2 hours in R10 media at 37 ºC. Cells were counted and an aliquot of cells (500,000 cells) were set aside for tetramer staining to confirm the percentage of the interested specific CTL in the PBMCs' population. Half of the rested cells were pulsed with 10 µl of 200 µg/ml peptide and incubated in a CO_2 incubator at 37 ºC for 3 hours. The other half were activated by suspension in R10/50 and incubated in a CO_2 incubator at 37 ºC for 3 hours. Cells were then transferred to T25-flasks and R10/50 were added to makeup 1 million cells/ml of the media. Cells were then incubated at 37 ºC in a CO_2 incubator for two weeks. Cells were fed with 2.5 ml R10/50 twice a week. Prior to cell sorting of specific CTLs, tetramer staining was setup to access the percentage of the expanded specific CTLs.

2.9.2 Tetramer Staining and Cell Sorting of Antigen-Specific CTLs

2.9.2.1 Principle

Tetrameric complexes of class I HLA molecule was used to stain CTLs directed against specific HIV-antigenic peptide. Tetramers were soluble oligomeric class I HLA molecules that bind to the TCR of the CTLs with high avidity. Tetramers were complexed with fluorochromes and cells were analyse on LSR II to identify the tetramer specific CTLs. Staining with tetramer helps to distinguished subset of specific CTLs from other CTLs in the PBMCs' population.

2.9.2.2 Procedure

Rested cells were first stained with 0.1 µl of blue viability dye (molecular probes invitrogen) and incubated in the dark for 10 minutes at room temperature. Thereafter, cells were washed with R10 and stained with 10 µl of class I HLA tetramer of interest and incubated in the dark for 30 minutes at room temperature. Wash step was repeated and cells were surface stained with 2 µl each of CD8-V500 (BD), CD3 AlexaFlour 647 (BD), CD4 AlexaFlour 700 (BD) antibodies and incubated in the dark for 20 minutes at room temperature. The cells were washed and suspended in the 200 µl of PBS for flow cytometry interrogation.

The stained cells were acquired on an LSR II (BD) and analysed using flowjo version 8.8.6. Cells were hierarchically gated on lymphocytes, dead cells and CD3+ T-cells, CD4+ T-cells and CTLs and the distinct tetramer-specific CTLs were gated from the CTLs population. Expanded specific CTLs were isolated by tetramer cell sorting. Prior to sorting of tetramer positive cells, cells were prepared by surface staining the expanded cells using 20 µl of class I HLA tetramer and incubated for 30 minutes at room temperature in the dark, followed by 2 µl each of CD8-V500 (BD), CD3 AlexaFlour 647 (BD), CD4 AlexaFlour 700 (BD) antibodies and incubated for 20 minutes at room temperature in the dark. Thereafter, HIV-specific CTLs were sorted on cell sorter (BD FACSAria, Germany).

GXR-VIA was setup to compare the inhibitory activity of the sorted CTLs targeting identical epitopes restricted by closely related class I HLA molecules. GXR cell lines expressing class I HLA molecules restricting identical epitopes were used as target cells in the assay. The different GXR cell lines were infected with the generated wildtype NL4-3 virus (objective 2), recombinant virus harbouring mutation of interest (objective 3). Sorted CTLs were co-cultured with the infected GXR cell lines and incubated at 37 ºC for 3 days. CTLs from healthy donor were also co-cultured with the infected and uninfected GXR cell lines as

controls in the experiment and incubated for 3 days at 37 ºC. The assay setup for objective 2 and 3 for each restricting cell line is illustrated in figure 13.

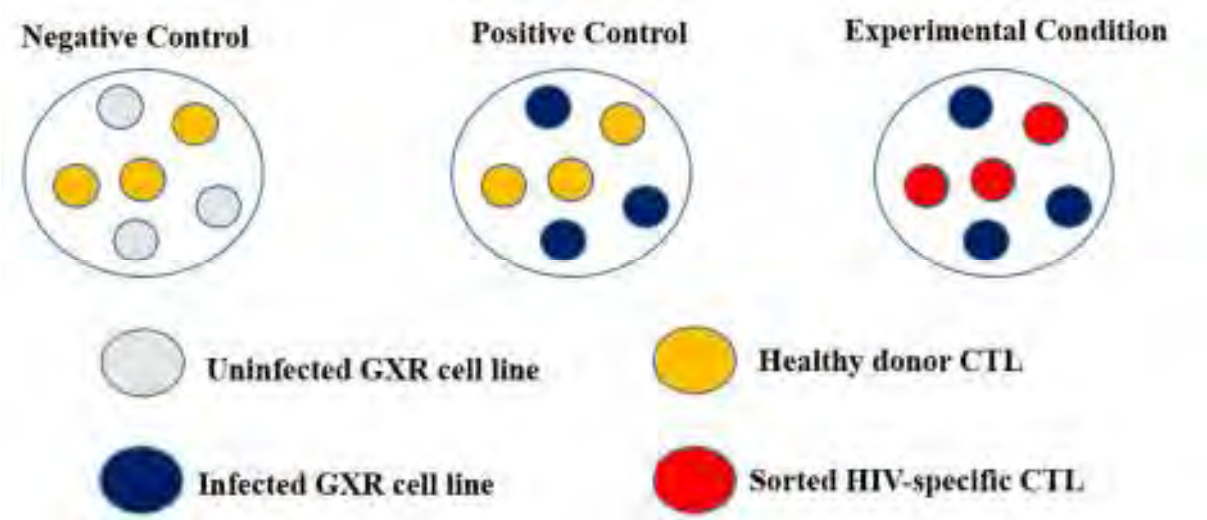

Figure 13: Experimental setup for objective 2 and 3. The diagram illustrate how GXR-VIA is setup to address objective 2 and 3. Infected and uninfected GXR cell line were co-cultured with CTLs isolated from healthy donor as control. Experimental condition includes infected GXR cell line with the sorted HIV-specific CTLs. This condition was setup side by side for the two closely related class I HLA alleles GXR-cell lines restricting identical epitopes under investigation with the tested HIV-specific CTL.

On day 3, cells population were harvested by spinning down the plate at 2,000 rpm for 6 minutes at 37 ºC. The pallets were fixed using 300 µl of paraformaldehyde and transferred into cluster tubes for flow cytometry interrogation. The inhibitory capacity of the HIV-specific CTLs recognizing identical epitopes restricted by one class I HLA molecule as opposed the closely related allele were estimated using the formula:

% inhibition = 100*[1-(% of Infected cells with HIV-specific CTLs / % of Infected cells with Healthy donor CTLs)]

2.10 Data Analysis and Statistical Considerations

All FCS data generated via flow cytometry were analysed using flowjo (version 9.0.2). All plotted graphs and statistical analysis were done using GraphPad Prism version 4.0. All tests were two-tailed, and P values of < 0.05 were considered significant.

CHAPTER THREE: Results

3.1 Validation of GXR-VIA with the Gold Standard ELISA-VIA

The viral inhibition assay (VIA) first described by (Bennett et al., 2008) has been shown to be a better option for identifying immune correlate of HIV-specific CTL. This assay involves infecting activated CD4+ T cells with HIV and co-culturing with CTLs. Thereafter, p24 antigen is measured over time using ELISA. ELISA-VIA has several advantages over ELISPOT and intracellular cytokine staining assays because it incorporates steps in viral entry, antigen processing and presentation and relies on the recognition of endogenously generated viral peptides within the target cells, as opposed to the loading of target cells with exogenous peptides at non-physiological concentrations. However, in its current format, the assay has several limitations including the fact that it is technically very challenging and labour intensive. ELISA-VIA requires separation of CD4+ T cells and CTLs and long term cells culturing. The autologous virus present in the target CD4+ T cells introduces biases to the p24 readout and the assay readout varies greatly because it is dependent on input virus strain and cellular activation. Furthermore, ELISA-VIA is very expensive.

Therefore, to overcome these limitations, we developed a GXR-VIA. The assay uses CEM-GFP reporter T-cell line (GXR-cell line) that has been genetically engineered to over express specific class I HLA molecule and expresses GFP upon HIV infection. The assay is less labour intensive and cheap to perform. We evaluated the performance of GXR-VIA compared to the gold standard ELISA-VIA and obtained comparable results.

3.2 GXR Cell Line Surface Staining of Class I HLA

To ensure the GXR cell line used in our study had comparable levels of class I HLA expression, we surface stained each cell line with pan-class I HLA-ABC (clone W6/32) antibody (BioLegend) before and after infection. All the GXR cell lines used expressed high levels (greater than 88%) of class I HLA molecules, (Figure 14).

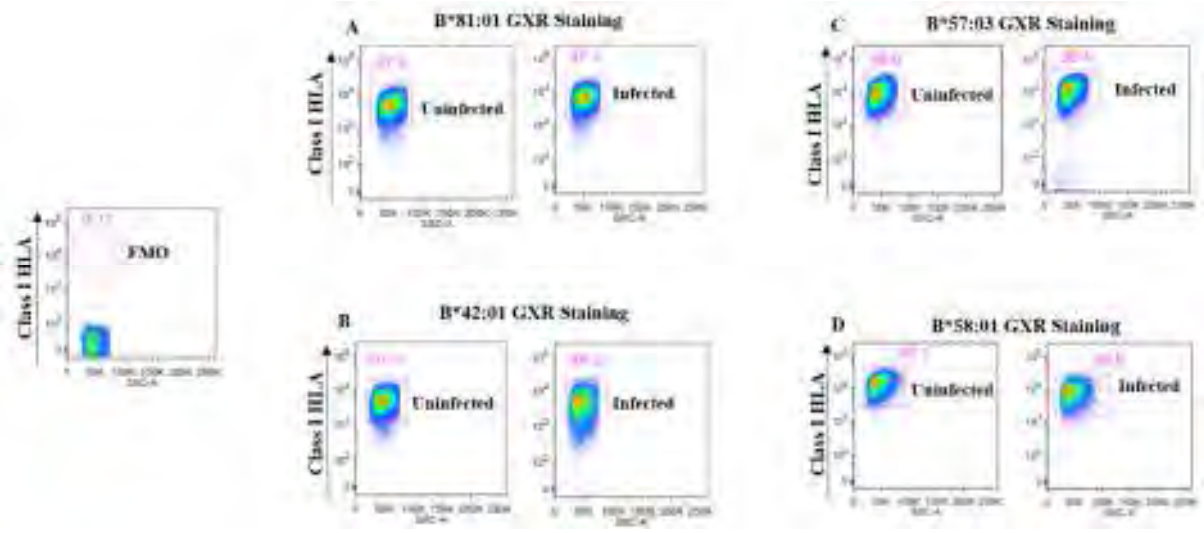

Figure 14: GXR Cell Lines Surface Staining for Class I HLA Molecules Expression. The flow data show the expression levels of class I HLA molecules before and after infection of different target GXR cell lines used in setting up GXR-VIA. (A) B*81:01 GXR cell line (B) B*42:01 GXR cell line (C) B*57:03 GXR cell line (D) B*58:01 GXR cell line.

3.3 GXR Cell Line Gating Strategy

The GXR cell lines used as target cells in the GXR-VIA express GFP upon HIV infection. Cells were gated on cell line, followed by cell line expressing GFP, (Figure 15). Reduction in the infected GXR cell line expressing GFP after co-culturing with CTL was used as readout to evaluate the CTL killing activity.

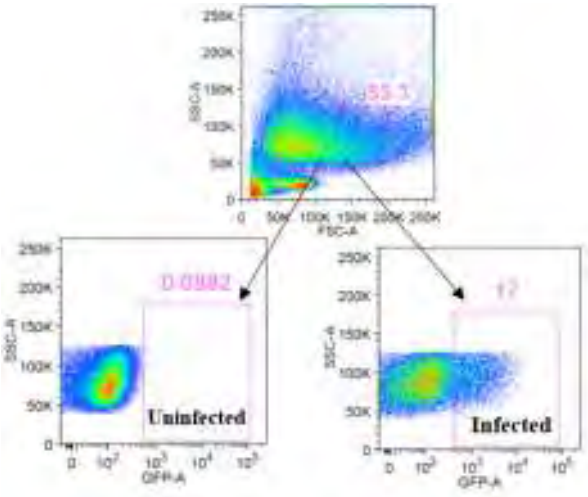

Figure 15: Gating Strategy. Cells were gated on cell line, followed by the infected cell line expressing GFP. The uninfected gate shows target population with less cells expressing GFP (0.0882) and the infected gate shows the infected target population with 17% of the cells expressing GFP.

3.4 Purity of CD8+ T Cells and CD4+ T Cells

Effector cells (CD8+ T cells) and target CD4+ T cells were isolated from the PBMC of 8 HIV infected subjects as described in the method section. The clinical characteristics of the assayed subjects are shown in Table 3. The percentage purity of the isolated CD8+ T cells and CD4+ T cells used to setup the two viral inhibition assays were routinely greater or equal to 93% and 78% respectively, (Figure 16).

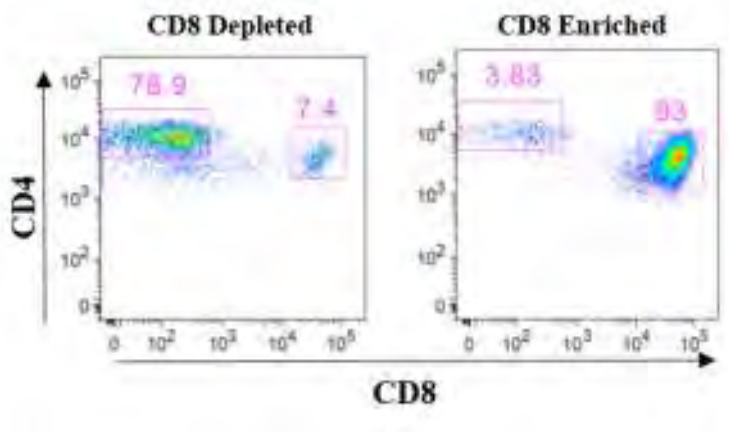

Figure 16: Purification of CD8+ T cells and CD4+ T cells. The diagrams show the percentage purity of effector and target cells routinely used in setting up the viral inhibition assays. The percentage purity of CD8+ T cells were routinely ≥ 93% and the percentage purity of CD4+ T cells used as target cells in ELISA-VIA were routinely ≥ 78%.

Table 3: Clinical Characteristics of the Subjects used to Validate GXR-VIA

Subject	CD4 count cells/µl	Viral Load copies/ml	Class I HLA-B		HAART
			B	B	
SK-199-B24	609	371	13:02	57:03	No
SK-318-B07	370	23600	07:02	57:03	No
SK-113-B16	351	<40	14:01	81:01	No
SK-358-B05	354	59200	08:01	57:03	No
SK-358-B10	200	7690	08:01	57:03	Yes
SK-081-B20	261	26727	13:02	81:01	No
SK-200-B02	567	14360	14:01	81:01	No
SK-421-B18	482	13356	15:03	58:01	No

3.5 Quantification of CD8+ T Cells Inhibitory Activity using GXR-VIA and ELISA-VIA

The GXR-VIA was compared with the gold standard ELISA-VIA. GXR-VIA and ELISA-VIA were concurrently setup using CD8+ T cells (CTLs) obtained from 8 HIV infected subjects, (Figure 17). GXR cell line and CD4+ T cells were respectively used as target cells in GXR-VIA and ELISA-VIA. CTLs' inhibition obtained using GXR-VIA and ELISA-VIA were converted to percentage inhibition and used in statistical analysis. The observed viral inhibitory results using GXR-VIA significantly correlated with the ELISA-VIA, (Figure 18). These data suggest that the GXR-VIA could be used to assess viral inhibitory of CTLs.

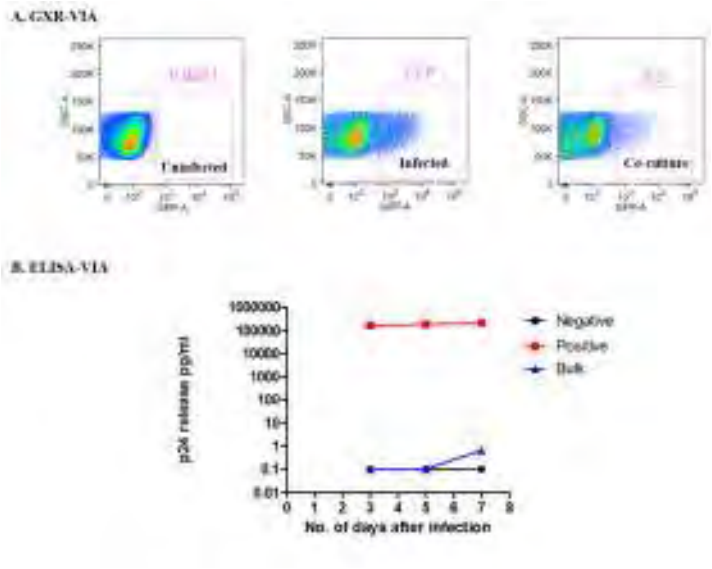

Figure 17: Representation of GXR-VIA and ELISA-VIA. The diagrams show the data for one of the assayed subjects: SK-200-B02. (A) GXR-VIA: Subjects' CTLs were able to inhibit viral replication by killing the infected target GXR cell line. The estimated inhibition value was 67.4%. (B) ELISA-VIA: The red line is showing the infected population (Positive control), the black line is the uninfected CD4+ T cells and the blue line showed the co-cultured population in which the estimated inhibition value was 67%.

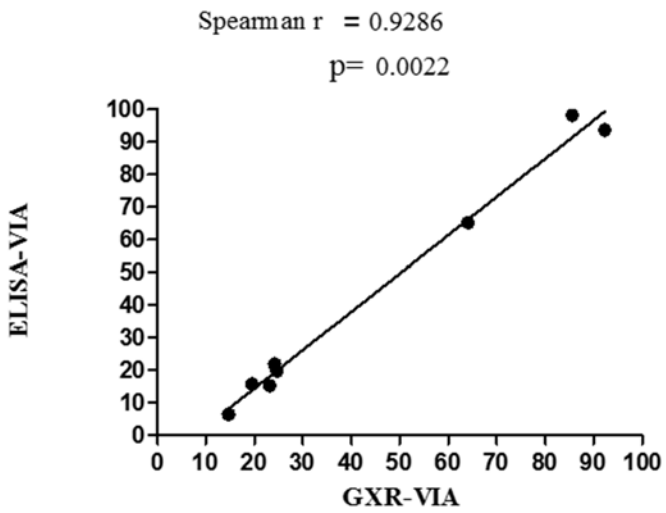

Figure 18: Correlation between GXR-VIA and ELISA-VIA. Significant correlation was observed when GXR-VIA and ELISA-VIA were concurrently setup using CTLs obtained from 8 HIV infected subjects. Spearman rank correlation was used to perform this analysis.

CHAPTER FOUR: Results

4.1 Comparative Analysis of Viral Inhibitory Activity of CTL Targeting Identical Epitopes Restricted by Different Class I HLA Molecules within the Same HLA Supertype

The diversity of HLA molecules is one of the major obstacles to the development CTL vaccine against HIV. Although studies have demonstrated that multiple class I HLA alleles can restrict identical epitopes due to the homology of amino acids within the major binding pockets of the peptide binding cleft (Sette and Sidney, 1999, Lund et al., 2004, Sidney et al., 2005, Sidney et al., 2008), it is not clear whether the identical epitopes restricted by closely related class I HLA alleles would induced functionally equivalent CTL responses. We therefore investigated the functional consequence of CTL targeting identical epitopes restricted by closely related class I HLA alleles using GXR-VIA.

We first focussed on the CTL targeting TL9 epitope restricted by class I HLA-B*81:01 or B*42:01 alleles within B7 supertype. TL9 Gag-epitope is an immunodominant epitope restricted by HLA-B*81:01, B*42:01 and B*0702 alleles (Leslie et al., 2006b). HLA-B alleles play a dominant role in the immune control of intracellular pathogens including HIV (Kiepiela et al., 2004b, Prugnolle et al., 2005), and B7 is the most prevalent HLA-B supertype (Sette and Sidney, 1999). HLA-B*81:01 and B*42:01 alleles are one of the most prevalent alleles within B7 supertype among the South Africans, worst affected with HIV pandemic (Kiepiela et al., 2004b, Leslie et al., 2006b).

We further investigated the inhibitory activity of CTL targeting identical IW9 Gag-epitope restricted by HLA-B*57:03 or B*58:01 within B57 supertype. We focussed on these alleles because studies have shown that individuals expressing HLA-B*57:03 or B*58:01 alleles mount an immunodominant responses to IW9 epitope during acute HIV infection (Altfeld et al., 2003, Altfeld et al., 2006, Crawford et al., 2009). HLA-B*57:03 and B*58:01 alleles are associated with slow progression to AIDS (Lazaryan et al., 2006, Kiepiela et al., 2007). In addition, HLA-B*57:03 and B*58:01 are prevalent among Sub-Saharan Africa population (Kiepiela et al., 2004b, Goulder and Walker, 2012, Prentice et al., 2013).

We first assessed the inhibitory activity of CTL targeting identical TL9 epitopes restricted by HLA-B*81:01 or B*42:01 alleles. We then investigated the functional consequence of CTL when *in-vivo* occurring mutant (Q182S)-TL9 epitope is restricted by HLA-B*81:01 or B*42:01 alleles. Lastly, we compared the Inhibitory activity of CTL targeting identical IW9 epitopes restricted by HLA-B*57:03 or B*58:01 alleles.

4.2 Inhibitory Activity of CTL Targeting TL9 Epitope Presented by HLA-B*81:01 or B*42:01 alleles

Four HIV infected subjects expressing B*81:01 and 4 subjects expressing B*42:01 were used to compare the inhibitory activity of CTL targeting TL9 epitope when presented by either HLA-B*81:01 or B*42:01 alleles. The clinical characteristics of these subjects are shown in table 4. TL9-specific CTL were assessed by tetramer staining. To increase the total number of TL9-specific CTL, PBMCs were cultured in the presence of TL9 peptide for 12 days as described in the method section. The specificity of the expanded cells was verified by tetramer staining, (Figure 19). The expanded TL9-specific CTL population was sorted using cell sorter (BD FACSAria, Germany).

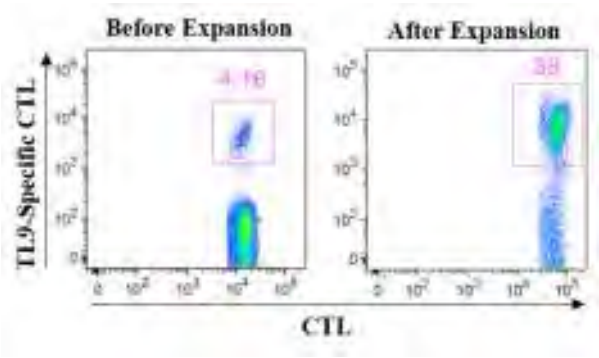

Figure 19: Expansion of TL9-specific CTL from one of the Studied Subjects. PBMCs were stimulated with exogenous TL9 peptide for 2 weeks to expand TL9-specific CTL. The percentages of TL9-specific CTL for this subject before and after expansion were 4.16% and 38% respectively.

Table 4: Clinical Characteristics of the B7 supertype Studied Subjects

Subjects	CD4 count cells/µl	Viral Load copies/ml	Class I HLA-B		HAART
			B	B	
SK-200-B02	567	14360	14:01	**81:01**	No
SK-316-B05	588	11200	82:02	**81:01**	No
SK-293-B01	624	188000	13:02	**81:01**	No
SK-416-B03	210	55200	15:18	**81:01**/81:02	No
SK-437-B08	337	64200	57:02	**42:01**	No
AS2-1034-B9	508	10874	45:01	**42:01**	No
AS2-0016-B13	457	14171	15:10	**42:01**	No
AS2-0483-B11	553	34671	15:03	**42:01**	No

The sorted TL9-specific CTLs were used as effector cells in the GXR-VIA, using GXR cell line overexpressing HLA-B*81:01 or B*42:01 alleles as target cells. Percentage killing when CTL was co-cultured with HLA-B*81:01 infected target cell line was compared with the percentage killing when CTL was co-cultured with B*42:01 infected target cell line, (Figure 20). Our results showed that CTL targeting TL9 epitope was able to inhibit viral replication when TL9 epitope was presented by B*81:01 or B*42:01 alleles. The percentage maximal inhibition at different target to effector ratios for one of the assayed subjects is shown in figure 21.

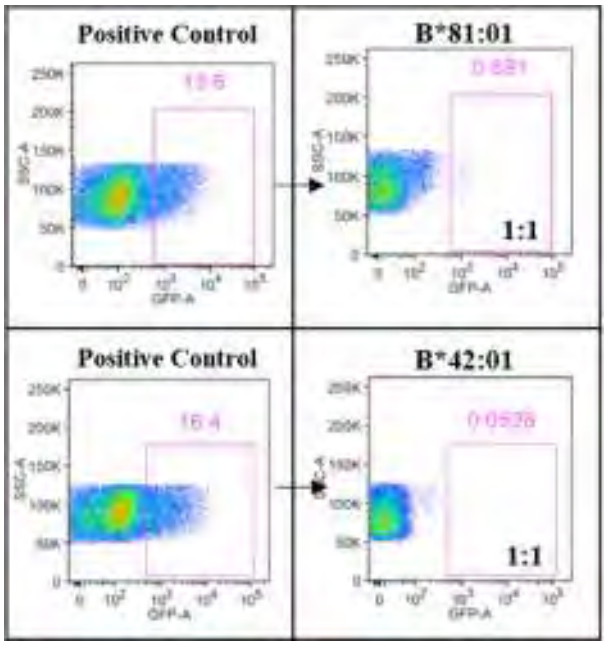

Figure 20: Inhibitory Activity of CTL Targeting TL9 epitope Presented by B*81:01 or B*42:01 Alleles. The diagrams show inhibition mediated by CTL from B*42:01 subject when TL9 epitope was presented by B*81:01 or B*42:01 alleles. The positive control consists of the infected target cell line with CTL from healthy donor, while the experimental condition consist of the infected target cell line with the tested TL9-specific CTL.

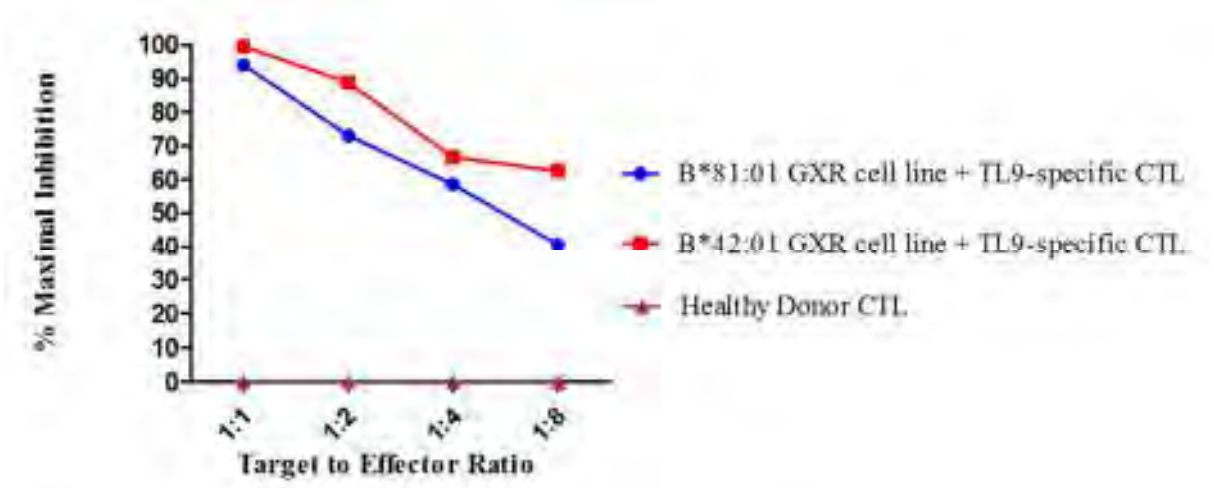

Figure 21: Representation of Maximal Inhibitory Activity of CTL Targeting TL9 Epitope at Different Target to Effector Ratios. Inhibitory activity of CTL from B*42:01 subject when presented with TL9 epitope by B*81:01 or B*42:01 alleles at different target to effector ratios is shown.

4.2.1 No Difference in the Inhibitory Activity of CTL Targeting Identical TL9 Epitopes Presented by B*81:01 or B*42:01 Alleles

Cytotoxic-T-Lymphocyte from the 8 subjects described in table 4.1 were assayed to assess the inhibitory activity of CTL when presented with identical TL9 epitopes by HLA-B*81:01 or B*42:01 alleles. Interestingly, analysis showed no difference in the inhibitory capacity of CTL targeting identical TL9 epitopes presented by HLA-B*81:01 or B*42:01 alleles at different target to effector ratios (Figure 22). In summary, these data suggest that TL9-specific CTL from B*81:01 and B*42:01 subjects are able to mediate viral inhibition when TL9 epitope is presented by either B*81:01 or B*42:01 alleles.

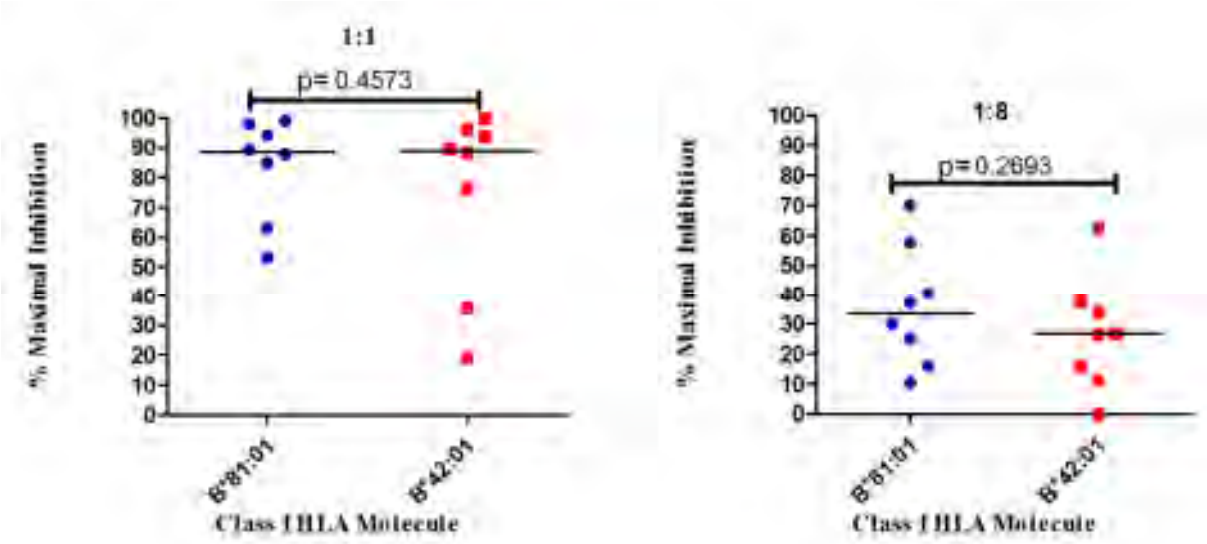

Figure 22: Comparison of the Inhibitory Capacity of CTL Targeting Wildtype TL9 epitopes Presented by B*81:01 or B*42:01 Alleles. CTL from 8 subjects described in table 4.1 were assessed for their inhibitory capacity when presented with TL9 epitope by B*81:01(Blue) or B*42:01(red) alleles. Inhibitory capacity of CTL is not statistically different when TL9 epitope is presented by B*81:01 or B*42:01 alleles. P-Values were estimated using paired t test.

4.3 Inhibitory Activity of TL9-specific CTL Presented with *in-vivo* occurring mutant (Q182S)-TL9 Epitope by HLA-B*81:01 or B*42:01 Alleles

Mutant (Q182S)-TL9 epitope is one of the variant epitopes that is frequently selected by subjects expressing HLA-B*81:01 or B*42:01 (Leslie et al., 2006b, Ntale et al., 2012). We therefore assessed the functional consequence of CTL when *in-vivo* occurring mutant (Q182S)-TL9 epitope is presented by HLA-B*81:01 or HLA-B*42:01 alleles.

We first generated recombinant virus harbouring mutation Q182S within TL9 epitope as described in the method section, and assessed its infectivity. To confirm the presence of the mutation in the generated virus stock, sequence was aligned with HXB2 reference sequence and WT-NL4-3 (Figure 23).

Target GXR cell lines were infected with the generated recombinant virus to measure the infectivity levels. Infectivity levels on day 3 and 5 were used to determine the relative percentages of maximal infection (Figure 24). Recombinant virus infectivity compared to the WT-NL4-3 virus was estimated for day 5. Calculated infectivity level of mutant Q182S recombinant was 49%, suggesting that recombinant virus harbouring Q182S mutation was able to infect target cells, although at lower level compare to un-mutated virus.

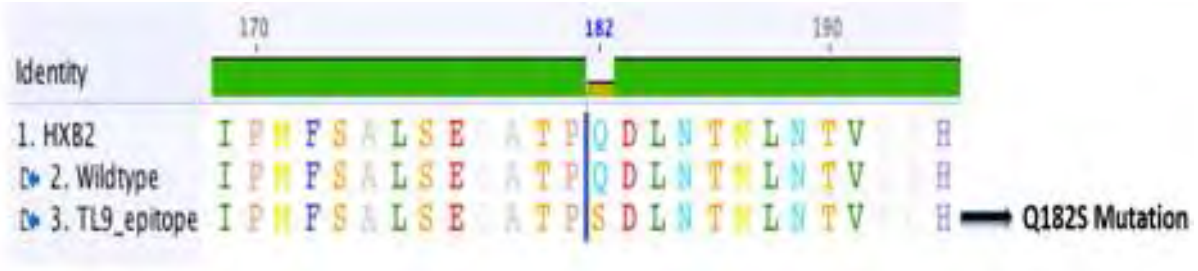

Figure 23: Sequence of Recombinant Virus Harbouring Q182S Mutation within the TL9 epitope. The sequence on row 3 shows change in the amino acid with reference to the HXB2 and WT-NL4-3 sequence. Glycine (Q) was changed to Serine (S) at position 182 within the TL9 epitope. Sequences were aligned using Geneious R8 version.

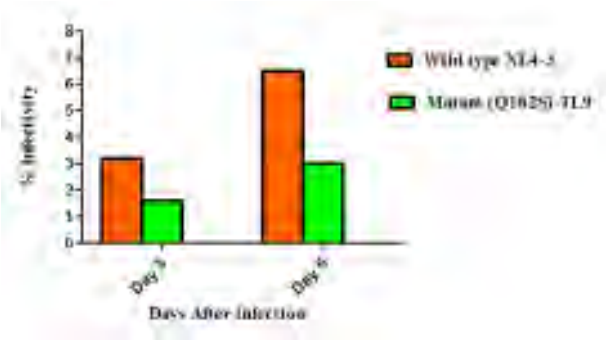

Figure 24: Percentage Infectivity of the Generated Recombinant virus as Compared to Wild Type (WT) NL4-3. Green bars are showing the infectivity levels of the recombinant virus stock harbouring Q182S mutation compared to WT-NL4-3 virus (red bars) for day 3 and 5 after infection.

4.3.1 No Difference was Observed in the Inhibitory Capacity of TL9-specific CTL Presented with *in-vivo* occurring mutant (Q182S)-TL9 Epitope by B*81:01 or B*42:01 Alleles

TL9-specific CTL from the 8 subjects described in table 4 were then investigated for their functional consequences when presented with mutant (Q182S)-TL9 epitope. Interestingly, our data showed that TL9-specific CTL were able to inhibit viral replication in both B*81:01 or B*42:01 infected target cell lines presenting mutant (Q182S)-TL9 epitope, (Figure 25). Estimated percentage maximal inhibition at different target to effector ratios for one of the subjects is shown in figure 26.

Statistical analysis showed no difference in viral inhibitory activity of TL9-specific CTL presented with mutant (Q182S)-TL9 epitope by B*81:01 or B*42:01 alleles, even at low target to effector ratio (Figure 27). These data suggest that TL9-specific CTL from B*81:01 and B*42:01 subjects are capable of recognizing mutant (Q182S)-TL9 epitope when presented by these two closely related alleles.

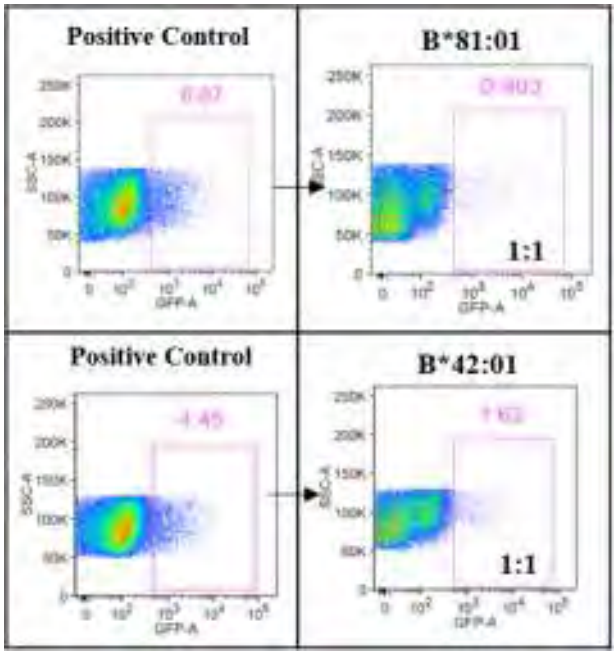

Figure 25: Inhibitory Activity of TL9-specifc CTL when Presented with mutant (Q182S)-TL9 Epitope by B*81:01 or B*42:01 Alleles. The flow data show TL9-specific CTL from B*42:01 subject mediating killing of infected target cells presenting mutant (Q182S)-TL9 epitope in the context of B*81:01 or B*42:01 alleles.

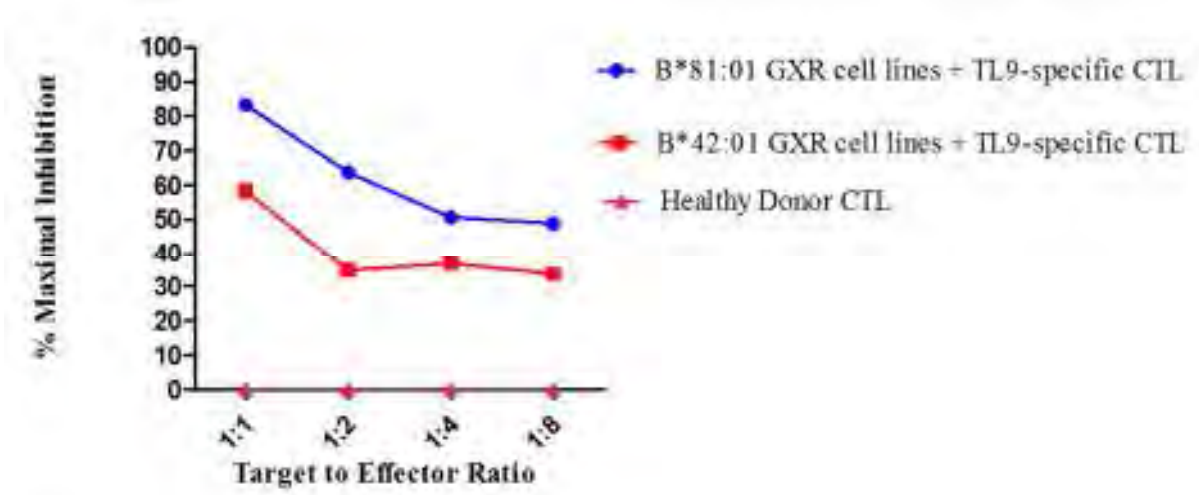

Figure 26: Maximal Inhibitory Activity of TL9-Specific CTL Presented with mutant (Q182S)-TL9 Epitope by B*81:01 or B*42:01 Alleles. Inhibitory activity at different target to effector ratios is shown for CTL obtained from HLA-B*42:01 subject.

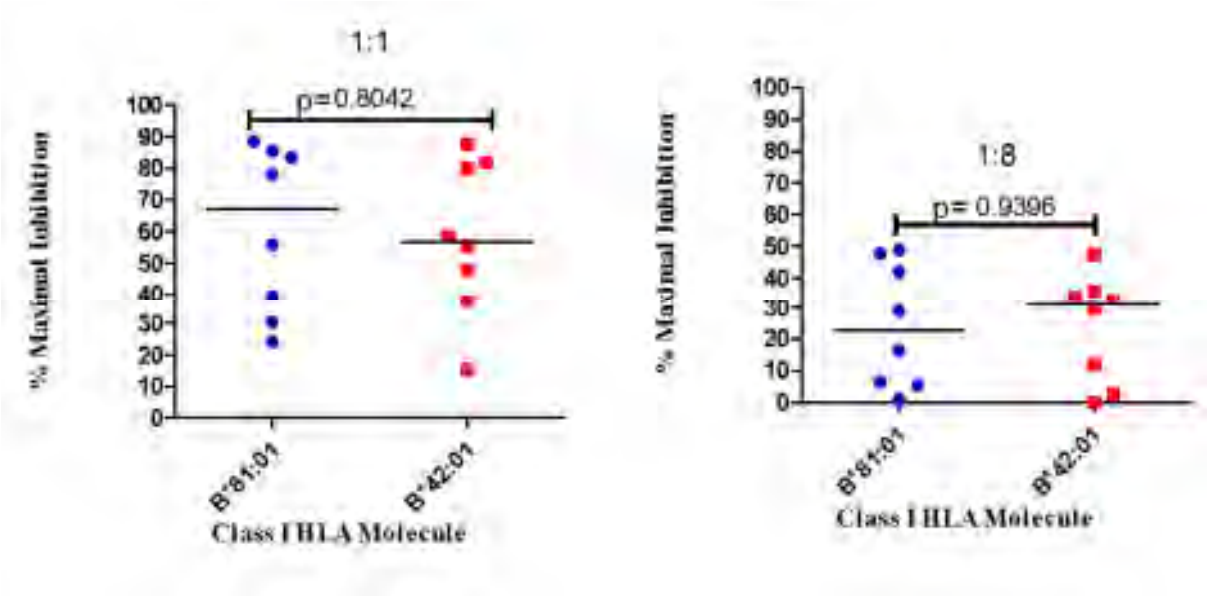

Figure 27: Comparison of the Inhibitory Activity of TL9-Specific CTL when mutant (Q182)-TL9 Epitope is Presented by B*81:01 or B*42:01 Alleles. TL9-specific CTL from the 8 subjects described in table 4.1 were assessed for their inhibitory capacity when presented with in-vivo occurring mutant (Q182S)-TL9 epitope. No difference was observed in the inhibitory capacity of the TL9-specific CTL presented with mutant (Q182S)-TL9 epitope presented by HLA-B*81:01 (blue) or B*42:01 (red) alleles at different target to effector ratios. All p-Values were estimated using paired t test.

4.4. Inhibitory Activity of CTL Targeting IW9 Epitope Presented by HLA-B*57:03 or B*58:01 Alleles

Reponses to p24 Gag IW9 epitope have been frequently associated with B*57:03 and B*58:01 alleles expressing subjects (Matthews et al., 2008). We compare the inhibitory activity of CTL when presented with IW9 epitope by B*57:03 or B*58:01 alleles. We were able to expand IW9-specific CTL from 4 HIV infected subjects expressing HLA-B*57:03 allele. The clinical characteristics of the 4 subjects are shown in table 5. We tested the inhibitory activity of IW9-specific CTL when the epitope was presented by HLA-B*57:03 or B*58:01 alleles.

Our data showed that B*57:03 CTLs targeting IW9 epitope presented by B*57:03 or B*58:01 alleles were able to inhibit viral replication in both target cell lines, (Figure 28). The percentage maximal inhibition at different target to effector ratios are shown in figure 29.

Table 5: Clinical Characteristics of the B57 Supertype assayed Subjects

Subjects	CD4 count cells/µl	Viral Load copies/ml	Class I HLA-B		HAART
			B	B	
SK-420-B11	455	688	15:03	**57:03**	No
AS2-0945-B4	912	1319	15:10	**57:03**	No
SK-215-B08	480	34800	0702	**57:03**	No
SK-355-B02	394	107000	15:03	57:01/**57:03**	No

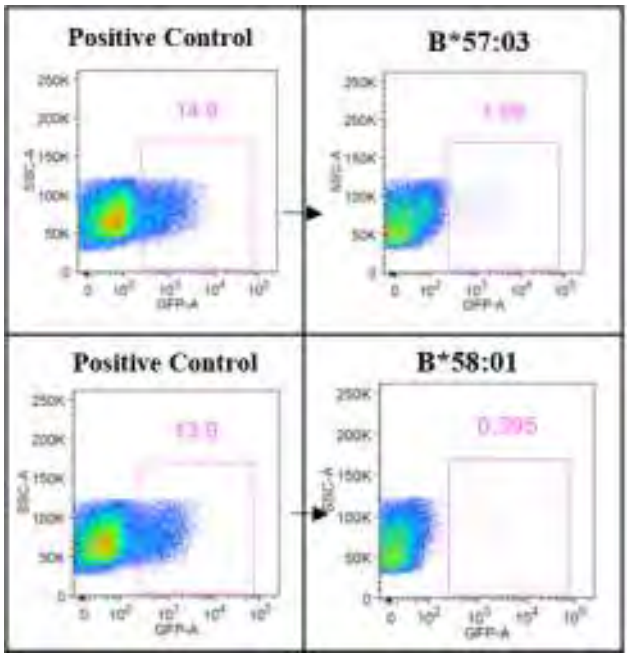

Figure 28: Inhibitory Activity of B*57:03 CTL Targeting IW9 Epitope. The diagrams show the inhibitory activity of one of the studied subjects' CTL presented with IW9 epitope by B*57:03 or B*58:01 alleles. B*57:03 CTL was able to recognise IW9 epitope presented in the context of B*57:03 or B*58:01 alleles and kill the infected cells expressing either of these alleles.

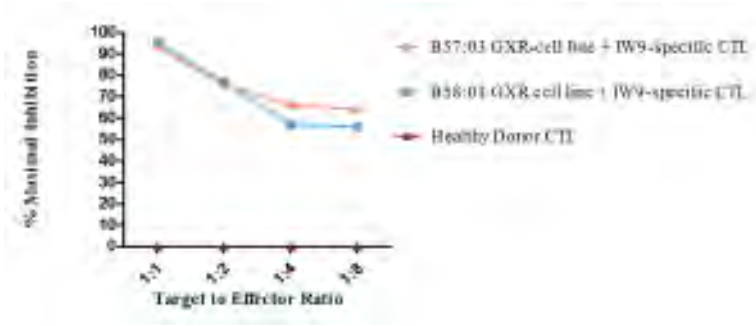

Figure 29: Maximal Inhibitory Activity of B*57:03 CTL Targeting IW9 Epitope. The Graph shows the Inhibition of B*57:03 CTL when B*57:03 or B*58:01 alleles presents IW9 epitope at different target to effector ratios.

Inhibitory activity of HLA-B*57:03 CTL was compared when B*57:03 or B*58:01 alleles present IW9 epitope. No difference was observed in the inhibitory activity of CTL targeting IW9 epitope presented by B*57:03 and B*58:01 alleles (Figure 30). Notably, a trend towards significance was observed at target to effector ratio 1:1. In summary, this data suggests that B*57:03 CTL can mediate inhibition of viral replication when IW9 epitope is presented in the context of B*57:03 or B*58:01 alleles.

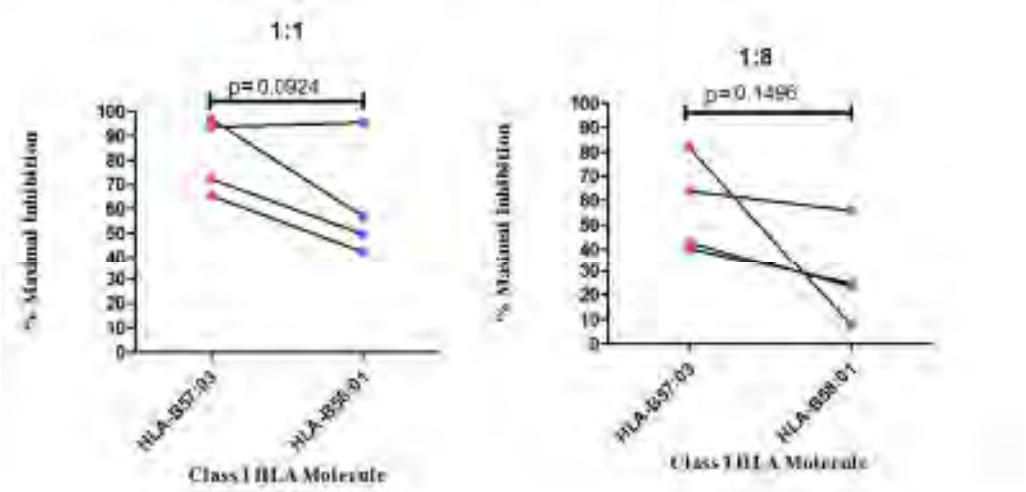

Figure 30: Comparison of the Inhibitory Activity of B*57:03 CTL Targeting IW9 Epitope Presented by HLA-B*57:03 or B*58:01 Alleles. No difference was observed in the inhibitory capacity of B*57:03 CTL targeting IW9 epitope presented by HLA-B*57:03 or B*58:01 alleles at different target to effector ratios. P-Values were estimated using paired t test.

CHAPTER FIVE: Discussion

Although several studies have provided evidences on the critical role of HIV-specific CTL in the immune containment of HIV infection (Walker and McMichael, 2012), developing a cross-reactive HIV CTL based vaccine remains an elusive goal. HIV-specific CTL exert potent antiviral effects that are mediated by two distinct mechanisms. The first requires direct contact of the CTL with infected target cells, resulting in cytolysis; the second is mediated by soluble inhibitory factors produced by CTL. Both processes require antigen-specific class I HLA restricted activation through the T-cell receptor (TCR) (Yang et al., 1997).

Class I HLA alleles have been shown to play a major role in immune mediated control of HIV infection (Goulder and Walker, 2012), but the extent of contributions of responses restricted by different class I alleles within a given individual has not been fully evaluated. Studies have also demonstrated that multiple class I HLA alleles can restrict identical epitopes (supertopes) due to the homology of the amino acids within the major peptide binding pockets of the peptide binding cleft (Sette and Sidney, 1999). However, it is not clear if supertopes presented in the context of closely related HLA alleles would induce functional equivalent CTL.

In this study, we investigated the inhibitory activity of CTL targeting identical epitopes presented by class I HLA alleles from the same supertype. We developed a new GXR-VIA that can assess the influence of class I HLA molecules on CTL inhibitory activity and evaluated the performance of the assay against the well-established ELISA-VIA. The GXR-VIA was used to evaluate the inhibitory capacity of CTL targeting identical epitopes restricted by closely related class I HLA alleles. We focussed on the two most common supertypes in Zulu/Xhosa ethnicity in KwaZulu-Natal, South Africa, namely; the B7 supertype (HLA-B*81:01 and B*42:01 alleles restricting TL9 epitope) and B57 supertype (HLA-B*57:03 and B*58:01 alleles restricting IW9 epitope). Furthermore, we assessed the functional consequence of mutant (Q182S)-TL9 epitope commonly selected by HIV infected individuals expressing B*42:01 allele (Leslie et al., 2006b) and B*81:01 allele (Ntale et al., 2012), on TL9-specific CTL.

GXR-VIA and ELISA-VIA were concurrently performed on 8 HIV infected subjects' CTL to assess their inhibitory activity. Our data showed no difference in the inhibitory activity of all the CTLs tested using GXR-VIA or ELISA-VIA. In addition, significant correlation was observed between GXR-VIA and ELISA-VIA. These data suggest that the GXR-VIA can reliably be used to measure viral inhibitory activity of CTL.

The unique benefit of GXR-VIA is its ability to evaluate the role of class I HLA restriction on CTL inhibitory activity. One notable advantage of the GXR-VIA over the ELISA-VIA is the relatively shorter assay duration of 3 days instead of 7 days for the ELISA-VIA, thus minimizing variations introduced by long-term culture, especially since multiple dilutions need to be tested. GXR-VIA is also significantly cheaper than ELISA-VIA.

The accurate assessment of the antiviral activity of HIV-specific CTL is a crucial step towards identifying the immune correlates of vaccine efficacy. It is possible that there is a hierarchy of efficacious HIV-specific CTL responses in individuals restricting identical epitopes (D'Souza and Altfeld, 2008).

Our data demonstrate no significant difference in the inhibitory activity of CTL targeting identical TL9 epitopes presented in the context of HLA-B*81:01 or HLA-B*42:01 alleles. The reasons behind this functional equivalence is not clear. Previous studies have identified TL9 epitope presented by closely related B*81:01 or B*42:01 alleles to be promiscuous (Leslie et al., 2006b), and the TCRs recognising promiscuous epitopes are highly cross reactive (Birnbaum et al., 2014), we can therefore speculate that there could be similarity in the biophysical properties of the TCRs of subjects with closely related HLA alleles. The ability of one TCR to bind multiple orientations of identical epitopes restricted by closely related alleles have also been proposed (Morris and Allen, 2012, Kloverpris et al., 2015). This could also be a contributing factor to the ability of CTL from B*81:01 or B*42:01 subjects to recognise TL9 epitope with comparable affinity.

Minor differences between closely related class I HLA alleles (micrpolymorphism), even of a single amino acid, can have profound impact on both T-cell immunity and disease outcome during a range of infections, including HIV (Gao et al., 2001b, Jin et al., 2002, Geldmacher et al., 2009, Goulder and Walker, 2012, Kloverpris et al., 2012a). This observation has been partly explained by selection of escape mutations that have different consequences on viral fitness (Matthews et al., 2008, Carlson et al., 2012, Kloverpris et al., 2012b). Interestingly, our data demonstrate that CTL from B*81:01 and B*42:01 background are able to inhibit HIV replication when promiscuous TL9 epitope is presented by closely related HLA-B*81:01 or B*42:01 alleles despite the potential differences between the closely related class I HLA molecule presenting promiscuous TL9 epitope. Overall, our data support the notion that promiscuous epitopes commonly restricted by closely related class I HLA alleles can be used to design supertype CTL based vaccine.

The CTL pressure has been associated with the appearance of escape mutations in HIV. Mechanisms of CTL escape fall into three categories; escape can affect epitope processing,

stability of peptides on class I HLA allele, or TCR recognition of the Peptide/HLA complex (Yokomaku et al., 2004). Of note, these categories are not mutually exclusive; for example, anchor residue mutations may alter the peptide secondary structure, affecting both HLA binding and TCR recognition (Reid et al., 1996). Mutation in residue Q182, located at the proximal to the promiscuous TL9 epitope binding, has been frequently identified as immune escape in the B*81:01 and B*42:01 positive individuals (Leslie et al., 2006b, Ntale et al., 2012). The implication of this mutation on the antiviral efficacy of CTL when TL9 epitope harbouring it is presented has not been fully evaluated.

We sought to determine the functional consequence of CTL when *in-vivo* occurring mutant (Q182S)-TL9 epitope is presented in the context of B*81:01 allele as opposed to B*42:01 allele. We first generated recombinant virus harbouring Q182S mutation within p24 Gag TL9 epitope and tested its infectivity relative to the wildtype NL4-3. In agreement with other studies (Friedrich et al., 2004, Peyerl et al., 2004, Matano et al., 2004, Fernandez et al., 2005, Martinez-Picado et al., 2006, Wright et al., 2010), we found that escape mutation at position Q182 within TL9 epitope has a minimal effect on the viral infectivity.

Next, we tested the functional consequence of mutant (Q182S)-TL9 epitope presented in the context of HLA-B*81:01 or B*42:01 alleles to TL9-specific CTL. Our data showed that the mutant (Q182)-TL9 epitope can be recognised by TL9-specific CTLs from B*81:01 and B*42:01 subjects when it is presented by either HLA-B*81:01 or B*42:01 alleles. Interestingly, the inhibitory activity observed when mutant (Q182)-TL9 epitope is presented in the context of HLA-B*81:01 allele is not different from the inhibitory activity observed when mutant (Q182)-TL9 epitope is presented in the context of HLA-B*42:01 allele. These data agree with the study that demonstrated the effectiveness of CTL to supress replication of HIV escape mutants (Pohlmeyer et al., 2013). Other studies have also proposed the flexibility of the CTL's TCR to recognizing mutant epitopes (Buseyne and Riviere, 2001, McKinney et al., 2004, Yu et al., 2005, Geldmacher et al., 2007). In contrast, some studies reported that substitutions in immunodominant epitopes might abrogate the ability of CTL to recognize mutant epitopes (Goulder et al., 1997, Iversen et al., 2006). Furthermore, variations of epitopes restricted by class I HLA allele are not efficiently cross recognized (Turnbull et al., 2006). Thus, our data suggest that TCR of TL9-specific CTLs from B*81:01 and B*42:01 subjects can cross recognize mutant (Q182)-TL9 epitope presented in the context of HLA-B*81:01 or B*42:01 alleles, thereby mediating inhibition of viral replication.

Growing numbers of studies suggest that CTL targeting Gag, particularly the HIV-p24 capsid protein play an important role in controlling viremia (Edwards et al., 2002, Zuniga et

al., 2006, Borghans et al., 2007, Kiepiela et al., 2007, Pereyra et al., 2008, Rolland et al., 2008). The closely related class I HLA-B*57:03 and B*58:01 alleles are notable for restricting immunodominant CTL responses that effectively supress HIV viremia and for being associated with improved long-term clinical outcomes of HIV infection (Kaslow et al., 1996, Migueles et al., 2000, O'Brien et al., 2001, Altfeld et al., 2003). Three of the epitopes that are most strongly targeted by HLA-B*57:03 and B*58:01 alleles are ISPRTLNAW (IW9; Gag 147 to 155) (Draenert et al., 2004a), KAFSPEVIPMF (KF11; Gag 162 to 172) (Crawford et al., 2007), and TSTLQEQIAW (TW10; Gag 240 to 249) (Leslie et al., 2004). IW9 is the well characterized p24 epitope frequently restricted early by HLA-B*57:03 and B*58:01 alleles (Matthews et al., 2008). Early CTL responses are thought to be more important for control of HIV infection than later CTL responses, both because they limit peak viremia and impose early fitness costs on the virus (Cao et al., 2003b, Streeck and Nixon, 2010).

We assessed the inhibitory capacity CTL targeting IW9 epitope presented in the context of HLA-B*57:03 or B*58:01 alleles. Surprisingly, we observed no statistical difference in the inhibitory capacity of IW9-spepcific CTLs from B*57:03 subjects when IW9 epitope is presented by B*57:03 or B*58:01 alleles.

Examinations of TL9 and IW9 supertopes presented by alleles within B7 and B57 supertypes respectively, as described above, suggest that these findings in which equivalence inhibitory activity of CTL targeting identical epitopes is observed, may be a general phenomenon for closely related class I HLA alleles.

Our study has notable limitations. Although the GXR cell lines we used as target cells have been genetically engineered to overexpress class I HLA of interest, the levels of expression of the specific class I HLA molecule in one target cell line as opposed the other target cell line is unknown and this could have introduced bias to the inhibition we observed in the context of one target cell line compared to the other target cell line. Samples size used in this study is low and this might have affected our data analysis. Another limitation is that we used expanded TL9-specific CTLs, this might have resulted in some non-specific inhibition that would have been considered as inhibition mediated by epitope presentation.

In conclusion, we have demonstrated that the inhibitory activity of CTL targeting identical TL9 epitopes presented by closely related B*81:01 or B*42:01 alleles is functionally equivalent. We have also demonstrated that TL9-specific CTL can mediate inhibition of viral replication when mutant (Q182S)-TL9 epitope is presented by B*81:01 or B*42:01 alleles. Furthermore, we have shown that IW9-specific CTL obtained from B*57:03 subjects can effectively inhibit viral replication when IW9 epitope is presented by B*57:03 or B*58:01

alleles. Thus, promiscuous TL9 and IW9 epitopes could be immunogenic. These findings are relevant for HIV vaccine approach that seeks to identify immunogenic epitopes that can be cross presented in broadly cross-reactive T cell based vaccine design.

CHAPTER SIX: References

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Appendix

Bovine Saline Albumin (BSA)

0.5g of BSA was added to 100 ml of distilled water.

Phosphate Buffer Saline (PBS)

One PBS table was dissolved per 100 ml distilled water. The solution was adjusted to a pH of 7.2. Thereafter it was autoclave for 10 min at 115 ºC.

Max Buffer

1 ml of BSA solution was added to 49 ml of PBS.

2% Paraformaldehyde (PFA)

10g PFA was added to 400 ml PBS (without Ca and Mg) and measured the pH to be between 7-7.4.

R10 Medium

To 500 ml RPMI (Sigma) add:
50 ml heat inactivated and filter sterilized fetal calf serum
5 ml L-glutamin
5 ml Penstrep Fungizone (100X)
5 ml Hepes
The medium was stored at 4 ºC with an expiry time of 14 days

R10/50

28 000 of interleukin-2 was added to 500 ml R10 medium to produce R10 medium containing 56 unit/ml of interleukin-2. The medium was stored at 4 ºC with an expiry time of 14 days.