UNIVERSITY OF KWAZULU-NATAL

Mapping immunodominant patterns and HLA class II restriction characteristics of HIV-specific CD4+ T cell responses in acute and chronic HIV-1 subtype C infection

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Abstract

Increasing evidence suggests that virus-specific CD4+ T cells contribute to immune-mediated control of HIV-1 infection. However, precise details of CD4+ T cell contribution to immune protection against HIV have not been adequately defined and most of the existing data was predominantly generated in clade B HIV-1 infection. Understanding the contribution of CD4+ T cell responses in clade C infection is important for developing vaccines that would be efficacious in sub-Saharan Africa which carries the highest burden of the HIV epidemic in the world. Therefore this study focused on the role of CD4+ T helper cells in the immune response to clade C HIV-1 infection. We tested the hypothesis that HIV-1-specific CD4+ T cell responses and protective class II HLA alleles are important determinants of effective immunological control of HIV-1 infection.

Firstly, CD8 depleted PBMCs were used in an IFN-γ ELISPOT assay to conduct a comprehensive analysis of virus-specific CD4+ T cell responses in acute and chronic HIV-1 clade C infection. Thereafter the host genetic effects of class II HLA-DRB1 alleles on HIV viremia were assessed using the HLA-DRB1 restriction assay, where HLA class II-restriction characteristics of detectable responses were defined. Lastly, functional differences of HIV-specific CD4+ T cells were further characterized using flow cytometric analysis.

In our study, Gag and Pol regions of the HIV proteome were found to be the most frequently targeted in acute HIV-1 infection (69% of total responses), with CD4+ T cell targeting across the proteome remaining relatively stable over time. In chronic HIV-1 clade C infection, dominant HIV-1-specific CD4+ T cell responses were detectable against a limited number of epitopes. Epitopes in the Gag region were the most targeted by CD4+ T cells (30/40 peptides), with OLP 41 in the Gag p24 region being the most dominant epitope targeted (15% of responses). There were no significant differences observed between total or Gag-specific

CD4+ T cell responses and contemporaneous viral load. Interestingly, responses rarely targeted the envelope region in clade C infection, in contrast to multiple epitopes targeted in this protein in previous clade B studies. Functional analysis demonstrated that IFN-γ, IL-2 and TNFα were the most secreted cytokines by HIV-specific CD4+ T cells in 18/25 individuals, with IFN-γ being the most dominant response in individual subjects. The HLA class II DRB1 restriction in clade C HIV infected individuals showed epitope promiscuity, consistent with previous studies in clade B infection. The HLA-DRB1*13:01 allele variant was associated with the highest frequency of responders (22%) in our cohort and restricted the highest number of HIV-specific peptides (9/15).

Together, our data identify immunodominant regions of HIV-specific CD4+ T cell responses and their association with viral control during clade C infection. Furthermore, our findings will inform studies aimed at elucidating the underlying mechanism by which CD4+ T cells modulate effective CD8+ T cell and B cell responses. Additionally, these data suggest that epitope promiscuity among class II HLA molecules should be taken into account for vaccines designed to induce CD4+ T cell responses. This information will be critical to vaccine efforts designed to induce these responses, as well as potential therapeutic manipulation of immunity in persons with acute and chronic HIV-1 infection.

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Research Output

Conference attendance

- Awarded a partial scholarship based on abstract merit to present a poster at the **Gordon Research Seminar and Conference in Immunochemistry and Immunobiology** between 31 May - 6 June 2014 in Newry, Maine, United States of America

- Awarded a full scholarship based on abstract merit to present both an oral and poster at the **5th Infectious Diseases in Africa Symposium and Primer Course** from 21-26 October 2014 in Cape Town, South Africa.

- Awarded a partial scholarship based on abstract merit to present a poster at the **HIV Research for Prevention conference** between 27- 31 October 2014 in Cape Town, South Africa.

- Awarded the Bill and Melinda Gates Foundation Global Health Travel Award to attend the **HIV Vaccines Keystone Symposium** from 22 - 27 March 2015 in Banff, Alberta, Canada

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Most importantly, God, for never letting me down...

Abbreviations

APC	Antigen Presenting Cell
ART	Antiretroviral Therapy
BCIP	5-Bromo-4-Chloro-3'Indolyphophate p- Toluidine Salt
BSA	Bovine Serum Albumin
CA	Capsid (p24)
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
CMV	Cytomegalovirus
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

GIT	Gastrointestinal Tract
HAART	Highly Active Antiretroviral Treatment
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
ICS	Intracellular Cytokine Staining
Ig	Immunoglobulin
IFN-γ	Interferon gamma
kDa	Kilodalton
MA	Matrix (p17)
MAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
mRNA	Messenger Ribonucleic Acid
NBT	Nitro-Blue Tetrazolium Chloride
Nef	Negative regulatory factor
NC	Nucleocapsid (p7)
OLP	Overlapping Peptide
ORF	Open Reading Frame
P24	P24 Capsid Protein
PBS	Phosphate Buffered Saline
PBMC	Peripheral Blood Mononuclear Cells
PHA	Phytohemagglutinin
Pol	Polymerase
PVDF	Polyvinylidene Fluoride

Rev	Regulator of virion expression protein
RNA	Ribonucleic Acid
SFC	Spot Forming Cells
SIV	Simian Immunodeficiency Virus
Tat	Transactivator of transcription
TCR	T-cell Receptor
tRNA	Transfer Ribonucleic Acid
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U

List of Tables

List of figures

Table of Contents

CHAPTER 1: LITERATURE REVIEW

1.1 THE HIV-1 EPIDEMIC

The Human Immunodeficiency Virus (HIV), discovered in 1983, is the pathogen responsible for Acquired Immune Deficiency Syndrome (AIDS), which has fast established itself as a global pandemic (BarreSinoussi, 1996). The first clinical observance of AIDS was made in 1981 in the United States amongst injecting drug users and homosexual men with no known cause of weakened immunity. They presented with symptoms of *Pneumocystis* pneumonia and Kaposi's sarcoma, with both diseases previously being rare infections occurring only in people with very compromised immune systems (Barre-Sinoussi et al., 2013, Urmacher et al., 1982, Janier et al., 1984, Gottlieb et al., 1981). There was no official name for the disease with various terms being coined, one of which being GRID, which stood for gay-related immune deficiency. It soon became apparent that this disease was not limited to specific population groups and in 1982 the term AIDS was introduced. A lentivirus with close relation to the human T-lymphotropic virus (HTLV) was identified as the aetiological agent of the disease and later named HIV (Montagnier, 2002). AIDS is characterized by a conspicuous reduction of CD4+ T cells and ultimately a failure of the immune system to contain virus replication, leading to increased susceptibility to opportunistic infections and finally death (Fauci, 2008).

1.2 PREVALENCE

HIV continues to be a major global public health issue, having claimed more than 39 million lives so far. According to the World Health Organization, an estimated 35 million people globally were living with HIV-infection at the end of 2013, sub-Saharan Africa being the most affected region, with 24.7 million people living with HIV in 2013. In that same year, 2.1 million people became newly infected with the virus and 1.5 million people died from HIV-

related causes globally (UNAIDS, 2012). Currently there is no cure for HIV-1 infection; however, effective treatment with antiretroviral therapy (ART) can control the virus to enable people living with HIV to enjoy healthier and more productive lives. In 2013, 12.9 million people living with HIV were receiving ART globally, many of whom were from low to middle income countries. Access to treatment is still lower in children, where 1 in 4 children living with HIV have access to treatment as compared to 1 in 3 adults (UNAIDS, 2012). Arguably, prophylactic vaccination will be the most effective way of controlling the epidemic because of the cost and complexity of other preventative methods available, and because other currently available methods only slow down the replication or spread of the virus, while failing to clear it from the body and completely stop the spread to other people.

1.3 HIV GENETIC DIVERSITY

One of the major challenges for developing an efficacious HIV vaccine is the intrinsic diversity among circulating populations of HIV-1 in various geographical locations and the need to develop vaccines that can elicit enduring protective immunity across different strains. Varied levels of sequence diversity of the viral genome across all regions have been found through molecular analysis of HIV isolates. Sequence diversity of HIV can occur in numerous ways, these include, recombination, insertions and deletions, gain or loss of glycosylation sites and simple base substitutions (Fauci, 2008). There are three major groups of HIV-1 which represent separate introductions of the virus into humans from simians, with a fourth one being proposed recently (Van Heuverswyn and Peeters, 2007). The groups are M (main), N(new) and O (outlier) and P. Group M, responsible for most of the infections in the world comprises of nine subtypes, which are labelled as A, B, C, D, F, G, H, J and K , as well as an increasing number of both major and minor circulating recombinant forms (CRFs), with the strain previously referred to as subtype E, being the most common (Van Heuverswyn and Peeters, 2007) (Figure 1.1). CRFs are formed through a recombination and

creation of a virus with selective advantage by an individual infected with two subtypes. Subtype A and F of Group M have been further differentiated into sub-subtypes A1 and A2, and F1 and F2, respectively. *Pan troglodytes troglodytes,* is the chimpanzee subspecies identified as the natural reservoir of the M and N groups of HIV-1 (Julg et al., 2010). The HIV-1 O group, a fairly rare form of the virus originating in France, Gabon and Cameroon is shown to be most closely related to viruses found in Cameroonian gorillas.

Subtype C viruses account for more than 50% of infections globally, with majority of infections caused by subtype C being in sub-Saharan Africa, which carries the highest burden of two-thirds of all individuals living with the disease (Maenetje et al., 2010, Hemelaar, 2012).

The astonishing diversity of HIV, which is observed by the existence of numerous subtypes, circulating recombinant forms and the continuous evolution of the virus, poses a formidable hindrance to HIV vaccine development, where varied rates of disease progression, resistance to ART and overall responses to therapy compound the problems.

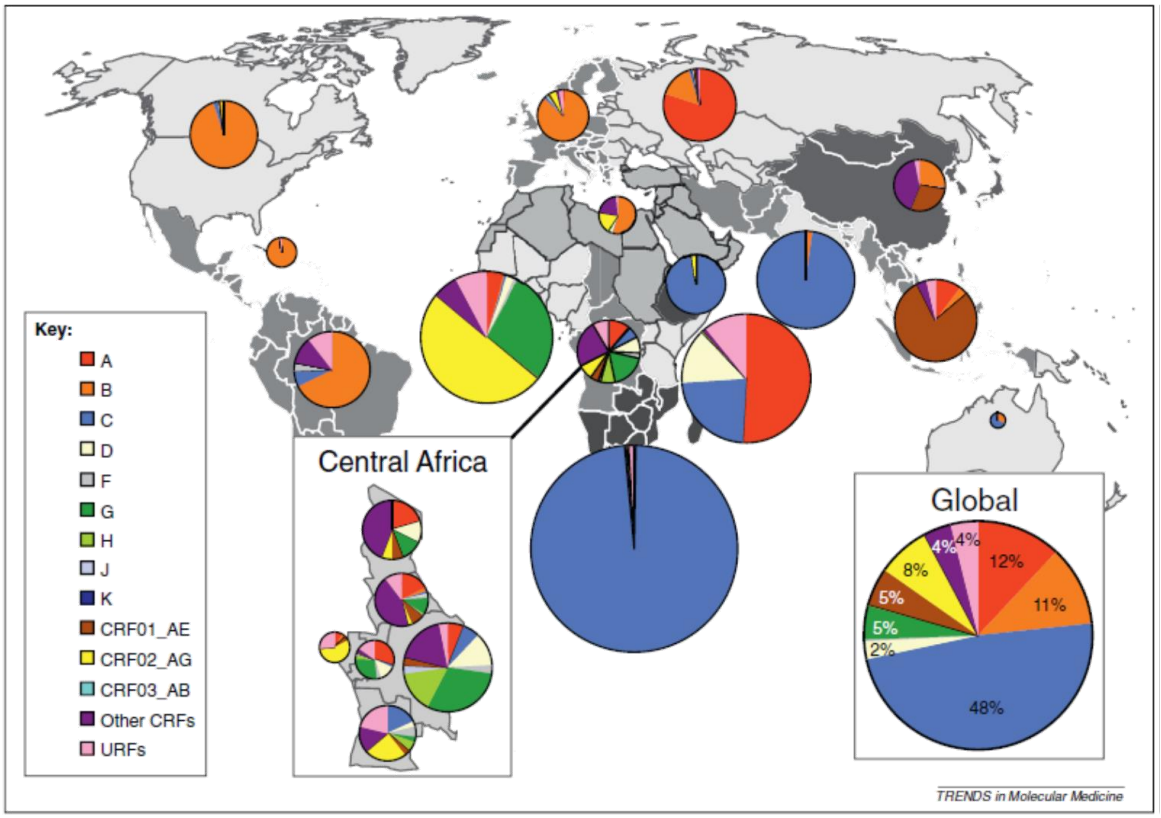


Figure 1.1. Worldwide distribution and genetic diversity of the major clades and recombinants of HIV-1. (Replicated from(Hemelaar, 2012))

1.4 MOLECULAR BIOLOGY OF HIV-1

1.4.1 STRUCTURE AND ORGANIZATION OF THE HIV-1 GENOME

HIV is an enveloped, single stranded RNA virus belonging to the genus *Lentivirus* of the *Retroviridae* family (BarreSinoussi, 1996). The HIV virion is spherical in shape, with a diameter of 1/10,000 of a millimetre. HIV, like other retroviruses contains a viral capsid, which includes the major capsid protein, Gag p24;the nucleocapsid protein p7/p9, the diploid single-stranded RNA genome, and the viral enzymes protease, reverse transcriptase, and integrase (Lever, 2009) (Figure 1.2). The viral capsid can be broken into three parts, viz. the viral envelope, the core and the matrix protein that lies between the two aforementioned

parts. The viral envelope is a phospholipid bilayer, which is derived from host cell membrane, which includes virally encoded membrane proteins (BarreSinoussi, 1996).

Approximately 72 copies of envelope glycoprotein complex form the spikes on the virus' surface (Gelderblom, 1991). Surface protein, gp 120 makes up the cap of the spike, while transmembrane protein, gp 41 makes up the stem of each spike. The layer below the viral envelope is known as the matrix, which is made up from protein Gag p17. The viral core, made up of Gag p24 is found beneath the matrix, and usually holds viral genetic material, consisting of two identical strands of RNA (Freed, 1998).

The entire retroviral HIV genome, encoded by approximately 9-kb RNA, consists of nine genes flanked by long terminal repeat sequences (LTRs) (Frankel and Young, 1998) (Figure 1.3). The genome consists of three classical retrovirus open reading frames (ORF) that encode the Gag (group-specific antigen), Pol (polymerase) and Env (envelope) polyproteins, which are then processed into individual proteins. In addition to these three retroviral genes, the HIV-1 genome contains genes encoding for six regulatory or accessory proteins i.e. Nef, Rev, Vif, Tat, Vpu and Vpr. These accessory proteins are particularly important for viral pathogenesis *in vivo* (Lever, 2009, Frankel and Young, 1998).

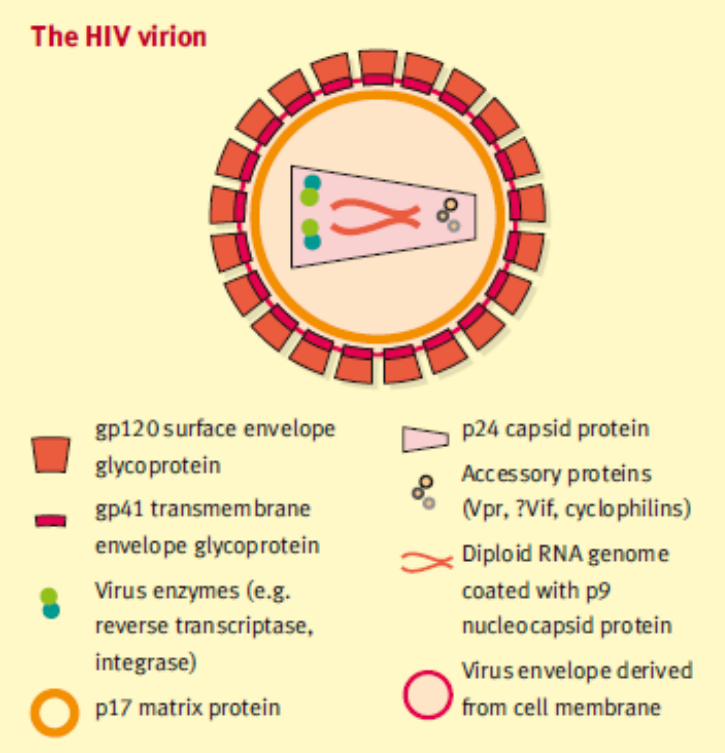


Figure 1.2. The structure of the HIV virion (Reproduced from (Lever, 2009))

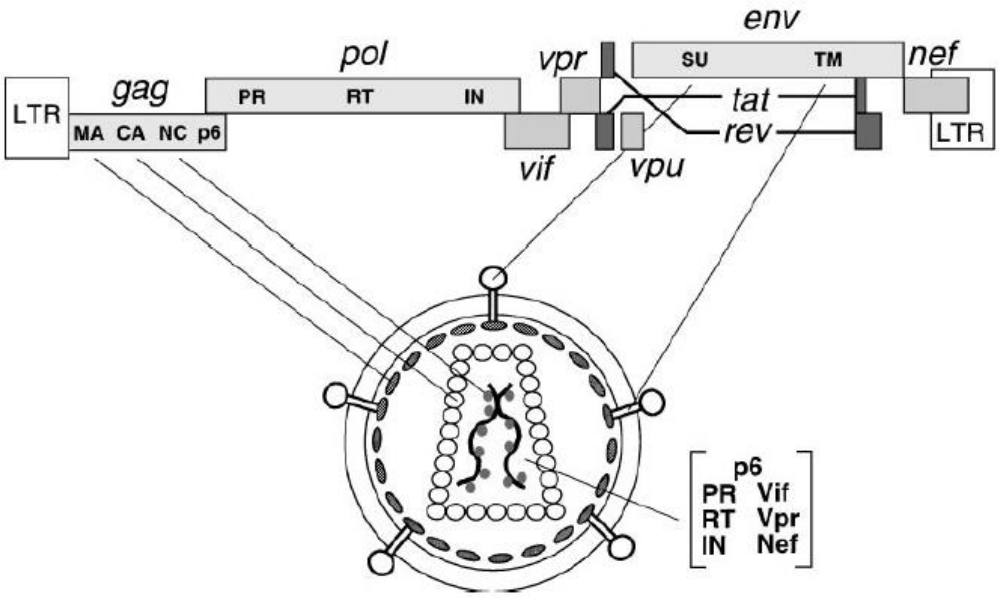


Figure 1.3.Organization of the HIV-1 genome and virion (Frankel and Young, 1998)

1.4.2 HIV GENE PRODUCTS

1.4.2.1 GAG

The *gag* gene encodes a $Pr55^{Gag}$, a polyprotein precursor which is cleaved by the viral protease to produce four Gag proteins viz. matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p7) and p6. The precursor protein, Pr55, is integrated into the budding virus, with cleavage occurring both during and after budding (Lever, 2009, Reicin et al., 1996). The matrix protein, a 132 amino acid protein lining the inner surface of the virion membrane envelope, serves a primary function of targeting Gag and Gag-Pol polypeptides to the membrane preceding viral assembly. Furthermore, the p17 matrix protein is important for anchoring the gp41 transmembrane protein (TM). The p24 capsid is a 24 kDa structural protein that condenses to form the conical capsid core of the virus particle, which encloses the viral genomic RNA. The p9 nucleocapsid is a 55 amino acid protein, forming a coat around the RNA genome in the virus particle, delivering the RNA into the assembled virion. It is also involved in additional functions, including reverse transcription through improving the binding of the transfer RNA (tRNA) to the viral primer binding site(Frankel and Young, 1998). Lastly, the p6, a 51 amino acid protein found at the C-terminal end of the Gag polyprotein, is known to enable budding of the virus from infected cells and further maturation of the viral progeny (Schubert et al., 2000). The p6 is also known to bind to the Vpr protein to incorporate it into the virus (Lever, 2009, Selig et al., 1999, Kondo and Gottlinger, 1996).

1.4.2.2 POL

The *pol* ORF encodes for the three viral enzymes, reverse transcriptase (RT), protease (PR) and integrase (IN)(Kaplan et al., 1994). The three Pol proteins need to come together as dimers (PR), heterodimers (RT) or tetramers (IN) to become functionally active in their

enzymatic activity. Initially, the Pol protein is formed as an extension of Gag by frame-shifting to produce the Pr160 Gag-Pol fusion polyprotein, which is integrated in the virus particle. This polyprotein is cleaved to produce the various enzymatic components of the virus. The functions of the components include cleaving Gag proteins within the particle (protease) and inserting dsDNA into target cell chromosomes (integrase and reverse transcriptase) (Lever, 2009). PR is one of the prime targets for drug design, where some of the current drugs used in HIV-1 treatment target the active cleavage site of the protease enzyme to inhibit the activity of the protease enzymes and therefore prevent viral reproduction, thus resulting in a significant reduction in plasma viral load(Frankel and Young, 1998).

1.4.2.3 ENV

The envelope protein is a 160 kDa precursor glycoprotein (gp160), cleaved by cellular proteases, unlike Gag and Pol which are cleaved by viral proteases, to form the gp120 surface protein (SU) and the gp41 transmembrane protein (TM) (Tagliamonte et al., 2011).These proteins make up the outer membrane of envelope as well as the core of the virion, and are critical for HIV binding to their host cell receptors and are important for the ensuing cell entry. TM plays the role of an anchor of the envelope glycoprotein complex into the cell membrane and next into the virus envelope. SU, attached to TM by non-covalent bonds, bears the detection site for the cell surface receptor proteins by which HIV attaches to and enters the cell (Tagliamonte et al., 2011). The Env glycoprotein is able to evade antibody neutralization through its extremely variable amino acid sequences and glycosylation (Lever, 2009).

1.4.2.4 NEF

HIV-1 Nef is a 206 amino acid myristoylated protein of 27-35 kDa in size that is produced in large quantities early in the HIV life cycle. It is one of the first viral proteins that is expressed

following infection and has been shown to act as an important virulence factor in HIV pathogenesis *in vivo* (Lever, 2009). Despite being initially identified as a 3' ORF that negatively influences viral replication thus termed "Negative factor", abbreviated to Nef, studies have shown that this protein actually acts as an HIV infectivity stimulant and leads to viral replication (Malim and Emerman, 2008, Chengmayer et al., 1989).The role of Nef as a critical determinant of pathogenicity has been established through observed evidence of long-term survival in humans and rhesus macaques infected with HIV-1 or SIV (simian immunodeficiency virus) strains respectively that lack intact *nef* genes (Deacon et al., 1995, Kirchhoff et al., 1995). The Nef protein has been shown to down-regulate the expression of CD4+, major histocompatibility complex (MHC) class I proteins and the interleukin-2 (IL2) receptor from the surface of infected cells (Lever, 2009). Thus, causing a disruption in the typical cell to cell interactions in immune responses. Nef in SIV causes a down-regulation of the T cell receptor on lymphocytes, therefore resulting in a reduction of cell activation. This process is absent in HIV Nef with the risk of activation-induced cell death being seen as an important pathogenic difference between the SIV and HIV (Lever, 2009). Further functions of Nef include the ability to alter cellular activation pathways and induce apoptosis and the ability to enhance virion infectivity.

Lastly, Nef seemingly is involved in the recruitment of lymphocytes to infected macrophages, thus increasing spread of the virus. Importantly, the endocytosis of CD4+ T cells, the primary receptor of the virus from the surface of infected cells are accelerated in response to the Nef protein (Lever, 2009).

1.4.2.5 VPU

Vpu is another accessory protein employed by HIV-1 to reduce surface expression of the CD4 receptor on T cells. Vpu, a 16kDa, 81 amino acid long dimeric integral membrane protein acts similarly to Nef, greatly influencing dissemination, replication and persistence of

the virus. It is exclusively found in infected target cells and expressed ‘late’ in the viral life cycle. It augments virus export from the cell, reducing the formation of a complex of gp120 being produced within the CD4 glycoprotein and the infected cell, essentially, assisting the virus escape the cell during budding by weakening the interaction of the new envelope proteins with cell receptors. In recent times it has been demonstrated that Vpu plays an important role in counteracting the inhibitory effects of tetherin, a cellular protein, on virus budding (Figure 1.4) (Lever, 2009, Malim and Emerman, 2008).

1.4.2.6 TAT

Tat is a 23-kDa regulatory protein of 86-101 amino acids in length, appropriately named for its primary function as a trans-activator of transcription from the HIV-1 long terminal repeat (LTR) promoter. It binds to the "Tat-responsive region" (TAR) in the newly transcribed virus mRNA. The binding process is initiated in order to form complexes with cellular transcription factors and together initiate transcription and elongation by RNA polymerase (Sodroski et al., 1985). This interaction greatly increases cellular RNA polymerase II activity, enhancing the generation of viral mRNA and essentially amplifying the amount of viral protein.

1.4.2.7 VIF

Vif is a 23-kDa accessory protein standing for 'viral infectivity factor'. Recent studies have shown that Vif interacts with and promotes the degradation of APOBEC-3G, which is a cellular cytidine deaminase that would usually attack viral genetic material (Figure 1.4). The human protein ABOBEC-3G and APOBEC-3F are loaded into virions in the absence of Vif, where they cause hypermutation of the HIV-1 genome following infection. Development of therapeutic interventions that restore the function of APOBEC could be an important consideration of future therapeutic strategies (Malim and Emerman, 2008).

1.4.2.8 VPR

Viral protein R (Vpr) is a 14 kDa size and 96 amino acid long accessory protein which has been shown to promote cell cycle arrest via a ubiquitin ligase mechanism and furthermore is thought to influence the expression of receptors for natural killer (NK) cells on infected cells. Vpr is involved in the process of transporting the "uncoated" virus to the host cell nucleus, following fusion and entry (Romani and Engelbrecht, 2009).

Other primate lentiviruses, i.e. HIV-2 and simian immunodeficiency virus (SIV) possess an additional protein, Vpx (Puigdomenech et al., 2013). Vpx has been shown to aid in replication of HIV-2 and some SIV in myeloid cells, triggering the destruction of an early-acting restriction factor and promoting synthesis of viral DNA in non-dividing cells. The restriction factor is SAMHD1, a deoxynucleoside triphosphohydrolase that cleaves deoxynucleotide triphosphates (dNTPs). SAMHD1 is not only active against HIV-2 and SIV but also HIV-1 that lacks Vpx. SAMHD1 has been demonstrated to negatively regulate virus-induced immune responses in monocyte-derived dendritic cells (MDDCs), where silencing of SAMHD1 promoted HIV-1 recognition (Ryoo et al., 2014, Yang and Greene, 2014). SAMHD controls the sensitivity of MDDCs to HIV-1 infection during intracellular contact, thus affecting their ability to detect the virus and exhibit the appropriate innate immune response.

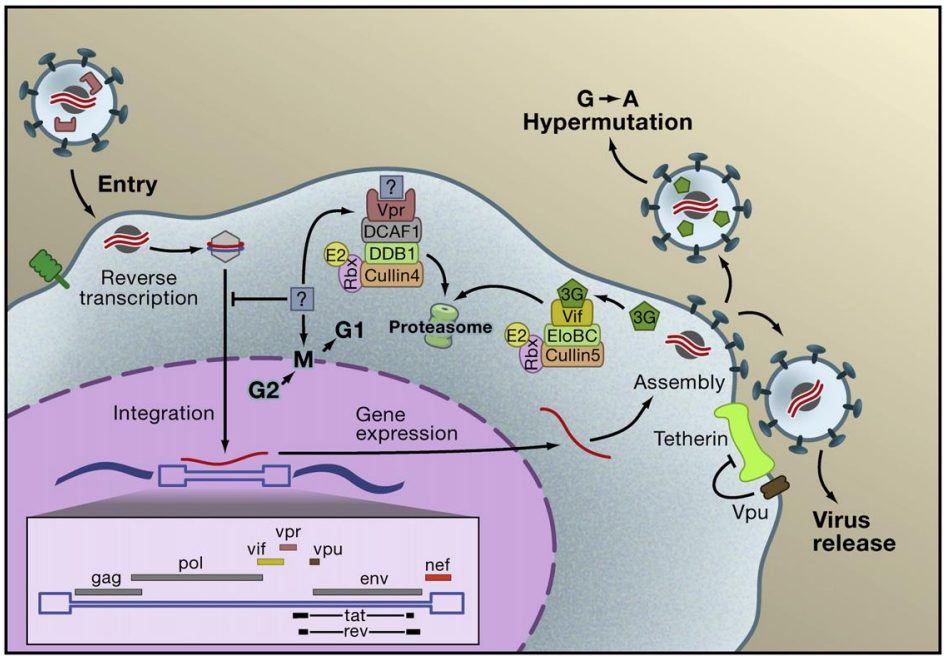


Figure 1.4. Vif, Vpu, and Vpr inhibit host cell restriction factors to promote efficient virus replication (Malim and Emerman, 2008)

1.4.2.9 REV

Rev is a 19 kDa phosphoprotein of 118 amino acid length is a regulatory protein essential for HIV-1 gene expression and viral replication. Rev is primarily localized in the nucleus, serving the main function of transporting viral mRNA transcripts (partially spliced and unspliced) that encode genomic RNA and the structural proteins, Gag, Pol and Env, from the nucleus to the cytoplasm. Rev controls the switch from "early" to "late" stage of the lifecycle (Pollard and Malim, 1998).

1.4.3 HIV LIFE CYCLE

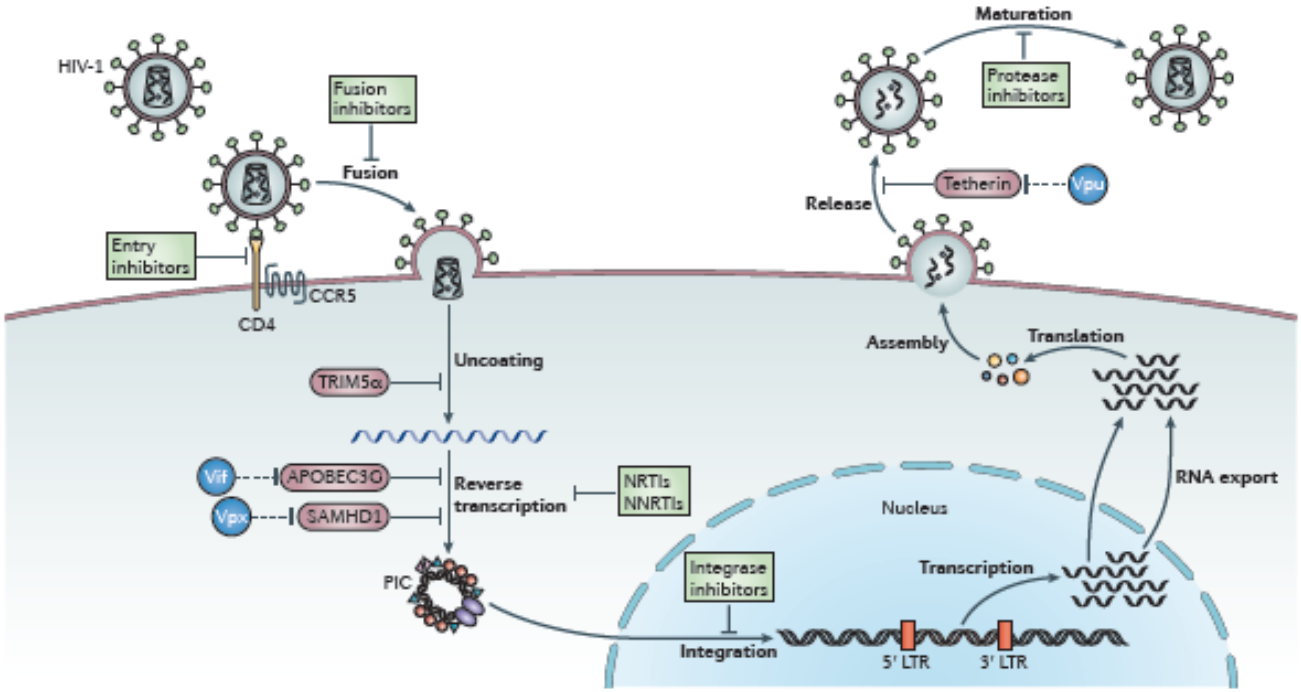


Figure 1.5.The HIV viral life cycle. (Replicated from (Barre-Sinoussi et al., 2013))

Although HIV can infect various cells the main target is the CD4+ T lymphocyte (CD4+ T cell). Upon infection of the CD4+ T cell, the virus undergoes multiple steps to generate viral progeny viruses. The HIV life cycle is as follows:

Binding and fusion

The process of gp120 binding to CD4+ and the appropriate co-receptor (typically CCR5 or CXCR4), triggers a conformational change in the envelope proteins, exposing the hydrophobic region of the TM protein, allowing for insertion into the target membrane (Kwong et al., 1998). This aligns the virus envelope to the cell membrane, resulting in the virus fusing with the cell membrane. Once fusion occurs the virus gains access to the cytoplasm, releasing genetic material into the cell (Barre-Sinoussi et al., 2013) (Figure 1.5).

Uncoating and reverse transcription

Once the HIV virion gets into the cell cytoplasm, the virus uncoats, releasing its nucleoprotein complex consisting of the matrix protein, reverse transcriptase, Vpr, integrase and the diploid viral genome (Miller et al., 1997). The reverse transcriptase (RT) enzyme reverse transcribes the viral RNA genomes into double stranded DNA, integrating the genetic material of the virus into the host DNA.

Nuclear import and Integration

The viral DNA and integrase are transported into the host cell nucleus after being assembled into the pre-integration complex (Figure 1.5), facilitated by Vpr and MA. The virus' new genetic material is then able to integrate itself into the host genome using the enzyme integrase, after which the viral DNA is referred to as 'provirus'. The provirus may stay inactive for extended periods or actively transcribe and then translate the genetic products for new viral replicates (Adams et al., 1994, Greene and Peterlin, 2002).

Transcription/Replication

When the host cell becomes activated, the virus uses its regulatory genes and various enzymes to create more genetic material and viral transcripts. *Rev*, *tat* and *nef* regulatory genes are expressed during the early stage of viral replication. *Nef* mRNA is particularly important, accounting for greater than 75% of all transcripts in newly infected cells. The *gag*, *env* and *pol* structural components are expressed as late gene products, requiring Tat for much more efficient transcription and Rev to transport unspliced mRNAs to the cytoplasm. Messenger RNA that has been suitably spliced does not require Rev, using the host cellular machinery instead (Malim and Emerman, 2008, Frankel and Young, 1998).

Assembly and Budding

Upon targeting and binding by the Gag matrix protein, viral assembly occurs. The assembly of infectious vial particles begins with the incorporation of a coated p7 nucleocapsid complex which includes two RNA molecules. With this the virus buds from the cell, integrated the virion comprising of Envelope glycoproteins and structural Gag proteins, replicative enzymes and viral accessory proteins. Cyclophilin, a HIV-1 cellular protein is also incorporated into the virus, protecting it from post-entry inhibitory effects of Trim 5α, another cellular protein that prematurely disassembles the viral capsid (Greene and Peterlin, 2002).

Viral maturation

Essential in the generation of mature and infectious virions is the cleaving of Gag and Gag-Pol precursors to form mature Gag and Pol proteins, which is assisted by the HIV protease enzyme which is activated during or after the virion has separated from the infected host-cell membrane (Kaplan et al., 1994).

1.5 THE HOST IMMUNE RESPONSE TO HIV-1 INFECTION

1.5.1 CLINICAL COURSE OF HIV-1 INFECTION

The natural history of HIV is well documented. It comprises of an acute/primary phase that usually lasts a few months, followed by an early/clinically dormant phase that characteristically lasts for 3-10 years, and finally an immune collapse which is characterized by AIDS (Figure 1.6) (Pilcher et al., 2004). Acute HIV-1 infection is the interlude during which HIV is detectable in the blood serum and plasma, but before antibody formation, which is regularly used to diagnose infection (Schacker et al., 1996). HIV replication during the acute phase, usually results in high levels of viremia and shedding of the virus at mucosal sites. Onset of early virus-specific immune responses is coincident with a decrease in viremia

(Lavreys et al., 2000, Vanhems et al., 1999). Resting memory CD4+ T cells expressing CCR5 have been suggested to be the first cells infected in studies conducted in SIV infected rhesus macaques. The virus becomes detectable in local lymph nodes one week into infection, indicating an important role of Langerhan cells and plasmacytoid dendritic cells (pDCs) (Fonteneau et al., 2004). The virus is then able to attack the main target cell, i.e. CD4+ T cells, particularly HIV-specific CD4+ T cells in the draining lymph nodes (Douek et al., 2002). The virus spreads to various other sites after a week, with the gut-associated lymphoid tissue (GALT) being the most notable, with large numbers of CD4+CCR5+ memory T cells residing within. As a result, there is an exponential rise in infected cells, with up to 20% of gut-associated CD4+ T cells being infected and 80% destroyed, possibly by Fas-mediated bystander killing (Mattapallil et al., 2005).

Even though there is strong evidence that the immune system is involved in the initial drop in viral load, more studies focused on elucidating the initial host-virus interactions are needed to allow deeper insights into HIV pathogenesis and disease progression (Speth et al., 2003, Yerly et al., 2001). Knowledge gained from such studies will inform vaccine design and improved therapies (Pope and Haase, 2003, McNeil et al., 2001, Oxenius et al., 2000).

The acute period, is followed by a clinical latency phase, also known as the chronic stage. It is at this point that viral and host factors merge to dictate a virologic set point (Pantaleo and Fauci, 1996). Reaching the middle or end of this period, viral load begins to increase and CD4 count begins to decrease, which usually signals the progression of HIV symptomatic disease, leading to the eventual and full blown AIDS (Pantaleo and Fauci, 1996). However, according to Brenchley *et al.* (2006), chronic activation of the immune system is a better indicator of disease progression rather than plasma viral loads.

The course of infection is characteristically different for various individuals. Individuals can be sub-divided into (a) so-called "typical" progressors, (b) rapid progressors, (c) long-term non progressors (LTNP) and lastly (d) long-term survivors (Pantaleo and Fauci, 1996). A large number of individuals fall under the category of typical progressors, where following primary infection, these individuals experience an extended clinical latency period, with progression to clinically noticeable disease or an AIDS defining illness occurring within eight to ten years. This progression is quite varied amongst individuals and is dependent on numerous factors including CD4+T cell counts or other opportunistic infections. Approximately 10-15 % of HIV-infected individuals fall under the category of rapid progressors where they experience a rapid progression to AIDS within as little as two years of primary infection. The period of clinical latency may be lacking in rapid progressors, with the initial burst of viremia and subsequent rise in viremia remaining inefficiently controlled. Long-term non progressors constitute less than 5% of HIV infected individuals, where disease progression is not experienced for an extended period. Clinically, long-term non progressors are asymptomatic, with CD4+ T cell counts remaining stable and within the normal range. It remains unknown whether these individuals experience the same kind of primary infection as the other groups, with this information being important in understanding whether long-term non progressors are more swiftly and effectively able to control the initial burst and ensuing dissemination of the virus. Lastly, long-term survivors are those individuals that experience HIV disease progression within a comparable time frame to typical progressors, however both by clinical and laboratory examination, although atypical, they remain stable for an extended time period. These individuals are thought to possess either virologic or immunologic mechanisms to prevent further disease progression (Pantaleo and Fauci, 1996).

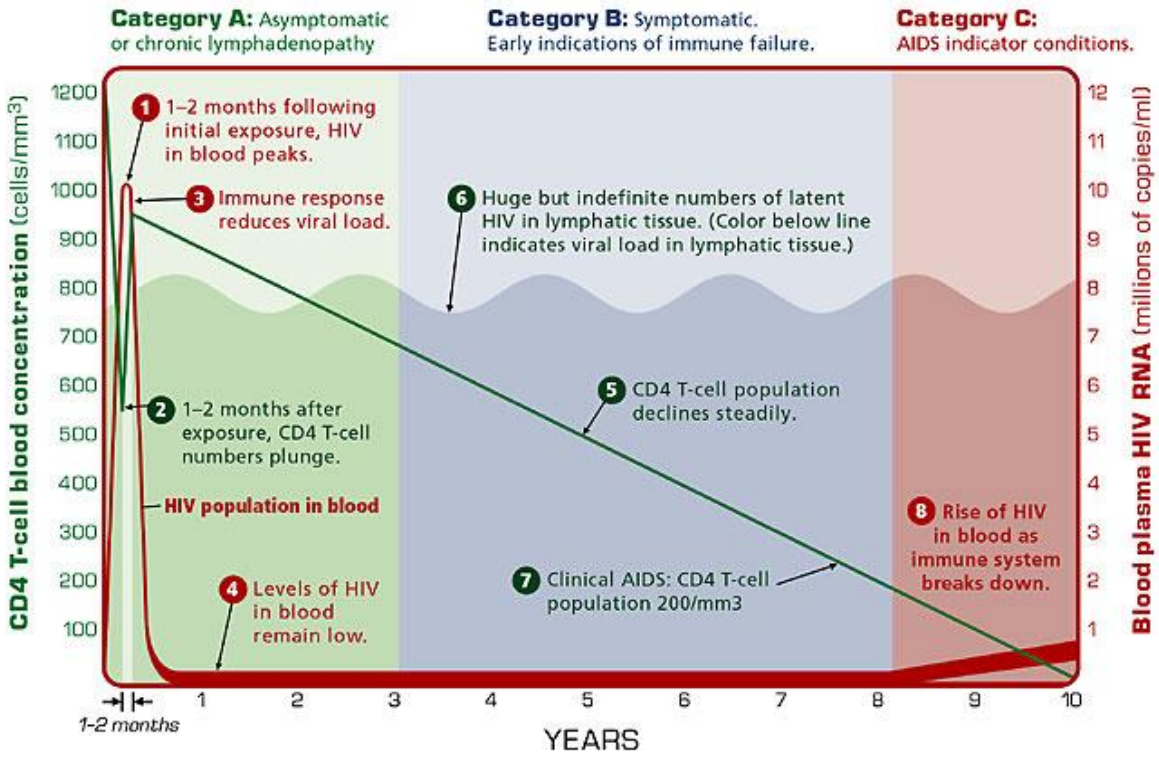


Figure 1.6. Stages of HIV-1 infection as indicated by CD4 T-cell blood concentration (cells/mm^3) and blood plasma HIV RNA (millions of copies/ml) (Reproduced from Online Textbook: HIV and AIDS: Progression of HIV Infection).

1.6 ANTIGEN PRESENTATION

The human immune response system recognizes pathogens, acting to remove, immobilize or neutralize them. The adaptive immune system is antigen-specific, responding to specific molecules on a pathogen. Immunological specificity and memory involve recognition of a specific invader, repeated cell divisions resulting in large lymphocyte populations and differentiation into subpopulations of effector and memory cells (Goldrath and Bevan, 1999). Host lymphocytes are able to recognize self-proteins and therefore once non-self is detected the appropriate response is triggered. An antigen is an important non-self marker which triggers the formation of large lymphocyte populations. T cells acquire receptors for self-

markers (MHC molecules) and for antigen-specific receptors upon initial differentiation and maturation and are thereafter released into the blood as naive T cells (Swain, 1983). These T cells ignore free-floating antigen and other cells that express MHC molecules. However, they will bind to antigen-presenting macrophages or other cell types. Antigen-presenting macrophages digest foreign cells, leaving the antigens intact. They then bind these antigens to MHC molecules on its cell membrane (Figure 1.7). These antigen-MHC complexes are recognized by certain lymphocytes, promoting repeated cell division and differentiation into effector and memory cells, all containing receptors for the antigen (Swain, 1983). Effector helper T cells secrete cytokines, stimulating T and B cells to divide and differentiate, while effector cytotoxic T cells recognize infected cells with the MHC-antigen complex (Gajewski et al., 1989, Glasebrook and Fitch, 1980). They then secrete perforins and other toxins in order to kill the infected cell. B cells receive an interleukin signal from the helper T cells which have been activated by a macrophage with the MHC-antigen complex, promoting rapid cell division, with effector B cells producing large amounts of free-floating antibodies. MHC molecules therefore play an important role in the process of antigen recognition and ultimate differentiation between self and non-self.

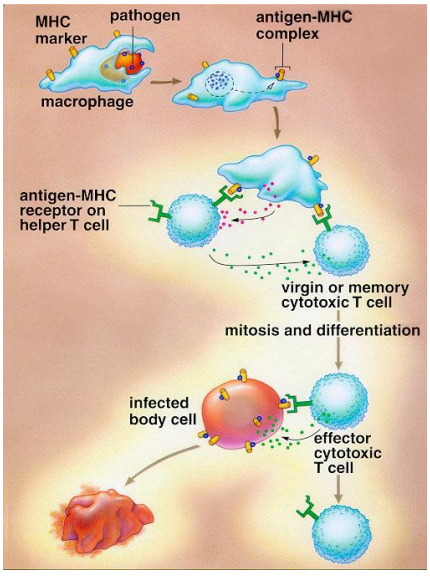


Figure 1.7. Immune system recognition and response to pathogens/antigens (Reproduced from Online Lecture Materials: University of Illinois, 2004).

1.6.1 MAJOR HISTOCOMPATABILITY COMPLEX (MHC)

The major histocompatibility complex (MHC), referred to as human leukocyte antigen (HLA) in humans, was initially discovered as the genomic region responsible for determining compatibility between donor and recipient in tissue and organ grafts (Zinkernagel and Doherty, 1974). These experiments showed that cytotoxic T lymphocytes (CTLs) were able to recognize the MHC molecule, with further experiments by McMichael and colleagues elucidating that virus specific CTLs are able to recognize both the antigen and a polymorphic MHC molecule (McMichael et al., 1977). The MHC is a cluster of genes that maps to the short arm of chromosome 6. The most important of the genes spanning the four million base pairs of DNA and over 128 expressed genes, are the MHC Class I and Class II genes, which encode the "classical" antigen-presenting MHC molecules. These genes are vital in regulating immunity and recognition of antigenic foreign peptides (Carrington and O'Brien, 2003). Genes within the MHC complex encode for various other factors including cytokines, enzymes involved in intracellular antigen processing, heat shock proteins, as well as proteins with unknown function. B and T cells only recognize antigens "presented" to them bound to an MHC molecule. MHC class I presentation is often referred to as the cytosolic or endogenous pathway as MHC class I molecules present peptides derived from cytosolic proteins. Conversely, MHC class II molecules are derived from extracellular proteins and the MHC class II pathway of antigen presentation is often called the endocytic or exogenous pathway (Ploegh, 1998).

In humans, this large group of genes is the most polymorphic loci in the entire human genome and is referred to as the Human Leukocyte Antigen (HLA). HLA gene products are central to acquired immune responses. The major HLA class I alleles can be divided into HLA-A, HLA-B and HLA-C, with the minor alleles including HLA-E, HLA-F, HLA-G and HLA-H. These are genetically inherited proteins that are present on the surface of every

nucleated cell of the body. The HLA class II genes, located within the HLA-D region, also on chromosome 6, include the gene products HLA-DR, DQ and DP (Campbell and Trowsdale, 1993).

Over 100 diseases have been shown to be associated with HLA loci, with the MHC complex shown to be an important genetic risk factor in infectious diseases as well as numerous autoimmune diseases and cancers, with HLA being linked to both susceptibility or resistance (den Uyl et al., 2004, Blackwell et al., 2009, Carrington and O'Brien, 2003, Borghans et al., 2007).

1.6.2 STRUCTURE OF MHC CLASS I MOLECULES

The MHC class I molecules are heterodimers, comprising a heavy alpha 45 kDa glycoprotein and a light chain, beta-2-microglobulin (12kD) (Rigney et al., 1998). The alpha chain can be divided into three extracellular domains, $\alpha 1, \alpha 2$ and $\alpha 3,$ in addition to the transmembrane region and cytoplasmic tail (Flutter and Gao, 2004, York and Rock, 1996). The class I molecule is expressed on the surface in association with β2 microglobulin, which is important for class I surface expression. The MHC-unique domains form the peptide-binding groove, which is formed by a β-pleated sheet structure and a sidewall consisting of two alpha-helices ($\alpha 1$ and $\alpha 2$) (Figure 1.8). The sequence polymorphism clustered in and around the peptide-binding groove is the most distinctive feature between MHC class I molecules. The polymorphism modifies their biochemical properties, dictating binding specificities, and therefore different MHC class I alleles bind unique peptides (York and Rock, 1996). The peptide binding groove houses a short peptide of approximately 7 to 15 residues long, with 9-11 residues peptides the most common, and the orientation of the bound amino acid residues always being the same. The N and C terminals are always positioned at respective ends, with the remaining residues, peptides associated with a particular MHC allele usually have conserved residues called 'anchors', housed in specialized small pockets found in the floor of

the groove (Klein and Sato, 2000). Polymorphisms in the peptide binding groove have a considerable effect on the peptide range associated with each MHC class I molecule, thus the interaction between MHC and peptide is known to be the most specific event in the antigen processing pathway (Gao et al., 2001). Peptide binding to the MHC class I molecule is essentially governed by anchor residues interacting with specific pockets.

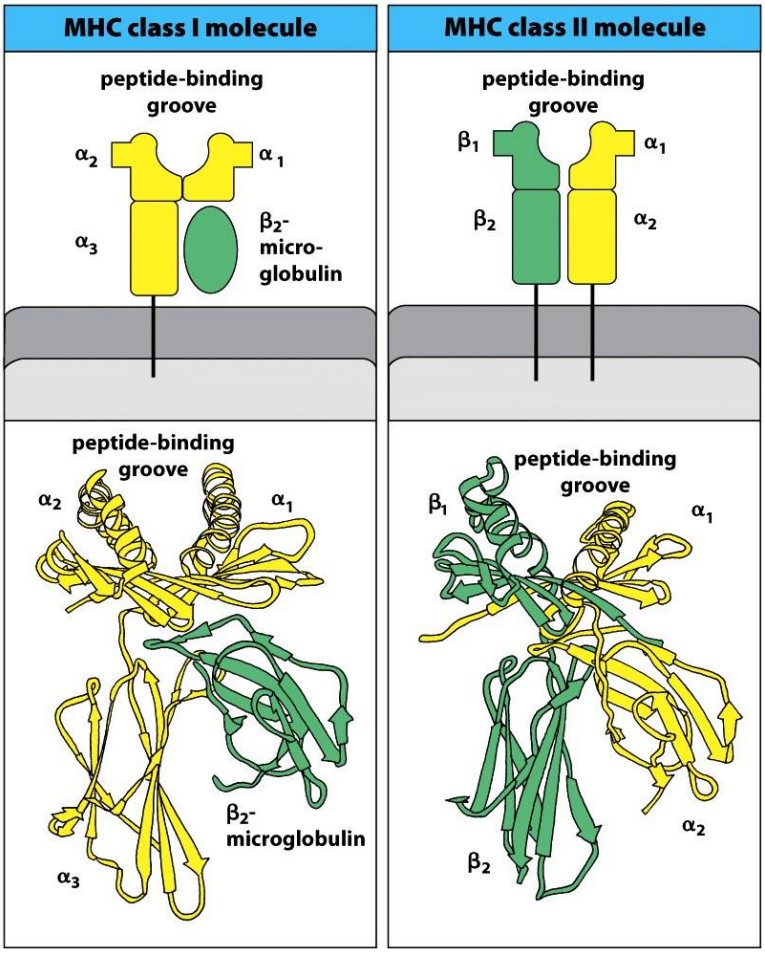


Figure 1.8. MHC Class I and MHC Class II structure. (Reproduced from Garland and Science, 2009).

1.6.3 STRUCTURE OF MHC CLASS II MOLECULES

MHC class II molecules comprise of two transmembrane glycoprotein chains of 34kDa and 29 kDa. Each chain has two domains, α1 and α2, and β1 and β2 respectively. The β2 domain contains the CD4binding site. MHC class II molecules are similar to class I molecules in their

three-dimensional structure, with allelic differences also being due to polymorphisms within and around the peptide binding groove (Figure 1.8). The peptide binding grooves in class II molecules allow for the ends of peptides to actually extend along the groove, rather than being embedded in the molecule. With this, the peptide-binding groove can contain peptides that vary in length, typically between 13-17 amino acids (or even longer), however peptide length is not restricted in class II molecules. Definition of anchor residues and prediction of peptide binding motifs is difficult in class II molecules dues to its binding pockets being more accommodating of various amino acid side chains (Rudensky et al., 1991).

1.6.4 ANTIGEN PRESENTATION PATHWAY OF MHC CLASS I and II

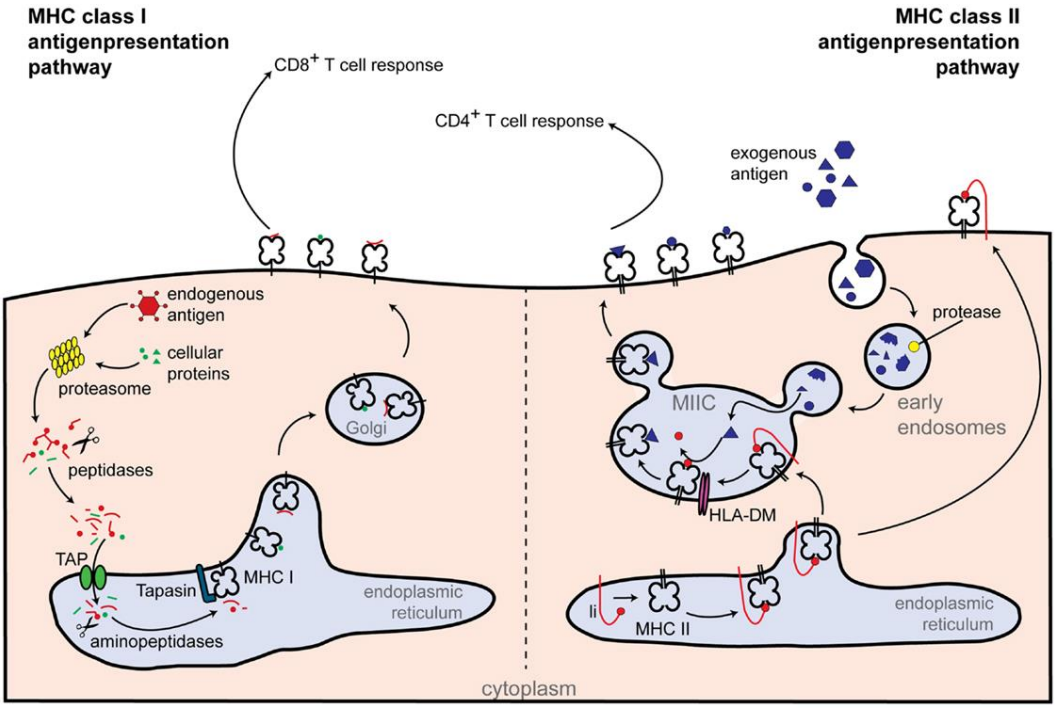


Figure 1.9.MHC class I and class II antigen processing pathways. (Reproduced from(Neerincx et al., 2013)).

MHC class I molecules present mainly endogenously processed peptides to CD8+ T cells. These peptides are derived from host proteins as well as intracellular pathogens and tumour specific antigens (Figure 1.9) (Yewdell et al., 2003). The immune system is then able to

monitor intracellular proteins for discrepancies, thus detecting viruses and bacteria concealed within the cell. Individuals cells can express up to six different MHC class I alleles, with each MHC molecule of a particular allele being able to potentially bind to billions of distinct peptides (York and Rock, 1996). The antigen processing and presentation pathway is what enables these extensive selection of peptides to load onto MHC molecule and present as peptide-MHC (pMHC) complexes on the cell surface.

Conversely, MHC class II molecules present antigenic peptides that are derived from both endogenous and exogenous proteins to CD4+ T cells (Jensen, 2007). The peptides that are presented by MHC class II molecules are derived from self and foreign antigens that are internalized into phagosomes and endosomes through various mechanisms, which include macropinocytosis, phagocytosis and receptor-mediated endocytosis among others (Figure 1.9). Class II molecules are only active in antigen-presenting cells (APCs), including B cells, macrophages, dendritic cells and thymic epithelial cells. Antigens are processed differently in each of these various cells; however the major components that are used are similar.

1.7 ROLE OF IMMUNE MEDIATED CONTROL OF INFECTION

The central function of the human immune system is to protect the host from invading pathogens and preserving the internal balance of the body. The internal world is referred to as self, with potential infectious agents from the external world recognised by the immune system as non-self. Some of the main functions of the immune system include: immunological recognition, generation of immune effector functions, immune regulation, immunological memory and limiting damage to self.

The first lines of defence adopted by the immune system are physical barriers in the form of skin and mucosal barriers/cells lining the air passages and the gut. Any irregularities in these protective physical barriers would enable the entrance of foreign material into the body. Once

either bacteria or viruses penetrate the internal world, the immune system becomes activated. The activated immune system follows a sequence of events to finally destroy the pathogen. The bulk of the infectious agents that passes through the first line of defence are immediately dealt with by the second line of defence i.e. the innate immune system. The innate immune system is a natural, non-specific line of defence, consisting of cells that engulf whole particles or release toxic substances that may kill off these invading pathogens (Janeway, 2001). These cells include natural killer (NK) cells, dendritic cells (DCs), macrophages, monocytes, neutrophils, basophils, T helper and B cells. The cells of the immune system recognise foreign objects through pattern recognition receptors (PRRs) which are found on the surfaces of micro-organisms bearing repeated patterns of molecular structures e.g. viruses' double stranded RNA. The invariant molecular structures called pathogen-associated molecular patterns (PAMPs) are produced only by the microbes and not host cells and are constitutive and conserved (Janeway, 2001). The innate immune system also includes intracytoplasmic proteins that bind to inactivate the pathogen- such as host restriction factors that bind HIV-1 proteins and restrict virus replication at various stages of the life cycle.

If the pathogen is not destroyed by the innate immune system, the third line of defence i.e. the acquired or adaptive arm of the immune system is activated. Adaptive immunity is the response of antigen specific lymphocytes to antigen. Host defences are mediated by B and T cells following exposure to antigen, thus demonstrating specificity, diversity, memory and self/non-self recognition. Unlike the innate immune system which relies on a set of germ-line encoded receptors to recognize non-self, the adaptive immune system relies on somatically generated antigen receptors which are clonally distributed on T and B cells (Medzhitov and Janeway, 1998). The adaptive immune system is based on clonal selection of lymphocytes and an activation of adaptive immune responses.

B cells synthesize a single type of antibody, proteins which recognize antigens as they develop and mature. Upon encountering specific antigens, B cells become primed for replication, consequent to receiving a signal from helper T cells, which are already activated by antigen-presenting macrophages. B cells differentiate into effector and memory B cells. Effector B cells produce antibodies which bind antigens and tag them for destruction by phagocytic cells and the complement system. B cells are unable to detect intracellular toxins or pathogens (Moir and Fauci, 2009).

T cells are the second subset of lymphocytes that mediate the adaptive immune response. All T cells express CD3 on the surface along with the T cell receptor (TCR).

CD8 which is expressed by subsets of CD3+ T cells make up a smaller proportion of CD3+ cells as compared to CD4 which is the major T cell subset in humans (CD4+ cells make up between 32-68 % of total lymphocytes). CD8+ T cells are involved in protecting the host from invading pathogens. These cells are referred to as cytotoxic T lymphocytes (CTL) and function by killing cells marked with foreign pathogens (Zhang and Bevan, 2011).

CD4+ T cells, known as T 'helper' cells, ensure the efficient functioning of the adaptive immune system, shaping an effective response against pathogens. Diseases that destroy or impede CD4+ T cells e.g. HIV, causes immune dysfunction, eventually leading to degeneration (Soghoian and Streeck, 2010).

T cell responses are known to play a major role in immune mediated control of HIV-1 infection.

1.8 HUMAN IMMUNODEFICIENCY VIRUS (HIV)-SPECIFIC T CELL RESPONSES

Strong cellular immune responses exhibited after HIV-1 infection have been shown to correlate with a decrease in viral load (Goulder and Watkins, 2004), however, the exact cell-mediated *in vivo* mechanisms of virus control are poorly understood. HIV-specific T cell responses play an important role in the decline of viral load during primary infection as well as in being able to influence viral load set point (Mellors et al., 1995, Mellors et al., 1996, Schacker et al., 1998). There has been evidence in humans showing that the appearance of HIV-specific CD8+ T cells coincides with a decrease in plasma viremia, during acute infection(Koup et al., 1994). Furthermore, the loss of HIV-specific CD8+ T cells has been associated with a swift progression to full blown AIDS (Klein et al., 1995).

There have been several studies in both human and murine viral infections that indicate the generation of effective adaptive B and T cell antiviral effector responses are critically dependent on the presence of virus-specific CD4+ T helper cells (Homann et al., 2007, Norris and Rosenberg, 2002, Norris et al., 2004, Schacker et al., 1996). Despite this, one of the big gaps in HIV vaccine related research to date has been the relative neglect of the potential role of HIV-specific CD4+ T cell immunity in shaping effective adaptive antiviral immune responses (Ranasinghe et al., 2012).

1.8.1 CD8+ T CELL RESPONSE TO HIV-1 INFECTION

It is believed that CD8+ T cells play an important role in immune mediated control of HIV. Their main role is the recognition and killing of infected cells harbouring intracellular pathogens such as HIV. One of the key observations arguing for the role of CTLs in the initial control of virus replication is the temporal association between the emergence of CD8+ T cells and the rapid decrease in viral load during the acute phase of viral replication (Borrow et al., 1994, Koup et al., 1994, Schmitz et al., 1999). Additional studies have been able

demonstrate that CD8+ T cells have the ability to effectively inhibit viral replication *ex vivo* (Saez-Cirion et al., 2007). The most critical evidence for the role of CD8+ T cells in controlling HIV derives from the rhesus macaque model in SIV infection where depletion of CD8+ T cells using a monoclonal antibody resulted in a subsequent rise in SIV viral load and progression to disease (Jin et al., 1999, Metzner et al., 2000, Schmitz et al., 1999). Moreover, the detection of viral escape mutations in the CTL-targeted epitopes during early infection implies that CD8+ T cells mount a strong selective pressure on the virus (Allen et al., 2004).

The progressive dysfunction and loss of HIV-specific CD8+ T cells have been implicated in increasing viral load and disease progression over time, together with the ongoing loss of CD4+ T cells. Other key attributes of CD8+ T cells, apart from the cytolytic activity previously mentioned, include polyfunctionality and functional avidity. The ability of CD8+ T cells to release a mixture of cytokines and chemokines in tandem have been shown to enhance cytolytic activity (Betts and Harari, 2008). Interestingly, polyfunctional CD8+ T cells have been shown to be elevated in a rare group of HIV positive individuals who maintain undetectable viral loads in the absence of any treatment, commonly referred to as elite controllers (Betts et al., 2006).

Detailed characterization of the specificity of CD8+ T cell targeting of different HIV peptides have failed to correlate breadth and/or magnitude of HIV-1 specific CD8+ T cell responses with better control of viremia. Yet, these studies suggest that targeting of Gag is typically associated with immune control (Julg et al., 2010, Zuniga et al., 2006), with studies performed in clade C infection suggesting that broader targeting by Gag-specific CD8+ T cells is associated with lower viral load (Kiepiela et al., 2007, Maenetje et al., 2010, Riou et al., 2014). Collectively, however, findings from various laboratories highlight that CD8+ T cell activity is influenced by quality as opposed to quantity alone.

In addition, host genetic effects and MHC class I molecules have been widely studied, with strong association between the rate of disease progression and the HLA class I alleles being demonstrated. The expression of particular HLA class I alleles restricting HIV-specific CD8+ T cell responses are associated with different clinical outcomes. Various alleles such as HLA-B*57 or B*27 being labelled 'protective' alleles, whilst the accelerated onset of AIDS is associated with HLA-B*35. Overall, strong associations between HLA class I expression and viral control implicate an important role for HLA class I-restricted CD8+ T cells in the setting of HIV-1 infection (Carrington and O'Brien, 2003).

Ultimately, HIV-specific CD8+ T cell responses are important in exerting an antiviral effect and in contributing to lowering viremia. However, although the role of CD8+ T cells has been widely studied in HIV-1 infection, very little is known about the role and contribution of CD4+ T cells to immune mediated control of HIV.

1.8.2 CD4+ T CELL RESPONSE TO HIV INFECTION

CD4+ T cells typically co-ordinate various processes of the adaptive immune system to ultimately shape an effective response against specific pathogens (Soghoian and Streeck, 2010). However, CD4+ T cells are also known to be the primary targets of HIV-1 infection, acting as 'host' cells to aid in replication of the virus. As the number of functioning CD4+ T cells decrease, the weaker an individual's immune system becomes, making them more susceptible to various opportunistic infections and illnesses. The CD4+ T cell count is used as an important indicator of disease progression and survival of the individual. This value, which is measured as CD4 cells per cubic millimetre of blood, is used as a measure of when a person is put on antiretroviral treatment. The public sector in South Africa currently uses the count of ≤ 350 cells/mm^3 as a guideline for treatment initiation, however are in the process of changing to the guidelines followed by the private sector and the international standard which is ≤ 500 cells/mm^3.

During acute HIV-1 infection, there is an extensive depletion of memory CD4+ T cells from the lymphoid system, particularly in the gut through direct targeting of these cells as well as through bystander activation-induced cell death. Although studies have shown a preferential infection of HIV-specific CD4+ T cells over other antigen-specific CD4+ T cells (CMV) in the peripheral blood, the majority of antigen-responsive HIV-specific CD4+T cells remain virus-free at any time, even during peak viremia (Douek et al., 2002).

The role of antigen-specific CD4+ T cells is controversial. Typically, CD8+ T cell responses are the dominant T cell responses in individuals infected with HIV-1, and this has been reflected in the study and volume of literature of HIV-specific CD8+ T cells. However, the maintenance of effector CD8+ T cell responses is critically dependent on CD4+ T helper cells (Janssen et al., 2003, Walker and McMichael, 2012).

HIV-specific CD4+ T helper cells have been shown to be play an important role in generating long-lasting antiviral memory of CD8+ T cells (Sun and Bevan, 2003, Sun et al., 2004). While antigen-specific CD8+ T cells have the ability to be primed without CD4+ T cell assistance, subsequent expansion following the reencountering of the antigen is inefficient without CD4+ T cell help (Janssen et al., 2003, Matloubian et al., 1994, Williams et al., 2006). The advanced loss of general CD4+ T cells and HIV-specific CD4+ T cells during HIV infection has been assumed to lead to the dysfunction of virus-specific CD8+ T cells and the unsuccessful suppression of these chronic viral infections (Day et al., 2006, Trautmann et al., 2006, Wherry and Ahmed, 2004).

Yet, surprisingly very little attention has been given to the role of HIV-1 specific CD4+ T cells in controlling HIV infection. HIV-specific CD4+ T cells have been shown to have direct killing activity, both by *in vitro* cultured clones (Norris et al., 2004) and *ex vivo* (Soghoian et al., 2012). Nevertheless, the development and specificity of HIV-specific CD4+ T cell

responses in acute and chronic HIV infection, and relation to the development of broadly neutralizing antibody responses remain to be defined. The identification of the specificities and efficacies of these responses is likely to be vital for HIV vaccine design, since any protein based vaccines will most likely induce some degree of HIV-specific CD4+ T cells. Indeed, it will be important to identify which HIV-specific CD4+ T cells are induced in natural HIV infection in order to successfully augment the efficacy of these responses in future vaccines. Currently, only a small fraction of HIV-specific CD4+ T cell responses have been identified and specificities of the individual peptide responses have not been defined. This study addresses this lack of information, particularly in a clade C cohort in South Africa, which carries the highest burden of the HIV epidemic in world.

1.8.2.1 ASSOCIATION OF STRONG CD4+ T CELL RESPONSES DURING CHRONIC HIV-1 INFECTION WITH EFFICIENT VIRAL SUPPRESSION

Recent studies have alluded to the role of HIV-specific CD4+ T cells in viral control in HIV-1 infection. One of the first comprehensive studies to be conducted at the population level was carried out by Ranasinghe et al. (2012), where CD4+ T cell responses to individual HIV peptides were elucidated, generating an immunodominance profile of these responses. The presence and specificity of HIV-specific IFN- γ positive CD4+ T cell responses were evaluated in a large cohort in chronic clade B infection. The study demonstrated that the breadth of these CD4+ T cell responses was inversely correlated with viral load. Responses targeting Gag protein were most associated with favourable outcome. There were differences in the immunodominance profile of individuals that were able to control viral replication and those that were termed chronic progressors. Furthermore, the ratio between Gag and Env responses was a strong marker of viral control in the various groups, where increased Gag responses was associated with a lower viral load, and greater Env responses being linked to a higher viral load and an unfavourable clinical outcome (Ranasinghe et al., 2012). This study and others (Kaufmann et al., 2004, Ramduth et al., 2005, Ramduth et al., 2009) highlight the important role of HIV-specific CD4+ T cell responses in immune-mediated control of HIV-1 infection. They also identified immunodominant CD4+ T cell epitopes that could potentially be included in future vaccines to augment protective T cell responses.

Similar patterns of responses as seen in chronic clade B infection have been seen in chronic clade C infection. Ramduth et al. (2009), showed that IFN- γ secreting Gag-specific CD4+ T cell responses were immunodominant in chronic untreated HIV-1 infection. Here again the responses were directed at numerous distinct epitopes and associated with control of the virus. This evidence, particularly in clade C infection leads to the research detailed in this

thesis, where HIV-1 CD4+ T cell epitopes were further identified and characterized to provide detailed information on the important role of CD4+ T cells in viral control and more importantly to highlight the need for clade specific vaccine development. Furthermore, although the Ramduth et al. (2009) study alluded to the impact of host genetic factors on HIV-1 specific CD4+ T cell responses in clade C HIV-1 infection; this was not examined in detail during their study. The research presented in this thesis aims to delineate the precise peptide-specificity and immunodominance of HIV-specific CD4+ T cell responses and investigate the presentation of these peptides by HLA class II molecules.

1.8.2.2 CD4+ T CELL RESPONSES DURING ACUTE HIV-1 INFECTION

The role of HIV-specific CD4+ T cells during acute infection has been less studied, with recent evidence by Schieffer et al., (2014) suggesting that the induction of HIV-specific CD4+ T cell responses during acute HIV infection does not fuel disease progression and is overall beneficial. Furthermore, longitudinal analysis showed that those epitopes targeted at high frequencies during acute infection were recognized at the same frequency by HIV-specific CD4+ T cells during chronic infection. In addition, generation of Gag-specific CD4+ T cell responses during acute infection was shown to be inversely correlated with viral load set point in subsequent chronic HIV-1 infection (Schieffer et al., 2014).

1.8.2.3 ASSOCIATION OF HLA-RESTRICTED CD4+ T CELL RESPONSES WITH HIV IMMUNE CONTROL

Host genetic factors have a great influence on HIV disease progression (Carrington et al., 2001, Cohen et al., 1997, Roger, 1998). These include genetic polymorphisms within the human leukocyte antigen (HLA) class I and class II loci (Gao et al., 2001, Pereyra et al., 2010), chemokine receptors serving as HIV-1 co-receptors (O'Brien and Moore, 2000, Connor et al., 1997) and the genes that encode for the transporters associated with antigen processing (TAPs) (Malhotra et al., 2001, Abele and Tampé, 2004, York and Rock, 1996).

Currently virus-specific CD4+ T cell responses in HIV-1 infection are poorly defined and there is a lack of information on HLA class II restriction of HIV-specific responses. HLA class I alleles have been characterized, with their genetic association with HIV control being well defined (Gao et al., 2001, Pereyra et al., 2010). However, recent studies have suggested that HLA class II alleles may also have an effect on the control of HIV (Figure 1.10) (Julg et al., 2011, Malhotra et al., 2001, Ranasinghe et al., 2013). Several HLA class II DRB1*13 alleles and the DRB1*13-DQB1*06 haplotype have been shown to bestow a degree of protection in terms of HIV disease outcome. Studies have shown that individuals expressing theses alleles, display the strongest HIV-specific CD4+ T cell responses (Ferre et al., 2010)(Figure 1.10). The DRB1*1303 allele in particular has been associated with reduced viral loads in both subtype B and C populations (Julg et al., 2011, Ferre et al., 2010). The DRB1*13 allele has been shown to confer protection in other infections as well, particularly hepatitis B, where presence of this allele has been associated with a greater frequency of clearance as well as better clinical outcome (Julg et al., 2011). Although not conclusive, the finding of strong associations between class II HLA alleles and breadth and magnitude of CD4+ T cell responses re-iterates the important role that CD4+ T cells have in immune mediated control of HIV.

Table 1.1. Role of particular HLA alleles/ haplotypes on HIV-1 disease transmission, both positive and negative associations, on various populations. (Adapted from Trachtenberg et al. (2001))

HLA Association with HIV-1 Transmission				
HLA Allele or Haplotype	**Risk Group**	**Cases and Controls**	**Population**	**Reference**
TRANSMISSION: NEGATIVE (PROTECTIVE) ASSOCIATION HLA CLASS II				
DRB1*13(*1301-3), *1501	perinatal	45 HIV+, 63 seroreverting infants	Mixed	Winchester 1995
DQB1*03032	mixed	52 HIV+ , 47 HIV-	Caucasian & African American	Roe 2000
TRANSMISSION: POSITIVE (SUSCEPTIBLE) ASSOCIATION HLA CLASS II				
DRB1*03011	perinatal	46 HIV+, 63 seroreverting infants	Mixed	Winchester 1995

1.9 ASSAYS TO DETERMINE EPITOPE SPECIFICITY AND FUNCTIONAL EFFICACY OF HIV-SPECIFIC CD4+ T CELLS

The main assays used to measure HIV-specific CD4+ T cells include the interferon (IFN)-γ ELISPOT, tetramer-staining for common HLA-restricted epitopes and intracellular cytokine staining (ICS) measured by flow cytometry. Evaluating antigen-specific T cells and their effector functions is vital to understanding overall T cell immunity in terms of assessing the effectiveness of specific immune therapies (Zhang et al., 2009). However, because of the low frequency at which these antigen-specific T cells occur in peripheral blood, consistent and reliable measurement of T cell immunity poses a major challenge.

The IFN- γ ELISPOT is based on the enzyme-linked immunosorbent assay (ELISA), where the main principle is detection of a cytokine (IFN- γ), through antigen induced secretion, which is bound by an immobilized antibody and later visualized using another enzyme-coupled antibody (Czerkinsky et al., 1983). The ELISPOT assay was initially developed for detection of antibody-secreting B cells but is now widely used as a comprehensive screening method for HIV-specific CD8+ T- and CD4+ T cell quantification and characterization (Corne et al., 1999). Vaccine clinical trials in particular, utilize both ICS and IFN- γ ELISPOT as the main assays to assess vaccine immunogenicity (Streeck et al., 2009). The ELISPOT is a rapid and cost-effective method used in the large scale detection and mapping of T cell responses. One of the limitations of the assay is that it works under the assumption that all HIV-specific CD8- or CD4+ T cells are identifiable by their ability to secrete IFN- γ post peptide stimulation. With this, some cells of interest may not be identified or the magnitude of particular responses may be skewed. In general, both ICS and the ELISPOT have not been able to show direct correlation with vaccine efficacy or protection in HIV-1 infection however they are still able to provide useful functional information.

There are various other assays that can be used to measure antigen-specific CD4+ T cells, both in number and function. However, each has pros and cons. One such assay is tetramer staining, a highly sensitive method used to screen for CD4+T cell responses to a pre-determined peptide-HLA class II combination. However, tetramers are expensive and generation of class II tetramers is extremely difficult (both commercially and 'in-house'). Furthermore, the assay gives no functional information, it only measures the quantity of HIV-specific T-cells to a given HLA-restricted epitope, although it can be combined in multi-parameter flow cytometry with functional read-outs such as IFN-γ and other cytokines upon peptide stimulation (Sun et al., 2003).

For this study, we utilized overlapping peptides, spanning the entire HIV proteome, the ELISPOT megamatrix format. This approach is the most rapid, efficient and cost-effective method available to allow for a broader view of the overall immune response to HIV. We used ICS to further elucidate functional characteristics by measuring various other cytokines.

1.10 SPECIFIC AIMS AND OBJECTIVES OF THE THESIS

The precise details of CD4+ T cell contribution to immune protection against HIV have not been adequately defined, leaving a gap in the information needed for an effective vaccine design. My study focused on the role of CD4+ T helper cells in the immune response to clade C HIV infection.

1.10.1 STUDY AIM

The aim was to carry out a comprehensive analysis of HIV-specific CD4+ T cell responses to HIV-1 subtype C infection and to elucidate these responses in terms of class II HLA restriction. Furthermore, we evaluated functional qualities of CD4+ T cells restricted by protective class II alleles in a large cohort of individuals infected with HIV-1 subtype C infection in KwaZulu-Natal, South Africa.

1.10.2 STUDY HYPOTHESIS

We tested the hypothesis that HIV-1-specific CD4+ T cell responses restricted by class II HLA alleles play an important role in immune mediated control of HIV-1 infection.

1.10.3 STUDY RATIONALE

Our aim was to generate data that may elucidate the underlying mechanisms by which CD4+ T cells responses impact immune control of HIV. This information will be critical to vaccine efforts designed to induce these responses, as well as potential therapeutic manipulation of immunity in persons with acute and chronic clade C HIV-1 infection.

1.10.4 SPECIFIC OBJECTIVES

Chapter 3 focuses on studies of HIV-specific CD4+ T cell targeting during acute and chronic HIV-1 infection using the IFN-γ ELISPOT assay to define the immunodominance hierarchy of frequently targeted peptides in clade C infection. The chapter further evaluates the functional qualities of CD4+ T cells using flow cytometric analysis. These analyses were aimed at shedding light on the role of HIV-specific CD4+ T cell responses in HIV-1 subtype C infection. Chapter 4 defines the class II HLA restriction characteristics of HIV-specific CD4+ T cell responses. Defining HLA restriction and epitope specificity will provide a greater understanding of the association between protective class II HLA alleles and T cell immunity. This chapter provides information on allele frequency in the cohort and HLA class II associations in controllers and progressors. Finally, the overall implications and future research directions of the research within this thesis are detailed in Chapter 5.

CHAPTER 2: METHODOLOGY

2.1 STUDY POPULATION

Samples from adult study participants of predominantly Zulu/Xhosa ethnic origin with both acute and chronic infection were recruited from the Sinikithemba Clinic based as McCord Hospital (chronic infection) and Prince Mshiyeni Hospital (acute infection) in the greater Durban area of KwaZulu-Natal, South Africa. The study was approved by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (BE190.13). All study participants provided written informed consent for participation in the study. All subjects were informed of their CD4+ T cell count and plasma viral loads. Subjects meeting the South African national criteria for treatment were referred for antiretroviral therapy, with only therapy-naive subjects being included in this study.

2.1.1 ACUTE SAMPLING STRATEGY AND PATIENT SELECTION

A total of 15 acute HIV-infected subjects were analysed in this study. Study participants testing negative or discordant by dual commercial rapid HIV-1 tests (Bioline, Standard Diagnostics; and Sensa, Hitech Healthcare) were initially recruited from HIV Counselling and Testing centers. Acute HIV-1 infection was defined by a positive HIV-1 RNA test, negative HIV-1 enzyme immunoassay (SD HIV1/2 enzyme-linked immunosorbent assay [ELISA] 3.0, Standard Diagnostics), and a negative or indeterminate Western blot (Genetic Systems, Bio-Rad). Centers for Disease Control and Prevention (CDC) criteria were used for the interpretation of Western blot results such that a positive sample had at least 2 of the major bands (gp160, gp120, gp41 and p24),with either gp160 or gp120 present as well as gp41 or p24. An indeterminate result was one in which these requirements were not met but 1 or more bands were present, or where the band intensity was less than the weak positive control. Negative results exhibited no reactive bands. The infection date was estimated as

occurring 14 days prior to the first positive HIV RNA and negative HIV antibody test. Blood samples were obtained from study participants at 2, 4, 6, 8, 12, 18, 26 and 52 weeks post-infection.

2.1.2 CHRONIC SAMPLING STRATEGY AND PATIENT SELECTION

A total of 55 chronically HIV-infected subjects were analysed in this study. Study participants comprised of antiretroviral naive, chronically HIV-1 subtype C–infected adults from the Sinikithemba cohort. Socio-demographic characteristics, plasma viral load measurements, and CD4 cell counts were obtained at baseline. Follow-up CD4+ T cell counts and plasma viral load measurements were performed at 3- and 6-month intervals, respectively.

2.2 VIRAL LOAD DETERMINATION, CD4+ T-CELL ENUMERATION AND HLA TYPING

Plasma HIV-1 RNA levels were quantified using the Roche Amplicor version 1.5 on the Roche Cobas Amplicor instrument according to manufacturer's instructions (Roche Diagnostics, Branchburg, NJ).The lower limit of detection of the assay was 50 RNA copies/ml plasma.

Absolute blood CD4+ T-cell counts were enumerated using the MultiTest TruCount kit and MultiSet software on a FACS Calibur flow cytometer (Becton Dickinson).

Genomic DNA samples were typed to an oligoalleleic 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 Dynal Biotech sequence specific priming (SSP) kits in conjunction with the previous SSO type. Sequence specific oligonucleotide probes were immobilized on a membrane strip. Classical HLA class

II and I alleles were identified by the banding patterns produced by the selective hybridization of the sample amplicon to the probes on each locus specific strip.

It should be noted that all viral load assays and CD4+ T-cell enumeration were performed by in house laboratories. While the HLA typing was performed by a collaborator, Carrington Lab, Center for Cancer Research at National Institute of Health, Frederick, Maryland, USA.

2.3 SEPARATION AND CRYOPRESERVATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs)

2.3.1 SEPARATION OF PBMC

2.3.1.1 PRINCIPLE

The Ficoll density gradient method was used for the isolation of PBMCs from whole blood samples by our in house laboratories. Whole blood was overlaid onto Ficoll-Histopaque 1077 (Sigma) and centrifuged in a Sorvall RT7 Plus centrifuge (Thermo Scientific). During centrifugation, those cells with greater density such as erythrocytes and granulocytes are trapped in the lower layer of the tube. Mononuclear cells migrate, forming a distinct opaque layer at the plasma/histopaque interface.

2.3.1.2 PROCEDURE

Histopaque 1077 (Sigma), Phosphate Buffered Saline (PBS) containing antibodies and R10 medium (RPMI 1640 containing 10% heat-inactivated fetal calf serum, 2 mM l-glutamine, 50 U of penicillin/ml, 50µg of streptomycin/ml, and 10mM HEPES) were brought to room temperature. Whole bloods obtained from patients were initially centrifuged at 1,500 rpm for 10 minutes. After spinning, 10 ml of plasma was removed and stored in 15 ml Sterilin tubes for viral load testing.

A 1:1 dilution of blood to PBS was then made, which was then gently layered over the Histopaque 1077 in a 1:1:1 dilution in a 50 ml Sterilin tube. The diluted blood, forming a layer above the Histopaque was centrifuged at 1,500 rpm for 30 minutes at room temperature. Following centrifugation, the opaque layer containing the mononuclear cells was aspirated and transferred to a second 50 ml Sterilin tube. 40 ml of PBS was added to the tube, which was then centrifuged for at 1,500 rpm for 10 minutes at room temperature. The supernatant was discarded and an additional 40 ml of PBS was added to the tube for a second wash at the same speed and duration. Once again the supernatant was discarded, with the pellet gently re-suspended and warm R10 media added to the tube. The sample was incubated at 37º C in a 5% CO_2 incubator, counted, with the necessary amount of cells being used in various immunological assays if need be or cryopreserved if not utilized immediately.

2.3.2 CRYOPRESERVATION OF PBMCs

PBMCs for cryopreservation were centrifuged at 1,500 rpm for 10 minutes at 4º C. The supernatant was discarded, the pellet gently re-suspended and the tube thereafter transferred into an ice box. Each cryovial contained 10 million cells (which were prior determined using either a Guava Cell Counter or haemocytometer). If freezing 10 million cells, 500µl of fetal calf serum (FCS) was added to the cells and thoroughly mixed. An additional 500µl of FCS and 20% dimethyl sulfoxide (DMSO) was then added into the tube in a drop-wise manner bringing the total DMSO concentration to 10%. The cells in the freezing solution were then transferred to cryovials, which were stored in freezing containers (Mr. Frosty's - Nalgene) overnight at -80º C. Mr. Frosty's contain iso-propanol which cool at a rate of 1º C per minute, which is considered optimal for cell quality. Cells were moved from Mr. Frosty's after 24 hours and placed in liquid nitrogen for long-term storage.

2.3.3 THAWING OF CRYOPRESERVED PBMCs

Cryopreserved cells were removed from liquid nitrogen and rapidly thawed at 37º C in a water bath, until ice just began to become liquid. The cells were then re-suspended in 1ml cold R10 medium and transferred to Sterilin tube containing 9ml of cold R10 medium. The tube was centrifuged at 1,800 rpm for 6 minutes at 4º C. The supernatant was discarded, ensuring maximum removal of media containing DMSO freezing solution, due to toxicity of the chemical to cells once thawed. A second wash containing 10ml R10 medium was conducted at the same speed and duration as initially. The cells were thereafter re-suspended in 10ml cold MACS buffer solution prior to counting and CD8+ T cell depletion or 10ml R10 medium if cells were not to be depleted.

2.4 CD8+ T CELL DEPELETION USING MACS MICROBEADS

Post cold thawing procedure, PBMCs were CD8+ T cell depleted using Miltenyi MACS MicroBeads. A buffer solution i.e. a 0.5% BSA/PBS solution was freshly prepared and stored on ice or at 4º C prior to usage. Once cells were thawed and washed twice in R10 medium, they were re-suspended in 10ml cold buffer solution and centrifuged at 1,800rpm for 6 minutes at 4º C. 10µl of cell solution was added to 90µl of trypan blue at this stage and transferred to a haemocytometer for counting. After centrifugation, the supernatant was discarded and cells were re-suspended in 80µl of buffer solution and 20 µl of MACS MicroBeads. The cells were then incubated for 15 minutes at 4º C.

During the incubation period a Miltenyi MACS magnetic stand was set up, together with LS magnetic columns to be used in the separation. The column was wet using 1ml of buffer solution prior to adding the cells. Post incubation, 500µl of buffer was added to the cell solution. Cell solution was then passed through the LS column. The flow-through was collected and passed through the column again. 3ml of buffer solution was added to the original tube containing the cells, washing the tube for any remaining cells and thereafter

passing the solution through the column. Lastly, once all previous solution has passed through, 3ml of buffer solution was passed directly through the column. The flow-through (approximately 7ml of solution) was thereafter centrifuged at 1,800rpm for 6 minutes. The supernatant was discarded and the cells i.e. whole PBMC minus CD8+ T cells was re-suspended in 10 ml R10 medium and incubated at 37º C for resting until usage.

Flow cytometry confirmed at least 98% CD8-negative PBMC prior to use in the IFN-γ ELISPOT assay.

2.5 IFN-γ ELISPOT ASSAY

2.5.1 SYNTHETIC PEPTIDES AND PEPTIDE MATRICES

The IFN-γ ELIPOT assay was used to screen samples from all of the acutely and chronically infected subjects prior to conducting other immunological assays. The initial screening was carried out using a megamatrix of peptides. These peptides were produced at the Massachusetts General Hospital Core facility in 2001. The megamatrix involved the use of four hundred and ten 18-mer peptides overlapping by 10 amino acids that spanned the entire expressed HIV proteome. These were arranged into 72 pools of between 10 to 12 peptides in each pool such that an individual peptide was uniquely represented in two different pools. The peptides listed in tables 3-5 were used to set up the matrix, which has been designed to identify T-cell responses. The peptides in row A and B, C and D and E and F work in tandem. An example could be that if there was a T-cell response in row A, there would be one in row B as the particular OLP that the patient is responding to is present in both rows. By looking at table 1, the OLP can be identified. A particular example being a response to OLP 11, which will result in spot forming units in well A1 and B11(Note that peptides 108,109,110 and 132-144 do not exist in the clade C list).

Positive responses from initial megamatrix screening were confirmed using a separate ELISPOT assay at single peptide level as described in section 2.5.2.

Table 2.1. Positioning of OLP used in the megamatrix in rows A and B of 96 well ELISPOT plate

Pool A	OLPs	No of Peptides in pool	Pool B	Peptides												No. Peptides in pool
A1	1-11	11	**B1**	1	13	25	37	49	61	73	85	97	121	x	x	10
A2	12-22	11	**B2**	2	14	26	38	50	62	74	86	98	122	x	x	10
A3	23-33	11	**B3**	3	15	27	39	51	63	75	87	99	111	123	x	11
A4	34-44	11	**B4**	4	16	28	40	52	64	76	88	100	123	124	x	11
A5	45-55	11	**B5**	5	17	29	41	53	65	77	89	101	113	125	x	11
A6	56-66	11	**B6**	6	18	30	42	54	66	78	90	102	114	126	x	11
A7	67-77	11	**B7**	7	19	31	43	55	67	79	91	103	115	127	x	11
A8	78-88	11	**B8**	8	20	32	44	56	68	80	92	104	116	128	x	11
A9	89-99	11	**B9**	9	21	33	45	57	69	81	93	105	117	129	x	11
A10	100-107	8	**B10**	10	22	34	46	58	70	82	94	106	118	130	x	11
A11	111-121	11	**B11**	11	23	35	47	59	71	83	95	107	119	131	x	11
A12	122-131	10	**B12**	12	24	36	48	60	72	84	96	x	120	x	x	9

Table 2.2. Positioning of OLP used in the megamatrix in rows C and D of 96 well ELISPOT plate

Pool	OLPs	No. Peptides in pool	Pool	Peptides												No. Peptides in pool
C1	145-156	12	**D1**	145	157	169	181	193	205	217	229	241	253	265	277	12
C2	157-168	12	**D2**	146	158	170	182	194	206	218	230	242	254	266	278	12
C3	169-180	12	**D3**	147	159	171	183	195	207	219	231	243	255	267	279	12
C4	181-192	12	**D4**	148	160	172	184	196	208	220	232	244	256	268	280	12
C5	193-204	12	**D5**	149	161	173	185	197	209	221	233	245	257	269	281	12
C6	205-216	12	**D6**	150	162	174	186	198	210	222	234	246	258	270	282	12
C7	217-228	12	**D7**	151	163	175	187	199	211	223	235	247	259	271	283	12
C8	229-240	12	**D8**	152	164	176	188	200	212	224	236	248	260	272	284	12
C9	241-252	12	**D9**	153	165	177	189	201	213	225	237	249	261	273	285	12
C10	253-264	12	**D10**	154	166	178	190	202	214	226	238	250	262	274	286	12
C11	265-276	12	**D11**	155	167	179	191	203	215	227	239	251	263	275	287	12
C12	277-288	12	**D12**	156	168	180	192	204	216	228	240	252	264	276	288	12

Table 2.3. Positioning of OLP used in the megamatrix in rows E and F of 96 well ELISPOT plate

Pool	OLPs	No. Peptides in pool	Pool	Peptides												No. Peptides in pool
E1	289-300	12	**F1**	289	301	313	325	337	349	361	373	385	397	409	421	12
E2	301-312	12	**F2**	290	302	314	326	338	350	362	374	386	398	410	422	12
E3	313-324	12	**F3**	291	303	315	327	339	351	363	375	387	399	411	423	12
E4	325-336	12	**F4**	292	304	316	328	340	352	364	376	388	400	412	424	12
E5	337-348	12	**F5**	293	305	317	329	341	353	365	377	389	401	413	425	12
E6	349-359	11	**F6**	294	306	318	330	342	354	366	378	390	402	414		11
E7	360-370	11	**F7**	295	307	319	331	343	355	367	379	391	403	415		11
E8	371-381	11	**F8**	296	308	320	332	344	356	368	380	392	404	416		11
E9	382-392	11	**F9**	297	309	321	333	345	357	369	381	393	405	417		11
E10	393-403	11	**F10**	298	310	322	334	346	358	370	382	394	406	418		11
E11	404-414	11	**F11**	299	311	323	335	347	359	371	383	395	407	419		11
E12	415-425	11	**F12**	300	312	324	336	348	360	372	384	396	408	420		11

2.5.2 ENZYME-LINKED IMMUNOSPOT (ELISPOT) ASSAY

HIV-specific CD4+ responses were screened against 410overlapping-peptides (OLPs) spanning the entire HIV-1 clade C consensus proteome at the level of a single peptide per well in a modified IFN-γ enzyme-linked immunospot (ELISPOT) assay. CD8-depleted PBMC, were plated in 96-well polyvinylidene plates (MAIP S45, Millipore, MA, USA)pre-coated with 100µl MAb1-D1k anti-IFN-γ monoclonal antibody (0.5µg/ml) (Mabtech, Stockholm, Sweden) overnight at 4ºC. Prior to adding CD8-depleted cells the plate was washed 6 times with blocking buffer (PBS with 10% sterile heat inactivated fetal calf serum) to block non-specific binding sites. 90µl of R10 medium was added to each well. Thereafter 10 µl of a 33 µg/ml peptide stock solution (overlapping peptides) was added to each well, acting as a stimulus. The final concentration of each peptide used was 2µg/ml, with a single OLP tested per well.

A total of 100,000 CD8-depleted cells per well were added to each well in the presence or absence of specific stimulus, in this case HIV-1 derived OLPs in a final volume of 200 µl R10. Negative controls consisted of 4 wells of cells in medium without antigen or other stimuli. The addition of phytohemagglutinin antigen (PHA) (Sigma), at a concentration of 5µg/ml, served as a positive control for both cell viability and functionality of the immunoassay. The plates were incubated for 40 hours at 37°C, 5% CO_2 to elicit the maximal cytokine secretion.

Following the 40-hour incubation the plates were processed as follows. Cells were discarded and the plate washed with 100 µl PBS per well 6 times. Thereafter, 100µl biotinylated anti-IFN-γ MAb 7-B6-1 (0.5 µg/ml, Mabtech) detection antibody was added to each well. The plates were incubated for 90 minutes in the dark, at room temperature.

The plate was then washed 6 times again with 100 µl PBS per well. Thereafter, 100 µl streptavidin-alkaline phosphatase conjugate (0.5 µg/ml, Mabtech) enzyme was added to each well. The preparation of the aforementioned detection antibody and conjugate enzyme was prepared by adding 5 µl of antibody to 10ml of commercial PBS. The plate was incubated for 45 minutes in the dark at room temperature.

Lastly, the plate was once again washed for 6 times with 100µl of PBS per well. A combination of BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt) and NBT (Nitro-Blue Tetrazolium Chloride), where 100 µl of each NBT and BCIP was added to 10ml of Tris buffer and 100 µl of this solution was added to each well. This solution is ideal for staining applications as they yield an intense, insoluble black-purple precipitate. The plate was closely watched for the appearance of spots, with the reaction being stopped by washing the plate under running water before discoloration appeared in the negative well.

The AID ELISpot Reader (Autoimmun Diagnostika, Germany) was used to determine the spot-forming cells (SFC) per million CD8-depleted PBMC. The number of antigen-specific CD4+ T cells was calculated by subtracting the mean negative control values. An antigen-specific CD4+ T cell response was considered positive only if it was at least >3 times the mean background and also >3 times the standard deviation of the SFC within the negative controls, as per Ranasinghe et al. (2012). The breadth of responses is defined as the sum of IFN-γ-positive peptide responses within a given individual across the entire HIV proteome or within a specified protein.

2.6 HIV-SPECIFIC CD4+ T CELL LINES

Frozen CD8-depleted peripheral blood mononuclear cell (PBMC) samples from subjects with known CD4+ T cell responses was used to generate CD4+ T cell lines. CD8-depleted PBMC was simulated with 10 ug/mL of peptide at a concentration of 2 million cells on a 24-well

plate in H10 medium (RPMI 1640 containing 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 50µg of penicillin/ml, 50 µg of streptomycin/mL, and 10 mM HEPES). The cells were incubated at 37°C and 5% CO_2. After 2 days, the cells were washed and fresh H10 medium supplemented with 100 U/mL recombinant interleukin-2 (IL-2) was added. The CD4+ T cell lines were fed twice weekly with regular media exchanges.

2.7 HLA-DR RESTRICTION ASSAY

After 14 days, the T cell lines were simultaneously assessed for their specificity and HLA-DR restriction using a large panel of L cell line (LCL) mouse fibroblasts, each stably transfected with a single HLA-DR molecule, as previously described (Southwood et al., 1998). Each LCL was pulsed with 10 µg/mL peptide for 90 minutes at 37°C and 5% CO_2 and washed five times to remove free peptide. Clade C consensus sequence HIV overlapping peptides were used in the assay. 10,000 peptide-pulsed LCL was co-cultured in triplicate with 50,000 of each respective CD4+ T cell line per well on a pre-coated IFN-γ plate. As a negative control, each CD4+ T cell line was co-cultured in triplicate with the appropriate LCL in the absence of peptide. As a positive control, PHA was added at 2µg/mL. The plates were incubated overnight at 37°C and 5% CO_2 and processed as per usual ELISPOT protocol described above. The AID ELISPOT reader was used to determine the number of spot-forming cells (SFC) per 50,000 of the CD4+ T cell line. HLA-DR restriction was considered positive if it was at least ≥3 times the mean background and also ≥3 times the standard deviation of the negative control wells.

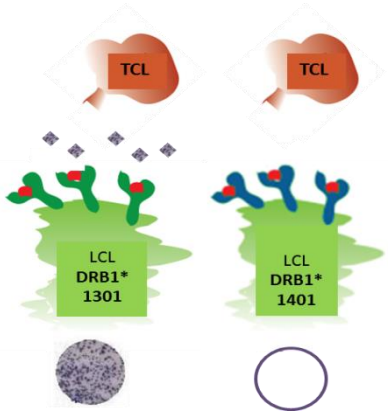


Figure 2.1. Graphical depiction of a typical response to co-culturing of T cell and L cell lines in a HLA-DR restriction assay (Ranasinghe et al., 2013).

2.8 INTRACELLULAR CYTOKINE STAINING

Intracellular cytokine staining is a flow cytometric technique used to detect single cell expression of cytokines, allowing for simultaneous detection, quantification and phenotypic characterization of antigen-specific CD4+ and CD8+ T cells in PBMC.

Post thawing of cells and a minimum two-hour resting period, cells were plated at a volume of 100µl per well (i.e. having the desired number of cells in 100µl). Peptide pools were used as specific antigen/stimulus; with 10µl being added at a concentration of 2µg/ml. PMA/Ionomycin was used as positive control with 10µl being added at a concentration of 2µg/ml. The stimulated cells were incubated at 37°C and 5% CO_2 for one hour. Following incubation Golgi stop protein transport inhibitor (BD Biosciences) (0.25µl) and Golgi plug protein transport inhibitor (BD Biosciences) (0.25µl) was added to each well. The plate was then incubated overnight at 37°C and 5% CO_2. Following incubation the cells were spun down at 2000rpm for 6 minutes. The supernatant was discarded and cells were washed with 200 µl PBS and centrifuged at the same speed. Live/dead fixable aqua dead cell viability dye (Invitrogen) was thereafter added to each well (1:5 dilution with PBS). Cells were incubated with 40 µl per well viability dye in the dark for 10 minutes. The cells were washed with 200 µl PBS and centrifuged at 2,000rpm for 6 minutes. The supernatant was discarded and a

surface stain (surface antibodies) was added to the cells and incubated in the dark for 20 minutes. The cells were washed in a 2% FCS/PBS solution after incubation and spun down at 2,000rpm for 6 minutes. Cytoperm/cytofix fixation permabilisation kit (BD Biosciences) was added to each well to fix the cells and incubated for 15 minutes. The cells were thereafter washed with perm wash (200 µl) and centrifuged at 2,000rpm for 8 minutes. Lastly, the intracellular staining antibodies were added to each well and incubated for 30 minutes. Post incubation, the cells were washed with 2% FCS/PBS solution and spun down at 2,000rpm for 8 minutes. The supernatant was discarded, cells were re-suspended in 300 µl 2% FCS/PBS and transferred to cluster tubes to be analysed by the LSRII flow cytometer (Becton Dickinson). A minimum range of 100,000 – 200,000 events were acquired. Flow data was analysed using FlowJo software (Treestar FlowJo version 9.5.2).

Table 2.4. Flow cytometry panel indicating antigens and fluorochromes used in functional analysis

	Marker	Antibody	Volume (1x) µl
Exclusion		LIVE/DEAD	
	Viability	Aqua	0.1
	CD14/CD19	V500	1.5
T cells	CD3	BV711	1
	CD4	APC C7	1
	CD8	Qdot605	0.3
Polyfunctionality	IL-2	FITC	2
	TNFa	Alexa700	1
	IL-17	V450	2
	IL-21	APC	2
	IL-13	PE	2
	IFN-γ	PE-CY7	1

2.9 STATISTICAL ANALYSIS

Statistical analysis and graphical presentation were performed using GraphPad Prism version 5.0 software. Spearman's Rank correlation was used to assess the relationship between immune responses and viral load. Statistical analysis of significance was calculated using

one-way ANOVA with Dunn's test for multiple comparisons. Statistical significance was set at $p < 0.05$.

CHAPTER 3: DEFININGIMMUNODOMINANCE HIERACHIES OF HIV-SPECIFIC CD4+ T CELL RESPONSES IN SUBTYPE C HIV-1 INFECTION

3.1 BACKGROUND

A hallmark of HIV-1 infection is the loss of CD4+ T cells. In particular, HIV-1-specific memory CD4+ T cells are preferentially susceptible to infection and are gradually depleted from the periphery and GALT (Douek et al., 2002). Studies in chronic infections, in both animal models and humans, have demonstrated that CD4+ T cells are required for optimal CD8+ T cell responses (Mattapallil et al., 2005, Janssen et al., 2003). However, the contribution of HIV-specificCD4+ T cell responses to viral control is unclear, particularly the relationship between HIV-specific T helper cell function and HIV-1 subtype C infection. Several studies have shown that HIV-specific CD4+ T cells play an important role in enhancing immunological control of HIV viremia by either providing help to CD8+ T and B cells or having direct antiviral effects (Chevalier et al., 2011, Norris et al., 2004, Soghoian and Streeck, 2010). Furthermore, studies have illustrated that there is a greater presence of HIV-specific CD4+ T cells in individuals that are able to spontaneously control viral replication without antiretroviral therapy (Zhang and Bevan, 2011, Rosenberg et al., 1997). Our current understanding of CD4+ T cell immunology in the setting of HIV-1 infection is skewed towards studies conducted mainly in subtype B infection, which is the predominant subtype in North America and Europe. However, in sub-Saharan Africa, which carries the highest burden of the epidemic, clade C virus is the predominant subtype. Our study provides additional insight to a previous study conducted on CD4+ T cell responses in subtype C infection by Ramduth et al. (2009). This study aims to identify HIV-specific CD4+ T cell responses to HIV subtype C infection at single epitope level. The identification of HIV-1

CD4+ T cell epitopes and a thorough understanding of the relationship between these responses and control of viremia is critical in designing a vaccine that could elicit CD4+ T cell responses protective against subtype C HIV-1 infection.

The first objective was addressed by the following goals:

(i) Evaluate the immunodominant regions of the HIV-1 proteome targeted by HIV-specific CD4+ T cells during the first year of acute HIV-1 infection and determine how these responses evolve over time.

(ii) Define the individual peptide sequences that are targeted by HIV-specific CD4+T cell responses in chronic HIV-1 infection. Here, overlapping peptide pools that span every encoded HIV protein was used, allowing for a broader view of the overall immune response to HIV as well as its association to the various parameters surrounding viral infection.

3.2 RESULTS

3.2.1 ACUTE HIV-1 INFECTION

Table 3.1. Clinical characteristics of acutely infected HIV-1 subtype C-infected adults.

Subcohort definition (viral load)	No. of subjects	Median viral load (copies/ml)	Median CD4 cell count (cells/µl) (range)	Gender % (male)	Gender % (female)	Race (% Black)	Median age (year)
Elite controller (EC) **<50 copies/ml**	0	—	—	—	—	—	—
Viremic controller (VC) **51-2,000 copies/ml**	1	1125	571	0	100	100	33
Low-viremic progressor (LVP) **2,001-10,000 copies/ml**	2	4757 (4,363-5,150)	690 (605-774)	0	100	100	25
Intermediate-viremic progressor (IVP) **10,001-50,000 copies/ml**	6	32718 (10,513-44,721)	622 (343-790)	50	50	100	29.5 (16-58)
High-viremic progressor (HVP) **>50,001 copies/ml**	6	153607 (72,104-437,020)	356 (171-524)	33	67	100	26.5 (21-66)
	15						

Our acute infection cohort consisted of 15 individuals divided into various groups according to viral load. Controllers were those individuals that were able to spontaneously control viral replication below a level of 2000 HIV RNA copies/ml in the absence of any antiretroviral therapy. Progressors were those individuals that indicated varying levels of viral replication and progression to clinically noticeable disease. The groups were further divided as seen in Table 3.1 above. All individuals were of Zulu/Xhosa ethnicity between the ages of 25 - 33 years old. Majority of the subjects were female.

3.2.1.1 Gag and Pol regions of the HIV proteome most frequently targeted in acute HIV-1 infection.

In order to assess the relative contributions of CD4+ T-cell responses to different viral proteins, we investigated the ability of peptide pools representing expressed gene products to induce IFN-γ production inCD8-depleted PBMC. We screened a total of 15 acutely HIV-infected subjects using the IFN-γ ELISPOT megamatrix assay with synthetic peptides spanning HIV-1 Gag, Pol, Env, Nef, Tat, Vpr, Rev, Vif, and Vpu regions. The magnitudes (strength of responses) of HIV-specific CD4+ T cell responses ranged from 3 to 1998 SFU/million (Figure 3.1). Interestingly, all subjects had detectable HIV-specific CD4+ T cell responses (15/15), and the vast majority of patients made a response to more than one protein (14/15, 93%) (Figure 3.1). Importantly, all subjects had strong Gag- and Pol specific T-cell responses, with these proteins contributing to 69% of the total responses made by all individuals, with Gag responses totalling 36% and Pol 33% (Figure 3.2). In contrast, Nef and Env were infrequently targeted, with only 10 and 9 % of responses, and very few responses were detected against HIV accessory proteins (Figure 3.2). These data suggest that the immunodominance of HIV-specific CD4+ T cell responses in acute clade C infection are biased towards Gag and Pol, which are highly conserved regions of the HIV proteome.

The role of HIV-specific CD4+ T cell responses and influence on disease outcome during acute infection has not been well characterized. However, our study was in agreement with recent work by Schieffer et al. (2014), which also showed an immunodominance of Gag-specific CD4+ T cell responses during acute clade B infection in a combined Berlin and Boston cohort. Unfortunately we could not determine CD4+ T cell responses at the single peptide level for acute infection due to limitations in sample availability; however the screening data represented in Figure 3.1 demonstrate the induction of robustCD4+ T cell responses targeting Gag during acute HIV-1 infection.

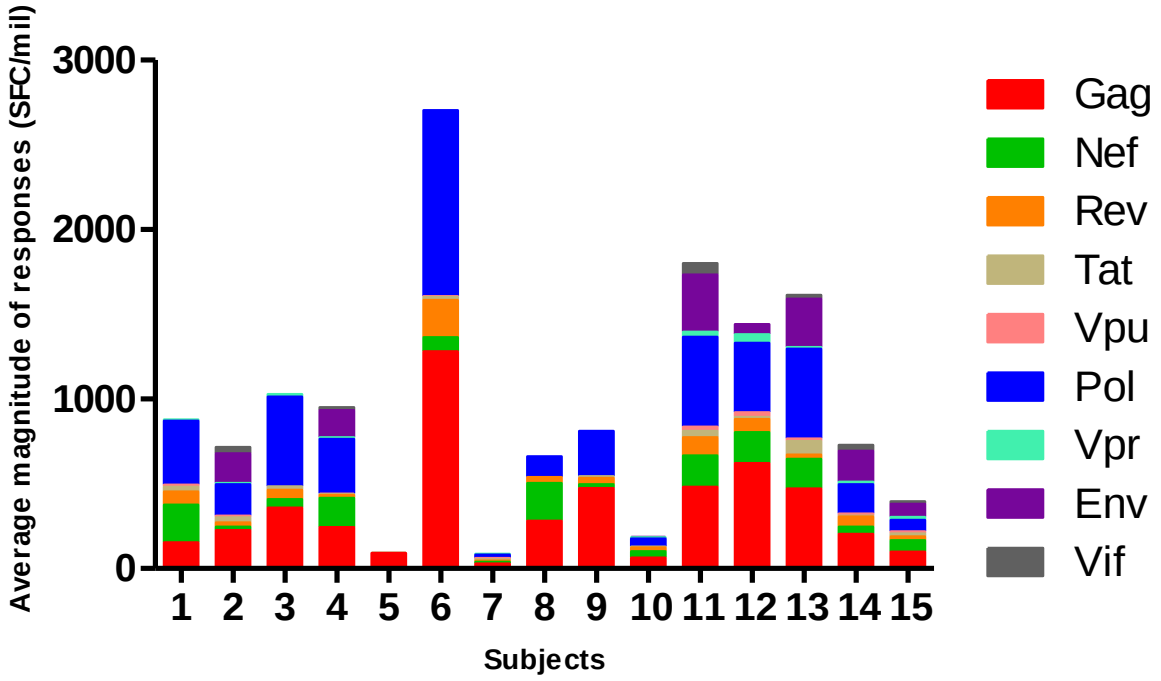
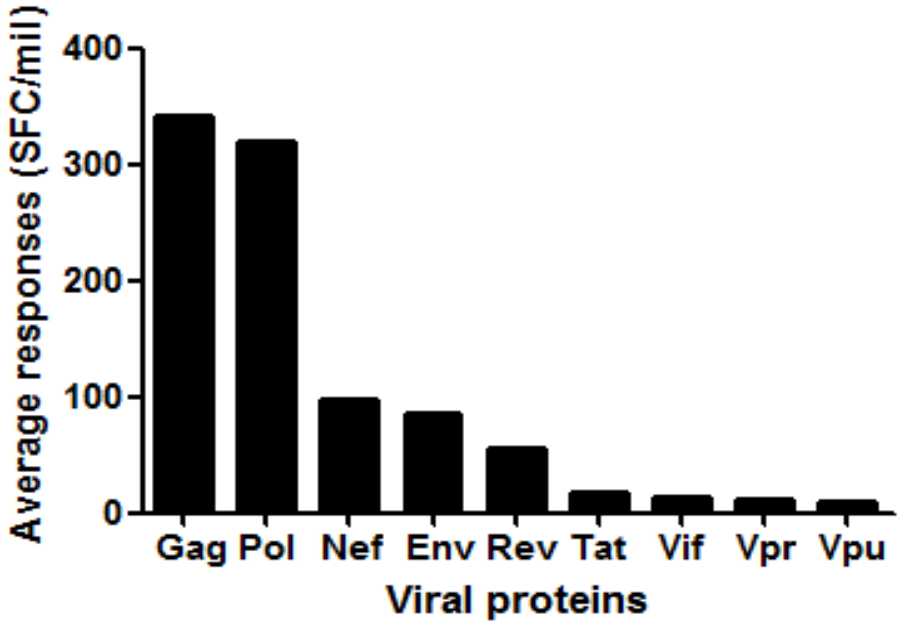


Figure 3.1. HIV protein targeting by CD4+ T cell responses during acute infection (n=15).

A



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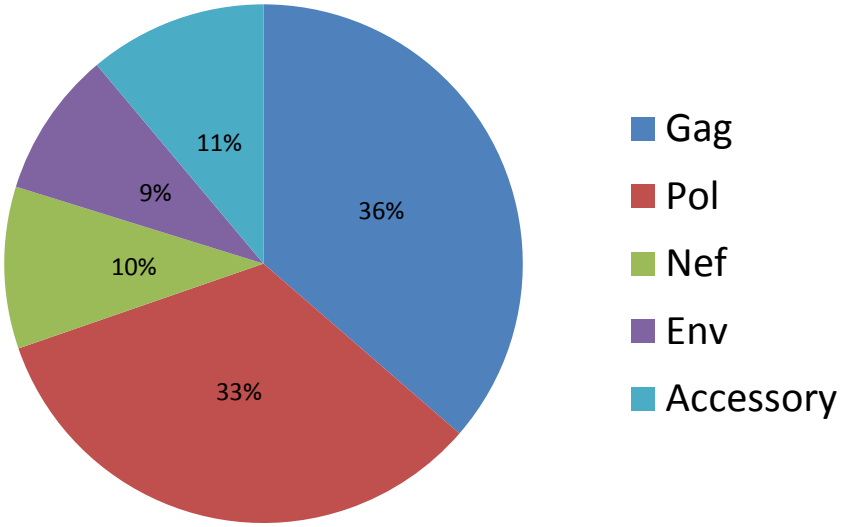

Figure 3.2. (A) Average CD4+ T cell response targeting of all subjects to various viral proteins in acute infection (n=15). (B) Percentage targeting of CD4+ T cell responses across HIV-1 viral proteome.

3.2.1.2 CD4+ T cell targeting across the proteome remains stable over time (range of 1 - 23% variation between time points).

In order to assess the evolution of CD4+ T cell responses over time, longitudinal analysis of CD4+ T cell responses in our acute cohort of 15 subjects was performed, with three separate time points assessed for 12 subjects and two time points analysed for 3 subjects (Figure 3.3). The average protein dominance of HIV-specific CD4+ T cell responses remained stable over time, with Gag-specific responses detected at an average magnitude of 249 SFC/mil, 387 SFC/mil and 402 SFC/mil over the 1-2, 3-11 and 12-36 months respectively. Proteins targeted at high frequencies as early as 4 weeks post infection were recognized at similar frequencies 1 year and even 3 years post infection, suggesting that CD4+ T cell responses continually target the same region of the virus. These data are consistent with observations by (Schieffer et al., 2014).

Interestingly, although the dynamics of HIV-specific CD8+ T cell responses are fairly well characterized very little is known about the evolution of HIV-specific CD4+ T cell responses. While it has been reported that Nef is the most targeted protein by HIV-specific CD8+ T cells in acute infection (Radebe et al., 2014), our study found that Gag and Pol were the most targeted regions of the virus by HIV-specific CD4+ T cells.

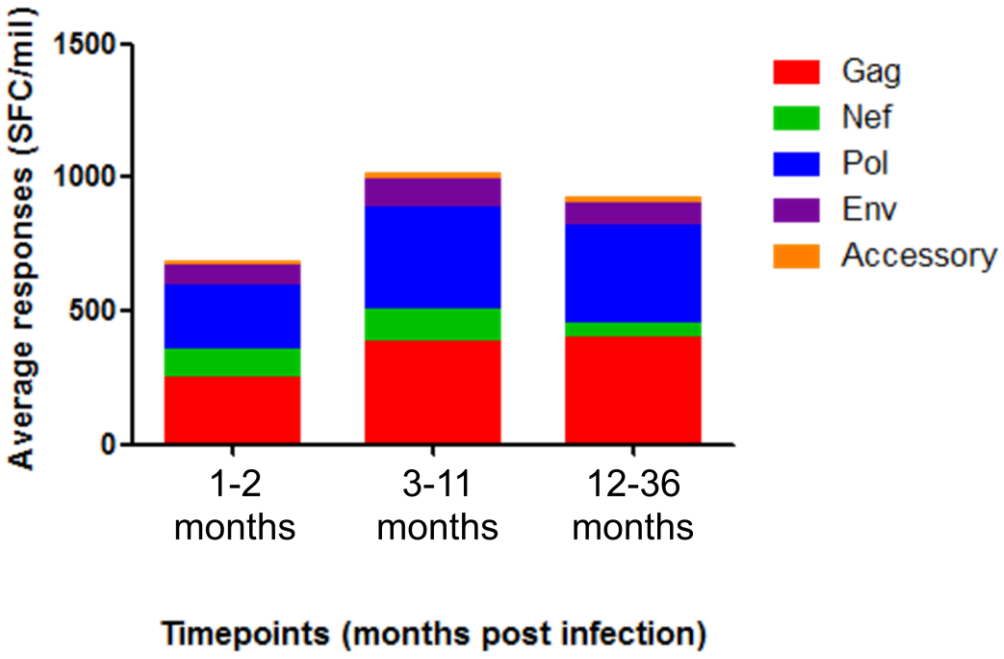

Figure 3.3. Average CD4+ T cell response targeting various viral proteins over a period of 1-36 months post infection (range of time points assessed across all individuals) (n=15).

3.2.1.3 Gag/Pol specific CD4+ T cell responses and their impact on viremia.

Having demonstrated in Figure 3.1-3.3 that Gag and Pol were the most dominant regions of the proteome targeted, we next wanted to analyse whether these responses had an impact on viral load set point. The average viral load between 3 and 12 months post infection was the measure of viral load set point used in this study. Figure 3.4 highlights a significant positive correlation between the total magnitude of CD4+ T cell responses (multiple time points) and viral load set point. Importantly, we found that the magnitude of Gag and Pol-specific CD4+ T cell responses (combined) was significantly associated with viral load set point ($p=0.006$, $r^2= 0.85$). These data suggest the greater the magnitude of Gag/Pol specific response, the higher the viral load set point. The higher viral load set point would indicate faster progression to disease, suggesting a potentially detrimental effect of the induction of Gag/Pol-specific CD4+ T cell responses in acute infection. This could be because HIV-

specific CD4+ T cell responses are preferentially targeted by HIV infection as reported previously (Douek et al., 2002).

Intriguingly, analysis of Gag and Pol individually, rather than combined showed no significant association with viral load set point for Gag responses (albeit close to significance; p= 0.08, Figure 3.5A). However, a significant positive correlation was detected for Pol-specific responses with viral load (p= 0.0013, Figure 3.5B), indicative that the association with high viral load set point was driven by the Pol protein. There was no association of Env-specific CD4+ T cell responses and clinical outcome (Figure 3.5C). It has been previously demonstrated that individuals that have higher Env-specific responses at baseline would initiate HAART significantly earlier than those individuals that had higher Gag-specific responses (Schieffer et al., 2014).

These results need to be interpreted with caution as the analysis of HIV-specific CD4+ T cell responses were conducted using pooled peptides. Results need to be confirmed in confirmatory ELISPOT analysis utilizing single OLPs.

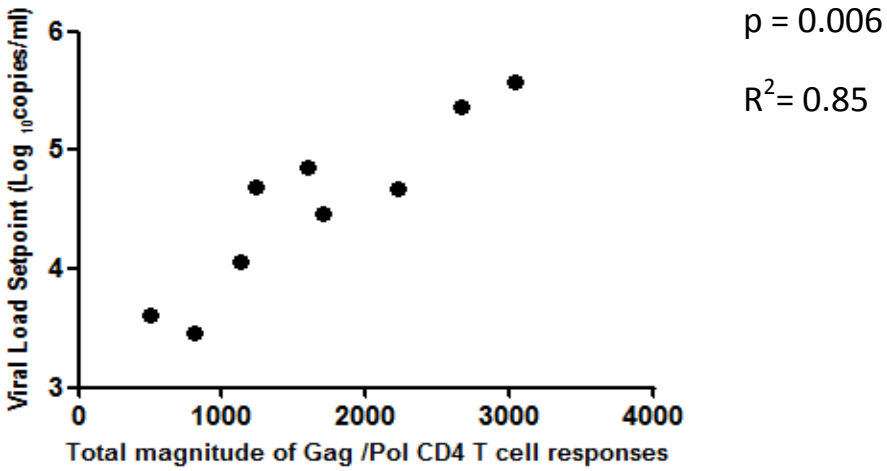

Figure 3.4. Relationship between magnitude of Gag/Pol specific responses and viral load set point (n=9), showing results for individuals where initial time of infection could be identified). Spearman's rank correlation was utilised for statistical analysis.

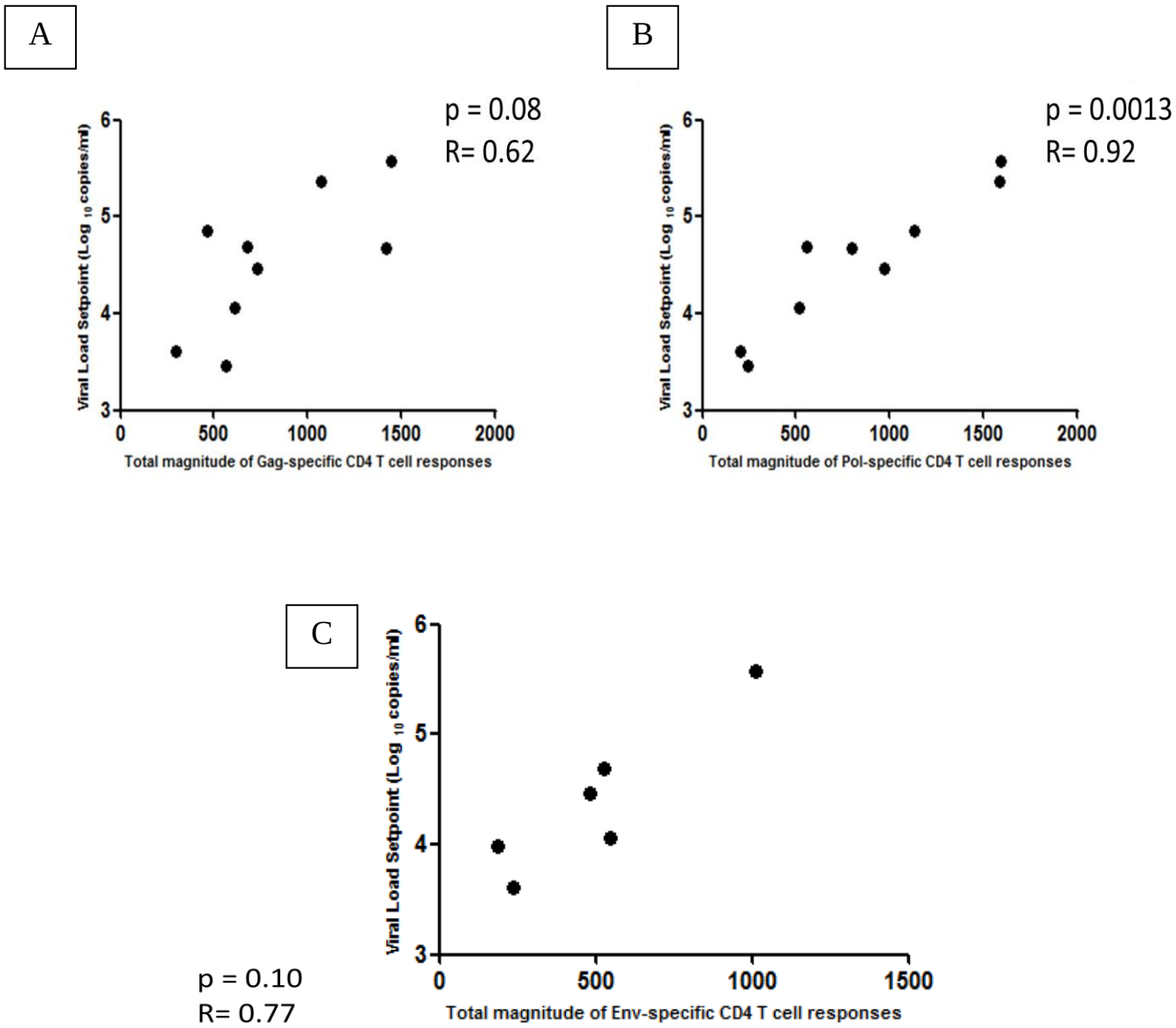

Figure 3.5. Relationship between magnitude of individual Gag (A), Pol (B) and Env (C) specific responses and viral load set point (n=9 for Gag and Pol-specific responses, and n=6 for Env-specific responses), showing results for individuals where initial time of infection could be identified. CD4+ T cell responses were measured across various time points (ranging from 1-36 months post infection). Spearman's rank correlation was utilised for statistical analysis.

3.2.2 CHRONIC HIV-1 INFECTION

Table 3.2. Clinical characteristics of chronically infected HIV-1 subtype C-infected adults.

Subcohort definition (viral load)	No. of subjects	Median viral load (copies/ml) (range)	Median CD4 cell count (cells/µl) (range)	Gender (%)		Race (% Black)	Median age (year) (range)
				Male	Female		
Elite controller (EC) <50 copies/ml	3	41 (20-50)	582 (340-699)	33	67	100	44 (43-55)
Viremic controller (VC) 51-2,000 copies/ml	12	1136.5 (235-2,000)	646.5 (210-1,100)	8	92	100	43 (32-68)
Low-viremic progressor (LVP) 2,001-10,000 copies/ml	11	6130 (2,100-9600)	513 (225-676)	9	91	100	38 (31-42)
Intermediate-viremic progressor (IVP) 10,001-50,000 copies/ml	16	24300 (11,933-49,000)	365 (217-773)	38	63	94	43 (35-59)
High-viremic progressor (HVP) >50,001 copies/ml	16	202000 (88,051-45,40000)	383.5 (75-934)	13	88	94	41 (22-51)
	55						

Our chronic infection cohort consisted of 55 individuals divided into various groups according to viral load. As with the acute infection cohort, controllers were those individuals that were able to spontaneously control viral replication below a level of 2000 HIV RNA copies/ml in the absence of any antiretroviral therapy. Progressors were those individuals that indicated varying levels of viral replication and progression to clinically noticeable disease. The groups were further divided as seen in Table 3.2 above. Majority of the individuals were of Zulu/Xhosa ethnicity between the ages of 38 - 44 years old. The cohort consisted of mainly female participants.

3.2.2.1 Screening of CD4+ T cell targeting across entire HIV-1 proteome in chronic infection.

To better understand the role of HIV-specific CD4+ T cell responses, we next investigated the presence and specificity of these responses in the setting of chronic clade C HIV-1 infection. For this analysis, we utilized a cohort of 55 chronically HIV-infected individuals (that was separate from the acute cohort). In the chronic cohort, HIV-specific CD4+ T cell responses were screened against a panel of 410 pooled peptides (megamatrix) spanning the entire HIV-1 clade C consensus sequence using the IFN-γ ELISPOT assay. Initial screening was conducted using pooled peptides due to limitations with sample availability. Results illustrate a representation of all proteins in all subjects tested, with Gag being the most dominant region targeted (100%, 31/31) (Figure 3.6).Pol was the second most dominant protein targeted, similar to what we observed in acute infection (Figure 3.7) Strikingly, all patients demonstrated more than one detectable HIV-specific CD4+ T cell responses.

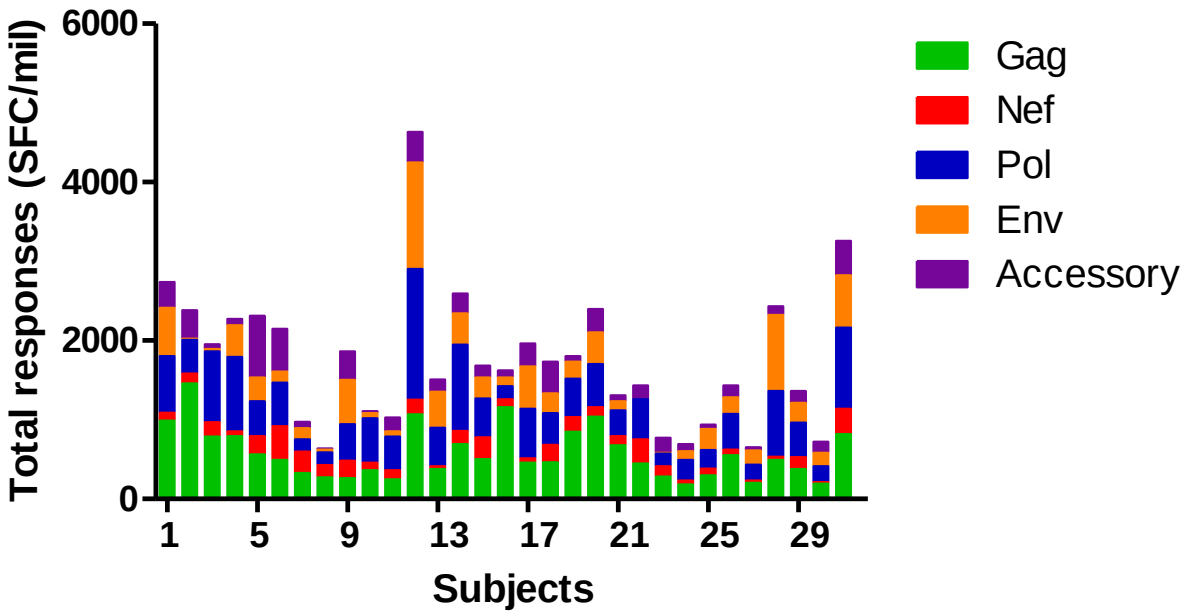

Figure 3.6. Megamatrix screening data indicating n=31 responders, subdivided into CD4+ T cell responses per patient across the HIV proteome.

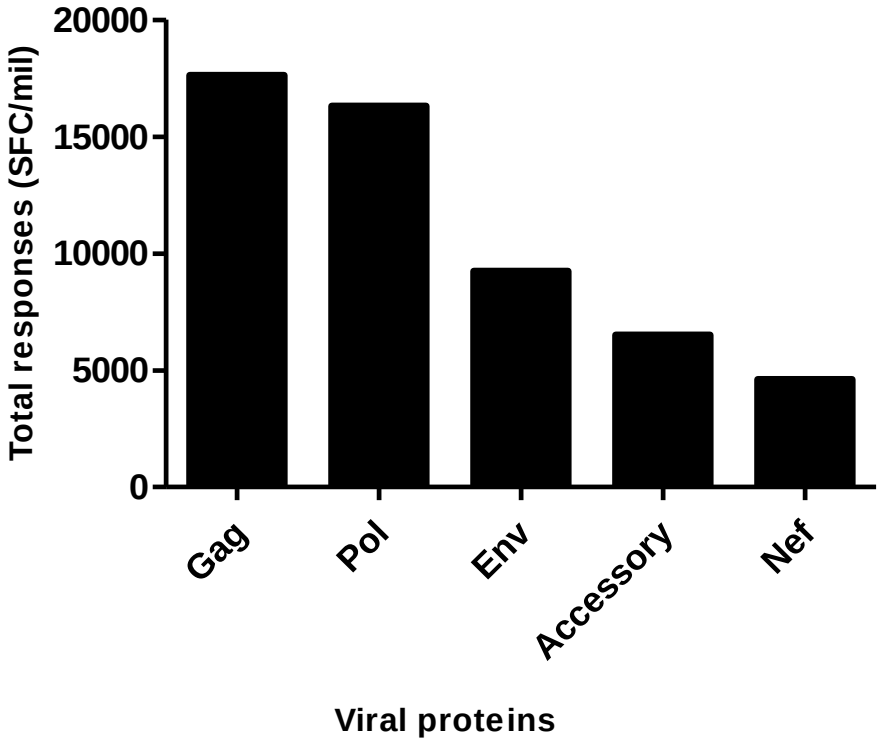

Figure 3.7. Megamatrix screening data indicating hierarchy CD4+ T cell responses per HIV-1 viral protein (n=31 responders).

3.2.2.2 No significant relationship between the breadth and magnitude of HIV-specific CD4+ T cell responses and disease outcome i.e. viral load and CD4 count

We next evaluated the relationship between HIV-specific CD4+ T cells and to two well defined markers of HIV disease progression viz. viral load and absolute CD4 counts (Mellors et al., 1997). Peptides identified in the megamatrix screening were tested at single peptide level, assessing the presence and specificity of HIV-specific IFN-γ CD4+ T cell responses in a cohort of 31 chronically infected individuals (subjects not exhibiting a response in the megamatrix screening were not tested with single OLPs). Peptide sequences that induce HIV-specific T cell responses in chronic HIV-1 infection were identified.

The breadth (number of responses) and magnitude (strength of responses) of overall HIV-specific CD4+ T cell responses did not correlate with contemporaneous viral load (Figure 3.8). Given the immunodominance of Gag responses, we examined whether Gag-specific CD4+ T cell responses impacted viral load and found no significant correlation. However, here too no significant correlation was observed between breadth and magnitude of Gag-specific responses and contemporaneous viral load (Figure 3.9). These data are contrary to what has been reported in other studies such as (Ramduth et al., 2009, Ranasinghe et al., 2012). Previous data suggested an inverse correlation between the total breadth of HIV-specific CD4+ T cell responses and viral load (Ranasinghe et al., 2012). Furthermore, the magnitude of responses to Gag also showed a strong inverse correlation with viral load (Ranasinghe et al., 2012). A possible explanation for our results could be our small sample number, where as we only studied 20 subjects, Ramduth et al. studied 373 subjects and Ranasinghe et al. studied 93 subjects. Other possible explanations for the difference could be the methods used, where Ramduth et al. used ICS to screen for Gag-specific IFN-γ CD4+ T cell responses.

Out of the 55 subjects tested in our chronic infection cohort, 35 individuals showed no HIV-specific CD4+ T cell response. We therefore investigated if there was a difference in the level of persistent viremia between individuals with detectable CD4+ T cell responses to those with no detectable responses. We examined the relationship between contemporaneous viral load and total CD4+ T cell responses (Figure 3.10 A) as well as Gag-specific responses (Figure 3.10 B) between the groups. There was no significant difference observed between both total response and Gag-specific response to contemporaneous viral load in these subjects. Once again, a limitation of these results is the sample size available for analysis.

In our chronic infection cohort we had 15 controllers and 40 progressors. We therefore investigated if durable CD4+ T cell responses were associated with prolonged virus

suppression. We evaluated the CD4+ count and breadth of total responses between the groups. Consistent with previous reports (Mellors et al., 1997), controllers had a significantly higher ($p = 0.01$) CD4 count as compared to progressors (Figure 3.11). However, no significant difference between the breadth of total responses was observed between controllers and progressors (Figure 3.12).

Overall, results from our chronic infection cohort illustrate that Gag-specific CD4+ T cell responses are the most dominant; similar to what is observed in the acute infection cohort. Furthermore, no significant relationship between the breadth and magnitude of HIV-specific CD4+ T cell responses and disease outcome (viral load) was observed. Further investigation using an increased sample size needs to be conducted.

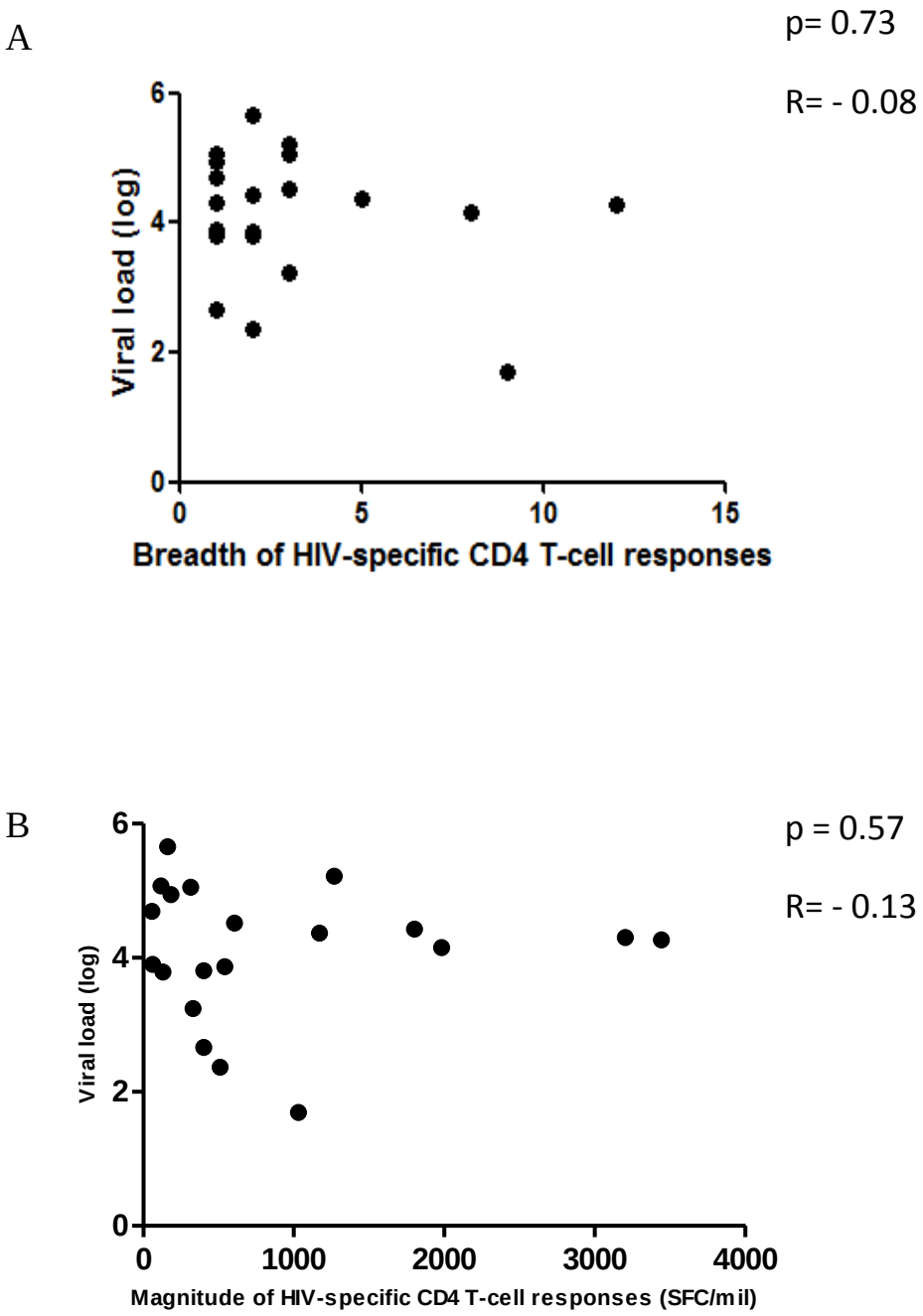

Figure 3.8. Characterization of the relationship between breadth (A) and magnitude (B) of HIV-specific CD4+ T cell responses to contemporaneous viral load (n=20). Spearman's rank correlation was utilised for statistical analysis.

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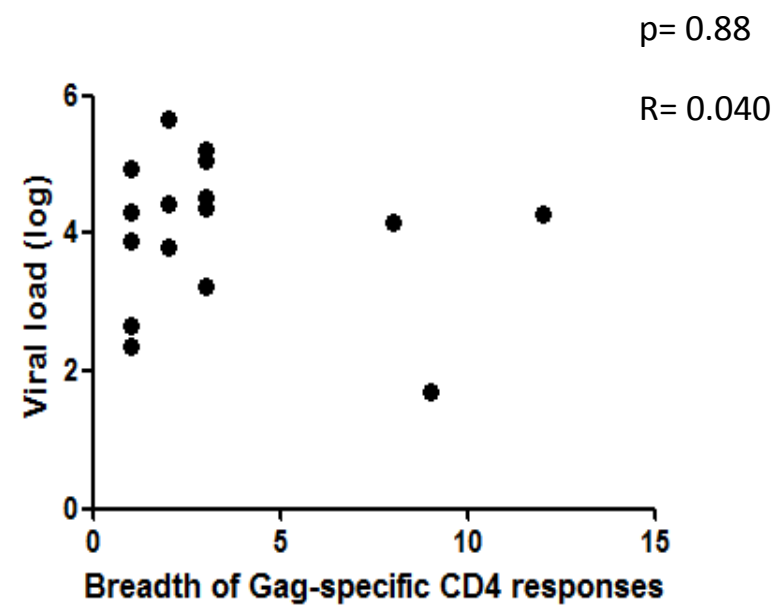

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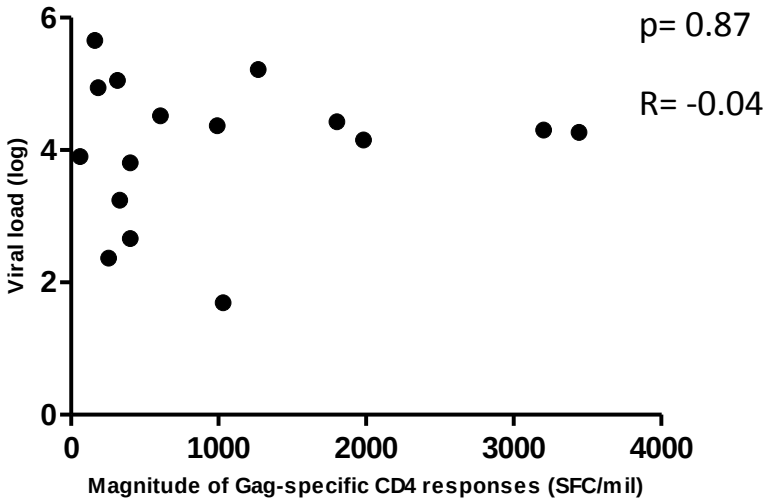

Figure 3.9. Characterization of the relationship between breadth (A) and magnitude (B) of Gag-specific CD4+ T cell responses to contemporaneous viral load (n=16). Spearman's rank correlation was utilised for statistical analysis.

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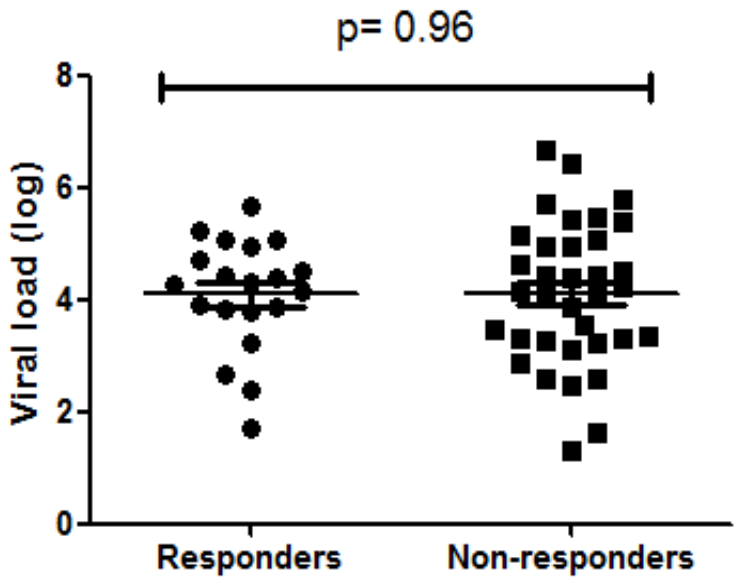

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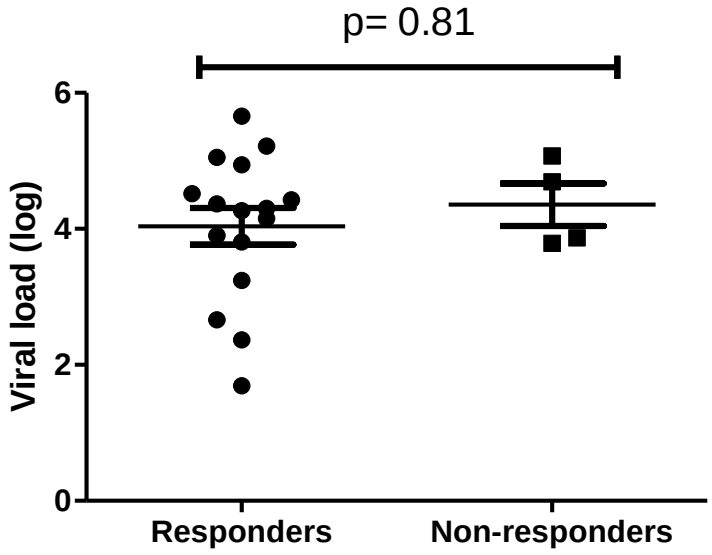


Figure 3.10. (A) Comparison of differences in viral load between responders (n=20) and non-responders (n=35). (B) Comparison of differences in viral load between Gag responders (n=16) and Gag non-responders (n= 4). Two-tailed t-tests were utilised for statistical analysis.

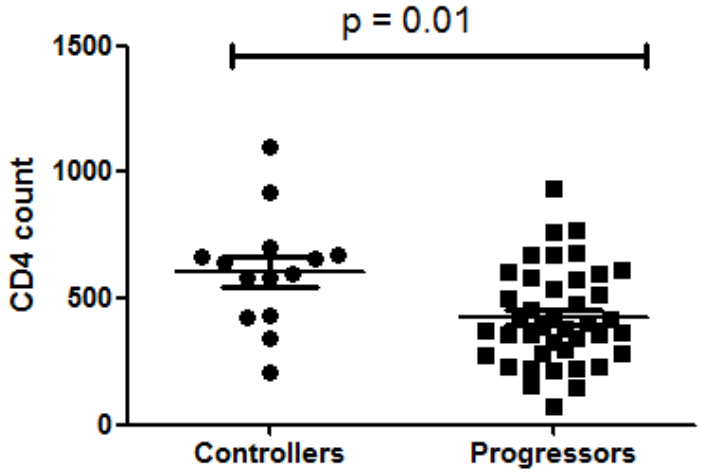


Figure 3.11. Comparison of differences in CD4 count between controllers (n=15) and progressors (n= 40). Two-tailed t-test was utilised for statistical analysis.

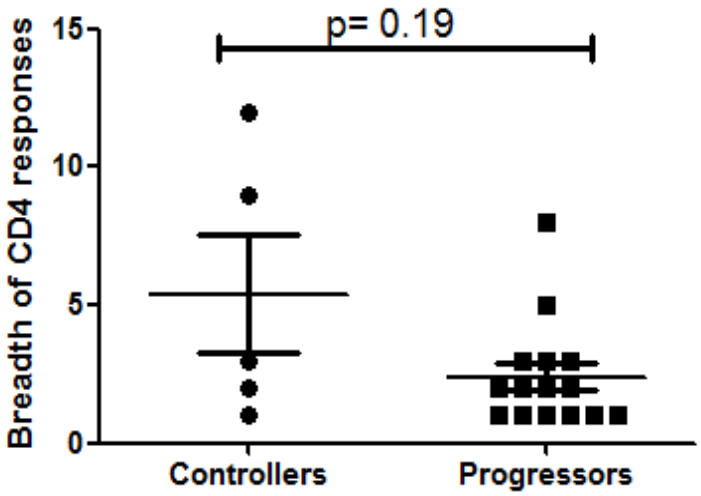


Figure 3.12. Comparison of differences in breadth of CD4+ T cell responses between controllers (n=5) and progressors (n=15). Two-tailed t-test was utilised for statistical analysis.

3.2.2.3 Immunodominance hierarchy of CD4+ T cell responses in chronic infection.

We next characterized epitope specificity of HIV-specific CD4+ T cell responses to individual peptides. Results from confirmatory IFN-γ ELISPOT assays identifying peptides at single peptide level were used to define the HIV-specific CD4+ T cell epitope targeting in chronic clade C HIV infection. HIV-specific CD4+ T cell responses in chronic clade C infection dominantly target the Gag region (Figure 3.12). The most dominantly targeted region in Gag was within the p24 protein (15/40 peptides). This region of Gag has also been shown to be immunodominant for HIV-specific CD8+ T cell responses and has been previously been associated with viral control (Streeck et al. 2007). The dual dominant targeting by CD8+ and CD4+ T cells could have a possible role in enhancing viral control. Our data showed that OLP-41 is the most dominantly targeted peptide making up 15% of all the measured responses in our cohort (Table 3.1). The second most commonly targeted epitope was within OLP 37 (9%) and the third was OLP 40 making up 9% of all detectable responses (Table 3.1). The p17 (9/40) region of Gag was the next most dominantly targeted region. Additional immunodominant epitopes were located within the Gag p15 (5/40) and Nef region of the protein (7/40 peptides) (Table 3.1). The results highlight a large number of detectable HIV-specific CD4+ T cell responses across the HIV proteome, dominantly targeting Gag and Nef (Figure 3.13). Concordant with what has been previously reported in, chronic, untreated clade C HIV-1 infection, IFN-γ – secreting Gag-specific CD4+ T cell responses are immunodominant and are directed at multiple distinct epitopes(Ramduth et al., 2009). Surprisingly, epitopes in the envelope region were rarely targeted in clade C infection as compared to multiple epitope targeting in this region in clade B (Ranasinghe et al., 2012). Further analysis into the lack of response targeting this part of the proteome needs to be conducted. One possible explanation for the paucity of detectable Env responses could be

because we used frozen PBMCs for our analysis, with the highly conserved responses being poorly preserved in frozen samples.

Table 3.3. Most frequently targeted peptides in clade C infection (% targeting of specific peptide in the tested population)

% targeting each CD4+ peptide	Protein	Peptide	OLP Sequence
15	p24	41	YVDRFFKTLRAEQATQDV
9	p24	37	WIILGLNKIVRMYSPVSI
9	p24	40	GPKEPFRDYVDRFFKTLR
7	p17	6	ASRELERFALNPGLL
7	p24	22	WVKVIEEKAFSPEVIPMF
7	p24	25	GATPQDLNTMLNTVGGH
4	p17	2	RGGKLDKWEKIRLRPGGK
4	p17	4	GKKHYMLKHLVWASREL
4	p17	5	KHLVWASRELERFAL
4	p17	7	ERFALNPGLLETSEGCK
4	p17	14	IEVRDTKEALDKIEEEQNK
4	p24	46	TILRALGPGASLEEMMTA
4	Nef	81	KKRQEILDLWVYHTQGYF
2	p17	11	TGTEELRSLYNTVATLY
2	p17	12	SLYNTVATLYCVHAGIEV
2	p17	13	LYCVHAGIEVRDTKEAL
2	p17	16	NKSQQKTQQKAAADKGKV
2	p24	18	GKVSQNYPIVQNLQGQMV
2	p24	21	PRTLNAWVKVIEEKAF
2	p24	30	LHPVHAGPIAPGQMREPR
2	p24	32	MREPRGSDIAGTTSTL
2	p24	38	IVRMYSPVSILDIKQGPK
2	p24	39	SILDIKQGPKEPFRDYV
2	p24	42	LRAEQATQDVKNWMTDTL
2	p24	44	MTDTLLVQNANPDCKTIL
2	p24	45	NANPDCKTILRALGPGA
2	p15	48	ACQGVGGPSHKARVLAEA
2	p15	57	WKCGKEGHQMKDCTERQA
2	p15	58	QMKDCTERQANFLGKIW
2	p15	62	FLQSRPEPTAPPAESFRF
2	p15	63	TAPPAESFRFEETTPAPK
2	Nef	67	MGGKWSKCSIVGWPAIR
2	Nef	76	EVGFPVRPQVPLRPMTFK
2	Nef	77	QVPLRPMTFKGAFDLSFF
2	Nef	78	FKGAFDLSFFLKEKGGL
2	Nef	80	GLEGLIYSKKRQEILDLW
2	Nef	82	LWVYHTQGYFPDWQNY
2	RT	168	GMDGPKVKQWPLTEEKIK
2	Integrase	252	GYIEAEVIPAETGQETAY
2	Vpr	272	IWKGPAKLLWKGEGAVVI

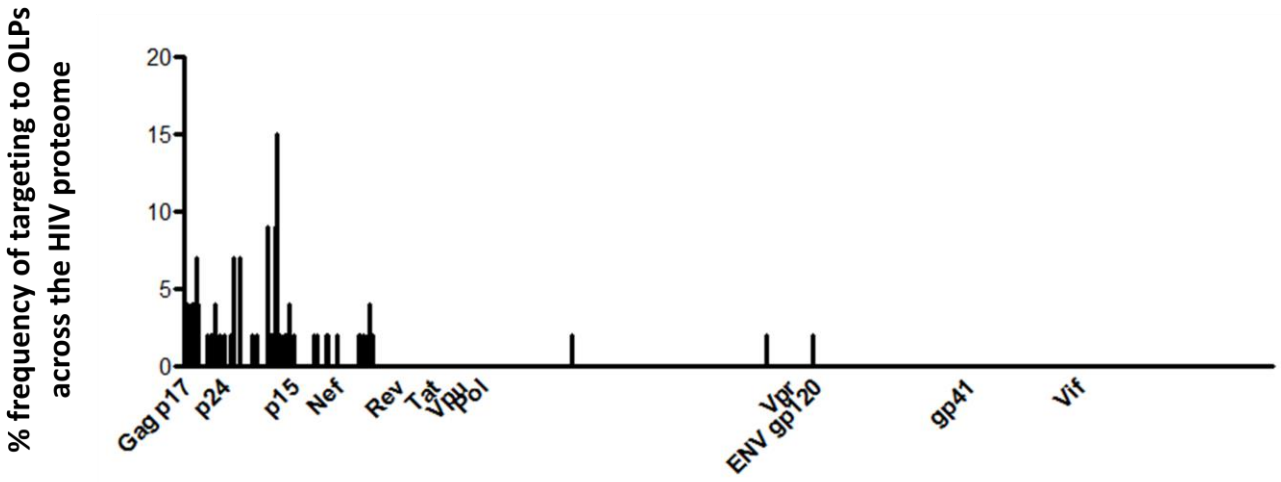


Figure 3.13. Percentage frequency targeting of HIV-specific CD4+ T cell responses to overlapping peptides across the HIV-1 proteome.

3.3 FUNCTIONAL QUALITIES OF CD4+ T CELLS IN CHRONIC SUBTYPE C INFECTION

3.3.1 BACKGROUND

Th cells play important roles in coordinating adaptive immune responses. They carry out functions principally through the secretion of cytokines and chemokines that activate and/or recruit target cells (Zhu and Paul, 2008). The four CD4 T cell subsets mediate various responses within an individual. Th1 cells are known to mediate immune responses against intracellular pathogens. Th2 cells promote host defence against extracellular parasites. Th17 cells mediate immune responses against extracellular bacteria and fungi. Lastly, Treg cells are critical in the maintenance of self-tolerance and in regulation of immune responses (Figure 3.14) (Zhu and Paul, 2008) .

More recently CD4+ T-follicular helper cells (Tfh) have been established as a distinct CD4 T cell type, shown to be essential for B cell help *in vivo,* highlighting the importance of these cells for protective immunity (Crotty, 2011). Tfh cells are essential for the development of antigen-specific B cells within germinal centres (GC). Tfh cells through the interaction of co-stimulatory receptors, provide important soluble factors (i.e. IL-4, IL-21) to promote survival, isotype switching and selection of high affinity memory B cells and plasma cells (Crotty, 2011). One of the major challenges surrounding Tfh cells is understanding the dynamics between Tfh cells in the lymph nodes as compared to those in the periphery, described as "peripheral Tfh". Studies by Boswell et al., (2014) have demonstrated that while sharing some characteristics with Tfh cells, peripheral Tfh cells may not embody a good substitute in studying T- and B cell dynamics within lymphoid tissue. All things considered, the relationship between CD4 T cells and B cells during HIV infection, specifically with relation to the generation of broadly neutralizing antibodies remains a key consideration in the study of HIV-1 infection.

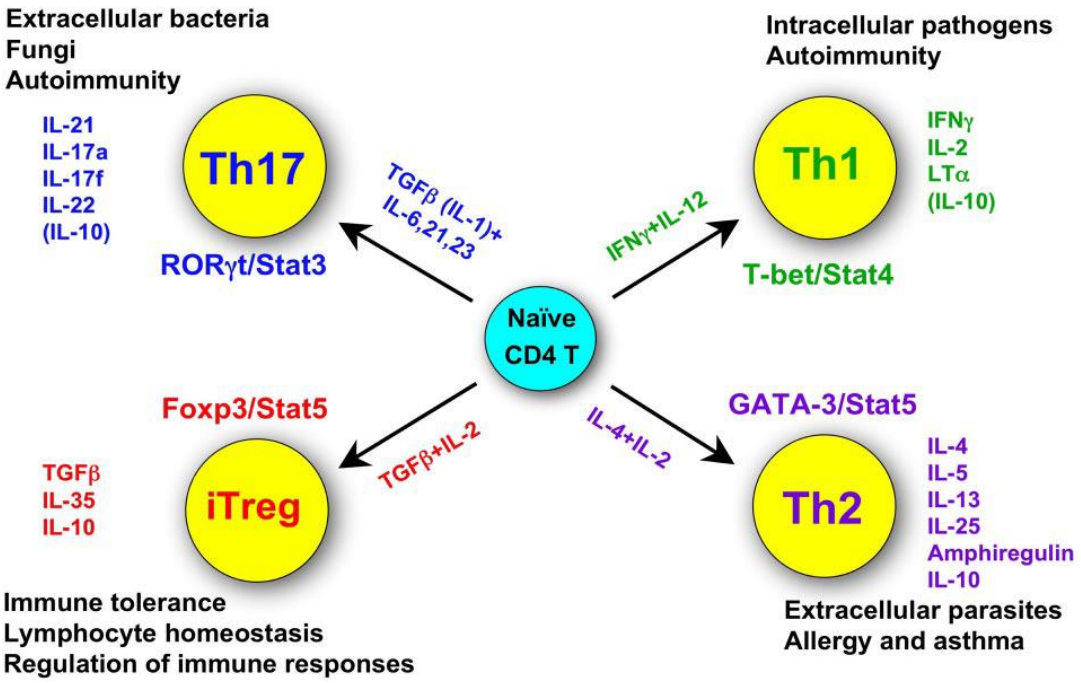


Figure 3.14. Summary of the four CD4+ T cell subsets; indicating functions, unique products, characteristic transcription factors and important cytokines responsible for their fate determination. Reproduced from (Zhu and Paul, 2008).

Here, in this study, we focus on the cytokines secreted by HIV-specific CD4 T cells to better understand their potential contribution to HIV-1 control through direct antiviral activity or 'helper' functions. We looked at the following cytokines in particular:

IFN-γ: one of the principal secretions of Th1 cells. IFN-γ is important in the activation of macrophages, increasing their microbicidal activity. IFNγ is also known to play an important role in the activation of cytotoxic T cells to destroy virally infected cells. Furthermore, IFNγ induces further production and secretion of IL-2 in activated APCs, thus strengthening the Th1 response and suppressing any Th2 response (Moir and Fauci, 2009, Suzuki et al., 1988).

IL-2: production of IL-2 is important for CD4 T cell memory. In addition, IFN-γ, IL-2 secreting cells are considered as the precursors of Th1 memory cells (Zhu and Paul, 2008, Darrah et al., 2007).

TNF-α: one of the greatly studied pro-inflammatory cytokines because of the key role it plays in the origin and progression of HIV-1(Bahia and Silakari, 2010).

IL-17: has the ability to induce many inflammatory cytokines. Furthermore, IL-17 recruits and activates neutrophils during host immune response to extracellular bacteria and fungi (Korn et al., 2007, Zhou et al., 2007).

IL-13: has been shown together with IFN-γ to be associated with viral suppression and a lack of HIV-1 disease progression when secreted by activated T cells (Bailer et al., 1999)

IL-21: is a stimulatory factor for Th17 differentiation, also acting on CD8 T cells, B cells, NK cells and dendritic cells (Korn et al., 2007, Veldhoen et al., 2008, Zhou et al., 2007).

Having provided examples of cytokines studied in this thesis, it is important to remember that the balance between different functions of a single cytokine, or between the synergistic and antagonistic actions of different cytokines, determines the ultimate effect of cytokine changes in an HIV infected person.

The second objective was then to:

(i) Evaluate functional qualities of CD4+ T cells using intracellular cytokine staining, flow cytometric analysis

3.3.2 RESULTS

Having elucidated IFN-γ responses to various regions of the HIV-1 proteome using the ELISPOT assay, we wanted to further assess the effector functions of HIV-specific CD4+ T cell responses to HIV-1 infection using intracellular cytokine staining. We evaluated functional qualities of CD4+ T cells of 25 HIV-1 chronically infected individuals for whom we had available samples using flow cytometric analysis. Peptide pools of the Gag, Nef and Env region of the proteome were analysed. This analysis enables a more accurate quantification of antigen-specific T cells.

Figure 3.15 illustrates a HIV-specific CD4+ T cell IFN-γ andTNFα response to Gag pool stimulation.

IFN-γ (Figure 3.16), IL-2 (Figure 3.17) and TNFα (Figure 3.18) were the most frequently secreted cytokines whereby 18 out of 25 individuals studied secreted at least one of these cytokines. Most of these responses were induced upon Gag stimulation. As shown in Figure 3.16, Gag stimulations induced moreIFN-γ compared to Env ($p < 0.01$). Significant differences were also observed in TNFα secretion between all stimulations (Gag, $p < 0.001$, Gag- Nef, $p < 0.01$ and Gag-Env, $p < 0.01$). However, there was no difference in responses for IL-2 production across different HIV proteins.

Collectively, these data confirm that Gag is the most immunodominant protein targeted by HIV-specific CD4+ T cells during the chronic stage of infection. Moreover, these data go beyond previous work on untreated clade C infection (Ramduth et al., 2009) to demonstrate the ability of Gag-specific CD4+ T cell responses to secrete multiple Th1 cytokines. These additional functional data allows for a better understanding of the role of HIV-specific CD4+ T cells in the setting of HIV-1 infection. Further work is now ongoing to assess whether specific cytokines are linked with immune control of clade C infection. Previous literature in

clade B infection has suggested that polyfunctional secretion (specifically IFN-γ+ and IL-2+) by HIV-specific CD4+ T cells is enhanced in elite controllers, although this is arguably a consequence, rather than a cause of superior immune control (Harari et al., 2004).

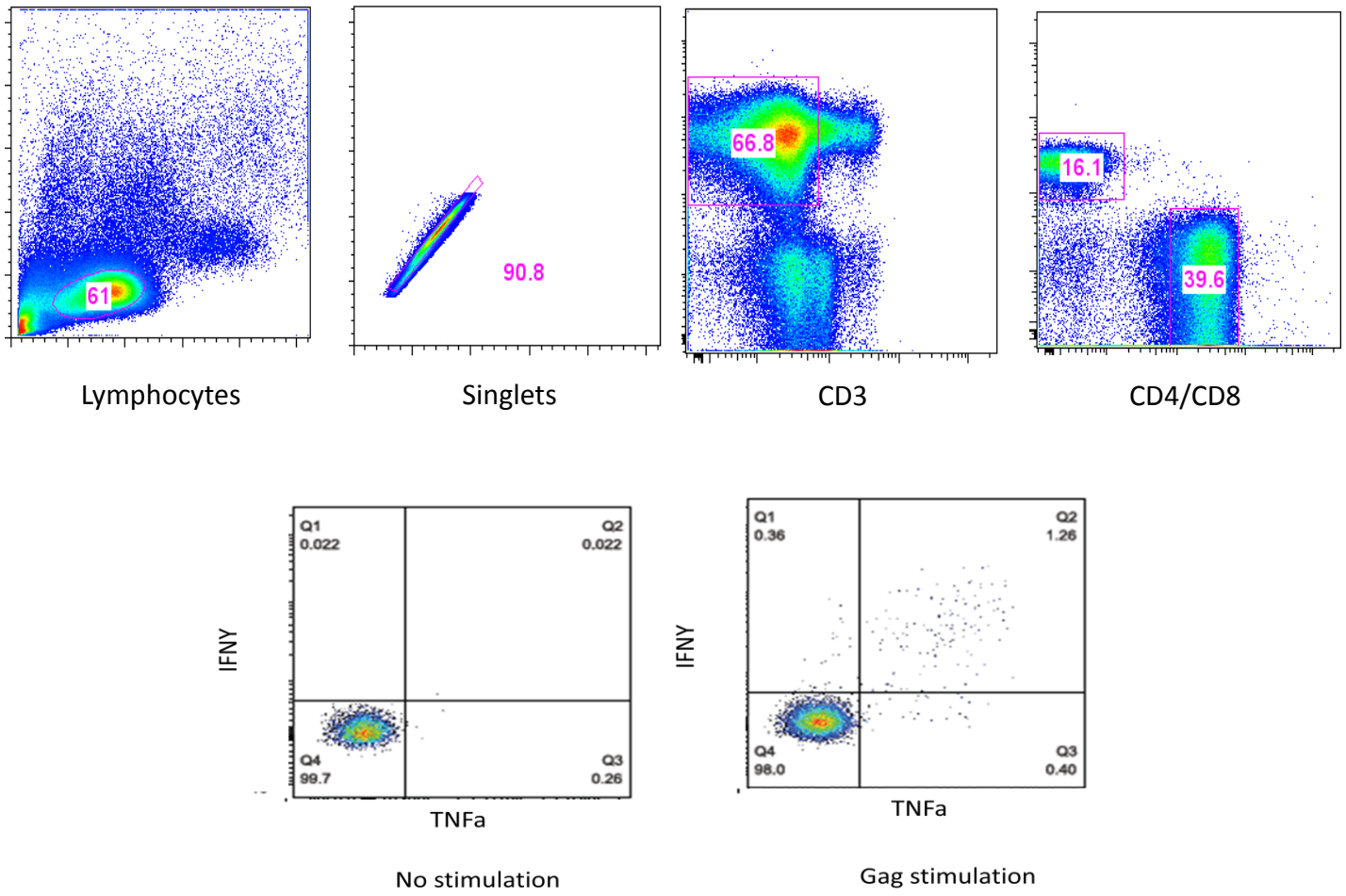


Figure 3.15. Gating strategy and graphical representation indicating a HIV-specific CD4+ T cell response to Gag pool stimulation.

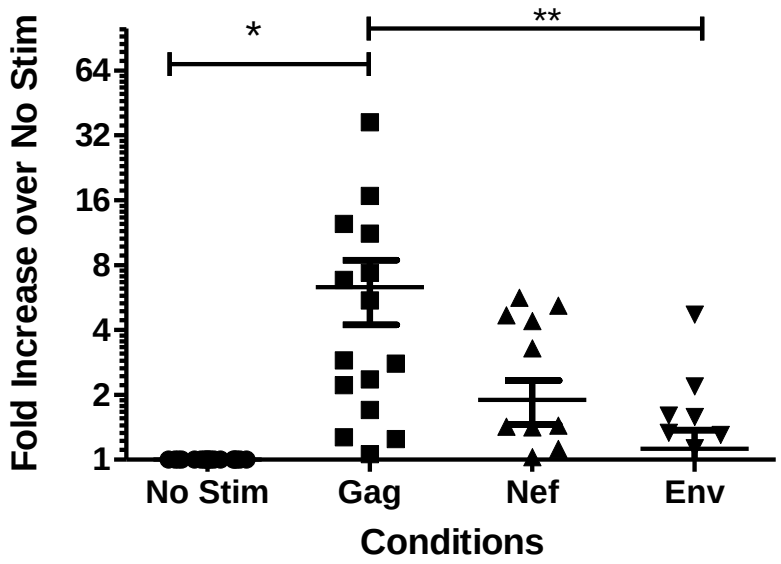


Figure 3.16. Functional response indicating IFN-γ secretion of HIV-specific CD4+ T cell responses to pooled peptides across the three regions of the HIV-1 proteome (n=18). One-way ANOVA was utilised for statistical analysis.

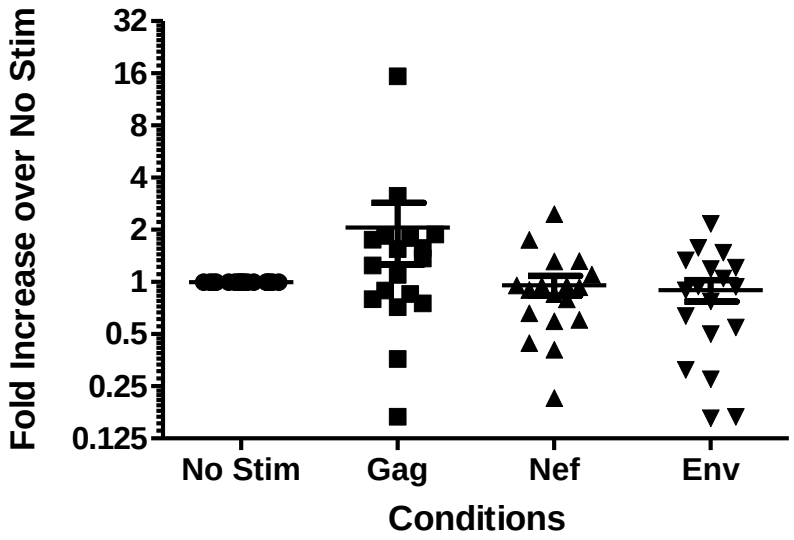


Figure 3.17. Functional response indicating IL-2 secretion of HIV-specific CD4+ T cell responses to pooled peptides across the three regions of the HIV-1 proteome (n=18).

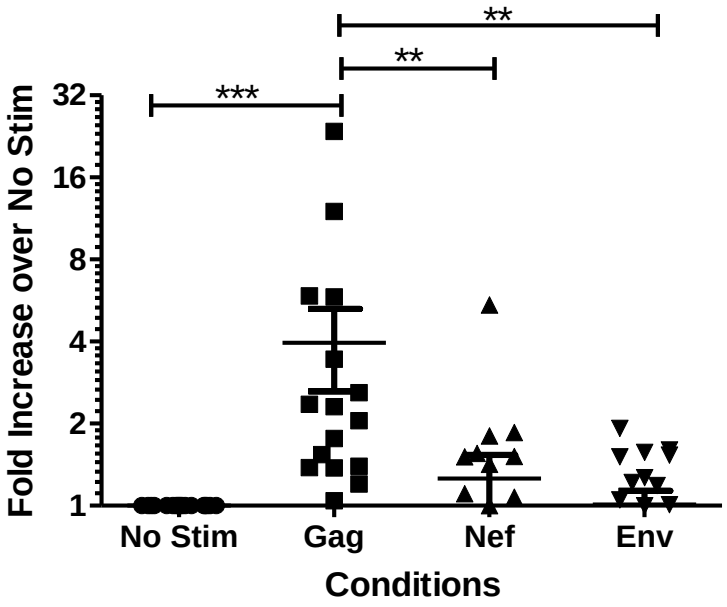


Figure 3.18. Functional response indicating TNFα secretion of HIV-specific CD4+ T cell responses to pooled peptides across the three regions of the HIV-1 proteome (n=18). One-way ANOVA was utilised for statistical analysis.

CHAPTER 4: HLA CLASS II RESTRICTION CHARACTERISTICS OF HIV-SPECIFIC CD4+ T CELL RESPONSES IN CHRONIC SUBTYPE C INFECTION

4.1 BACKGROUND

Having described the vital role that CD4+ T cells play in adaptive immunity during acute and chronic HIV-1 infection, we next studied the role of host genetics in the setting of HIV-1 infection. There is strong evidence of an association between CD8+ T cells restricted by HLA class I alleles and HIV disease outcome, with particular alleles being identified for their role in conferring protection or rapid progression to AIDS (Kiepiela et al., 2004, Carrington and O'Brien, 2003, Gao et al., 2001). HLA class II molecules present antigens to CD4+ T cells, with their role being considered central to the development and maintenance of HIV-specific CD4+ T helper cells (Julg et al., 2011). Despite knowledge of the potential role of HLA class II alleles in HIV-1 infection, there have been only a few studies that have identified a few class II alleles associated with slower HIV disease progression (Chen et al., 1997, Malhotra et al., 2001, Ferre et al., 2010, Ndung'u et al., 2005, Ranasinghe et al., 2013). Most studies have focused on specially selected patient subsets e.g. long-term non progressors. However, the role of HLA class II alleles in chronic progressive HIV infection, particularly in clade C infection is lesser studied and for the most part remains unknown.

The influential role of genetic effects on disease progression will allow for greater insight into HIV pathogenesis. Furthermore, analysis of clade B CD4+T cell epitopes binding to HLA class II molecules has revealed a high degree of promiscuity amongst the epitopes (Ranasinghe et al., 2013). This property may be beneficial in vaccine design, with these epitopes being able to induce responses amongst a wide array of HLA Class II types.

The HLA-DRB1 restriction assay utilized in these experiments has not been previously used to test restriction in clade C infection. The method was adopted from Ranasinghe et al.

(2013). In brief, frozen CD8-depleted PBMCs were used to grow 102 HIV-specific CD4+ T cell lines recognizing a wide range of peptide specificities from 27 HIV-infected individuals, with known CD4+ T cell responses and tested in functional HLA-DRB1 restriction assays. The T cell lines were successfully expanded and simultaneously assessed for their specificity to a particular peptide and HLA-DRB1 restriction using a panel of mouse L cell line fibroblasts stably transfected with a plasmid encoding a single recombinant human HLA-DRB1 allele. The HLA class II typing of individuals were previously elucidated and therefore restriction was tested against an L cell line stably transfected with a matching HLA-DRB1 variant.

The third objective was as follows:

(i) Define the class II HLA restriction characteristics and epitope specificity of HIV-specific CD4+ T cell responses using the HLA-DRB1 restriction assay.

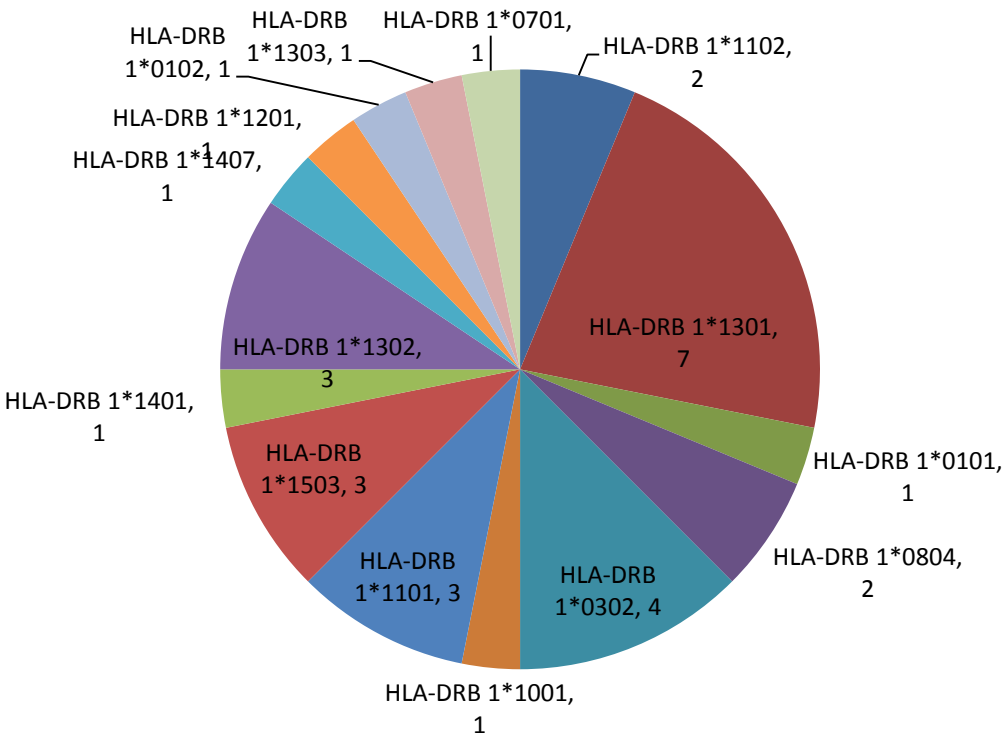
4.2 RESULTS

4.2.1 HLA-DRB1 13:01 most frequent allele among CD4+ T cell responders.

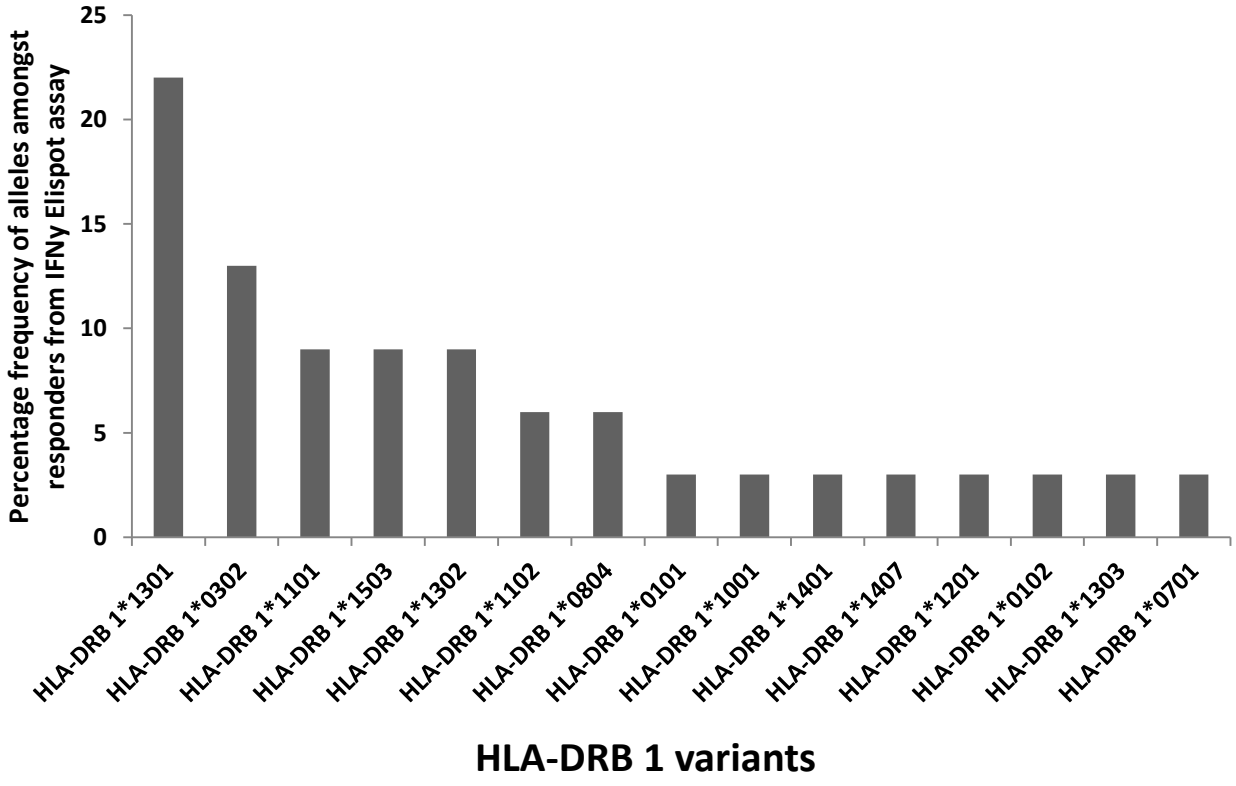
In order to perform HLA-DRB1 restriction studies, we first determined allele frequencies at the DRB1 locus in CD4+ T cell responders from our chronic infection cohort (n=20). DRB1*13:01 was the most common allele observed (n=7, 22% of responders) followed by DRB1*03:02 (n = 4, 13% of responders) (Figure 4.1). Our data suggests that individuals with the strongest HIV-specific CD4+ T cell responses possessed class II HLA-DRB1*13 alleles. Of the 20 responders, 34% had one of three HLA-DRB1*13 alleles i.e. HLA-DRB1*13:01, HLA-DRB1*13:02, or HLA-DRB1*13:03 (Figure 4.1). These results concur with what has been found in other studies of HLA class II frequencies in clade C HIV infection, where DRB1*13:01 and DRB1*03:02 were also seen at the highest frequencies (Ndung'u et al.,

2005). The DRB1*13 alleles has been reported to confer a protective effect (Ferre et al., 2010, Chen et al., 1997), as well as certain haplotypes of this allele being associated with an increased risk of AIDS progression (Kaslow et al., 1996, Kroner et al., 1995).

A


HLA-DRB 1*0701, 1
HLA-DRB 1*1303, 1
HLA-DRB 1*0102, 1
HLA-DRB 1*1102, 2
HLA-DRB 1*1201, 1
HLA-DRB 1*1407, 1
HLA-DRB 1*1301, 7
HLA-DRB 1*1302, 3
HLA-DRB 1*1401, 1
HLA-DRB 1*0101, 1
HLA-DRB 1*1503, 3
HLA-DRB 1*0804, 2
HLA-DRB 1*1101, 3
HLA-DRB 1*0302, 4
HLA-DRB 1*1001, 1

B


Percentage frequency of alleles amongst responders from IFNγ Elispot assay
25
20
15
10
5
0
HLA-DRB 1*1301
HLA-DRB 1*0302
HLA-DRB 1*1101
HLA-DRB 1*1503
HLA-DRB 1*1302
HLA-DRB 1*1102
HLA-DRB 1*0804
HLA-DRB 1*0101
HLA-DRB 1*1001
HLA-DRB 1*1401
HLA-DRB 1*1407
HLA-DRB 1*1201
HLA-DRB 1*0102
HLA-DRB 1*1303
HLA-DRB 1*0701
HLA-DRB 1 variants

C

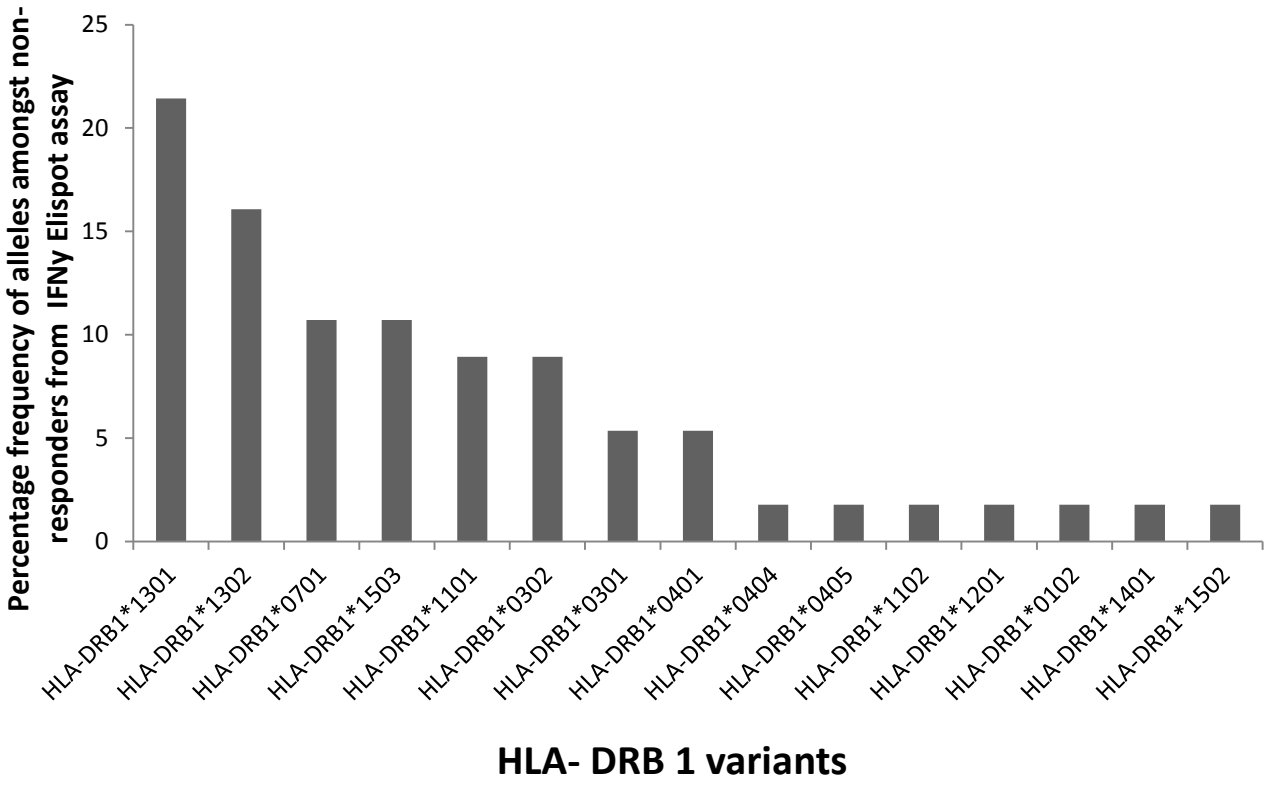


Figure 4.1. (A) Allele frequency among those individuals that displayed CD4 T cell responses in IFN-γ Elispot confirmatory assay (n=20). (B) Percentage frequency of various HLA-DRB1 allele variants in CD4 T cell responders (n=20). (C) Percentage frequency of various HLA-DRB1 allele variants in CD4 T cell non- responders (n=35).

4.2.2 HLA-DRB1 allele restriction characteristics of HIV-specific CD4+ T cell responses in subtype C infection.

Having defined the frequencies of these class II alleles in our chronic infection cohort, we next defined the role of DRB1 restricted HIV-specific CD4+ T cell responses on viral control. We observed increased levels of promiscuity, which is known to be a distinctive feature of antigen-specific CD4+ T cell recognition, with the presentation of a single peptide on numerous HLA class II variants expressed by various individuals (Figure 4.2). OLP-41 in the Gag p24 region, previously identified as the most frequently targeted epitope by HIV-

specific CD4+ T cells in clade B and C infection , exhibited the highest levels of promiscuity, restricted by 4 different HLA-DRB1 variants. The promiscuity in peptide-binding could be the open conformation of HLA class II, allowing for long peptides recognized by CD4+ T cells to extend beyond the HLA binding groove(Ranasinghe et al., 2013). In spite of the high degree of HLA-DRB1 binding promiscuity, marked differences was observed in the number of peptides that were restricted by each DRB1 variant (Figure 4.3A). Variants such as DRB1*03:01, DRB1*14:01 and DRB1*15:01 had no contribution to peptide restrictions as compared to DRB1*13:01 and DRB1*11:01 which had 9 and 4 HIV-specific peptides restricted respectively. It is important to note that these results could be biased by the number of subjects tested per variant, with a skewed testing of DRB1*13:01 due to aforementioned high allelic frequency in our cohort (Figure 4.3B).

The data obtained is in concordance with results obtained in a previous study, using the same methods, in a cohort of clade B HIV infected individuals from Boston and San Francisco, by Ranasinghe et al. (2013). Further detailed analysis into the relationship between HLA-DRB1 alleles and viral load need to be conducted to identify any associations between possession of a particular allele and disease outcome. Currently, the present data suggests an important role of HLA class II allele genetic associations with the functional responses of CD4+T cells. Further highlighting the important role that CD4+ T cells play in HIV infection.

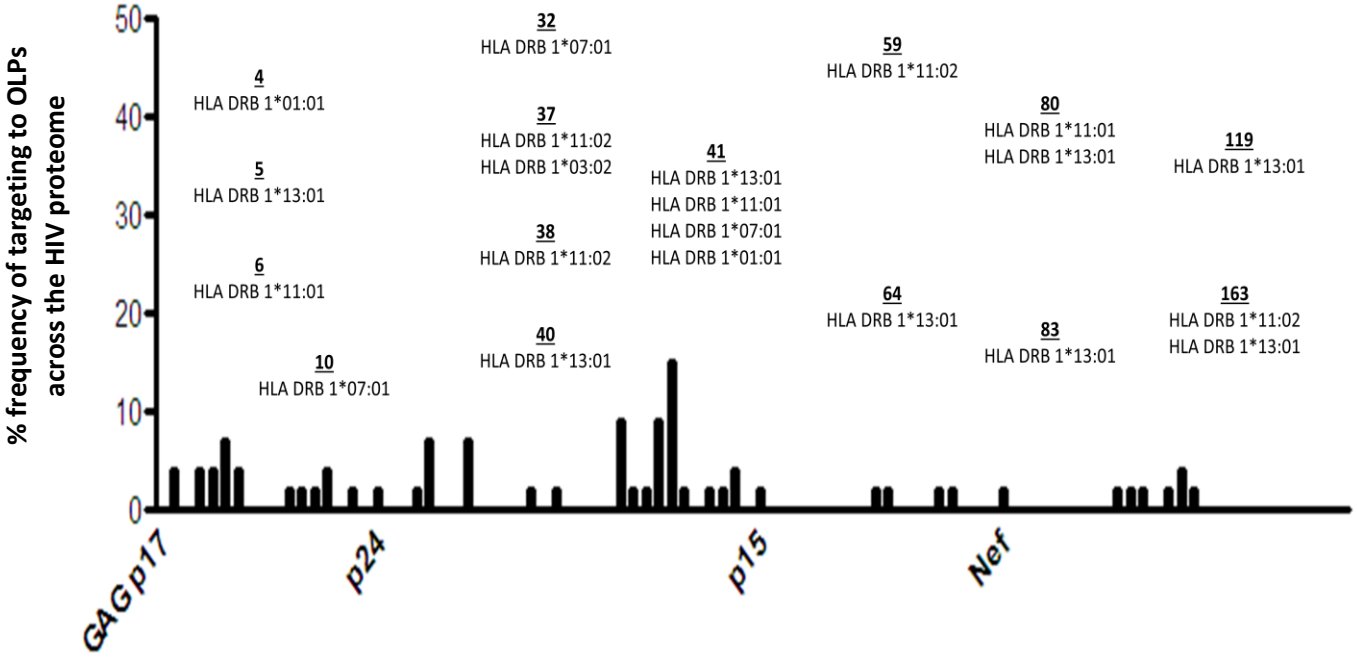
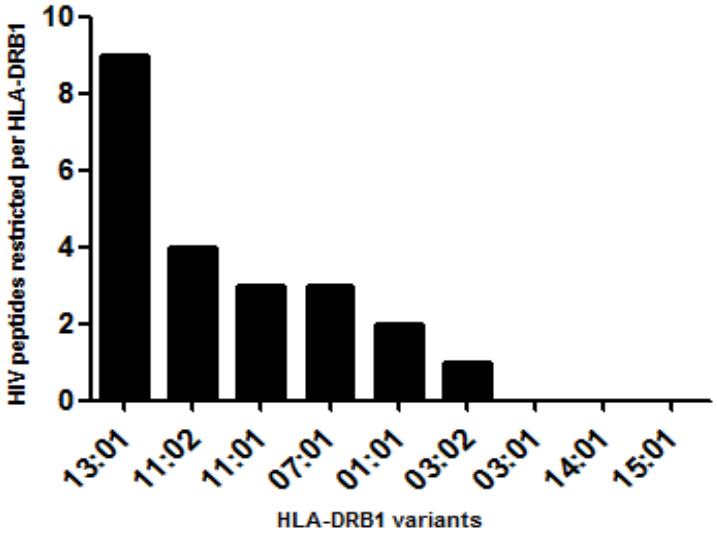


Figure 4.2. HLA-DR restriction of HIV-specific CD4 T cell responses (indicating responses in the Gag and Nef regions). The restrictions are indicated per peptide.

A



B

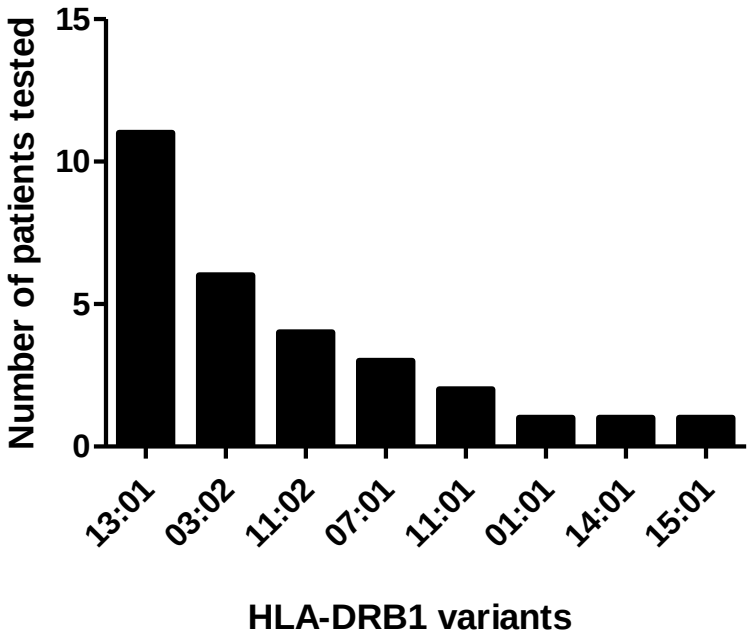


Figure 4.3. (A) The number of HIV-specific peptides recognized by each HLA-DRB1 variant tested. (B) Number of subjects tested per HLA-DRB1 variant.

CHAPTER 5: OVERALL DISCUSSION AND FUTURE DIRECTIONS

One of the major challenges for developing an efficacious HIV vaccine is the intrinsic diversity among circulating populations of HIV-1 in various geographical locations and the need to develop vaccines that can elicit enduring protective immunity across different strains. A growing body of literature has shown that effector CD4+T cells are an important factor in immune control of HIV-1 infection. However, precise details of CD4+ T cell contribution to immune protection against HIV have not been adequately defined and most of the existing data was predominantly generated in subtype B HIV-1 infection. Therefore, there is a gap in the information needed for designing an effective HIV vaccine with global utility.

The role of CD4+ T cell responses in the control of numerous chronic viral infections has been well characterized, yet little is known about the role of these responses in HIV-1 infection. Despite the significant role of CD4+ T cells in vaccine design and the course of natural infection, there have been very few studies that have characterized HIV-specific CD4+ T cells due to their preferential susceptibility to HIV-1 infection. Previous studies have suggested that the preservation of HIV-1 specific CD4+ T cell responses might be critical for the control of viral replication (Zhang and Bevan, 2011, Rosenberg et al., 1997). Therefore, this study and the work presented in this thesis aimed to assess the role of HIV-1 specific CD4+ T cell responses in HIV-1 infection. Furthermore, we wished to determine if the preservation of HIV-1 specific CD4+T cell responses might be a key factor in the overall immune responses in maintaining control over viral replication.

The study set out to obtain an in depth understanding of the role of CD4+ T cells in clade C HIV-1 infection. This was addressed by firstly mapping immunodominance patterns and identifying specific targeted regions and peptide specificities using the IFN-γ ELISPOT assay in both acute and chronic clade C HIV-1 infection. While the ELISPOT assay allows for the evaluation of breadth and magnitude of T-cell responses elicited, its major limitation is that it

relays on only one T cell effector function, which is the ability of virus-specific cells to release IFN-γ upon antigen-peptide stimulation. Therefore, to better understand functional characteristics and cytokine secretion of HIV-specific CD4+ T cells we complimented the ELISPOT assay with intracellular cytokine staining which allows for simultaneous measurement of multiple cytokines, chemokines and cytotoxicity function. Having obtained an understanding of immunodominance and functional characteristics of HIV-specific CD4+ T cells, we next determined the role of host genetics i.e. HLA class II alleles on disease progression, assessing restriction characteristics at the HLA-DRB1 locus. There are numerous studies highlighting the importance of host genetics in determining overall outcome of an HIV-infected individual, however, most of these studies have looked at CD8+ T cells and HLA class I alleles (Gao et al., 2001, Pereyra et al., 2010). We hypothesised that HIV-1-specific CD4+ T cell responses and protective class II HLA alleles are important determinants of effective immunological control of HIV-1 infection and have been able to demonstrate this to some extent.

Chapter 3 aimed to evaluate the immunodominant regions of the HIV-1 proteome targeted by HIV-specific CD4+ T cells during the first year of HIV-1 infection and to determine the evolution of these responses over time. Having previously alluded to well established knowledge that HIV preferentially infects and depletes HIV-specific CD4+ T cells, as well as to evidence suggesting that an effective CD8+ and antibody response is dependent on CD4+ T cell-mediated helper signals, it can be said that induction of HIV-specific CD4+ T cells during acute infection may in fact be a double-edged sword. As CD4+ T cells are the primary targets of HIV, increased generation of CD4+ T cell responses during the acute phase of HIV infection may cause an increase in the presence of target cells for HIV dissemination and hasten disease progression.

With various studies conducted on the specificities, breadth and magnitude of HIV-specific CD4+ T cell responses and the association with viremia in chronic infection, albeit in clade B HIV infection, detailed knowledge of these responses in early infection is poorly understood. We wanted to assess the development of HIV-specific CD4+ T cell responses in clade C infection during the acute phase, particularly the first year and map the progress of these responses over time. A diverse pattern of CD4+T-cell recognition across the HIV-1 proteome was evident during primary infection. We found that the dominant regions targeted during early infection were Gag and Pol, with Gag and Pol-specific responses found in 14 out of 15 individuals tested. Furthermore, the overall HIV-specific CD4+ T cell responses during acute infection until 36 months post infection remained relatively stable over time in terms of frequency and magnitude. Previous studies looking at HIV-specific CD4+ T cell responses during acute HIV-1 infection are overall consistent with our findings (Schieffer et al., 2014). Possible explanations provided for the stability of frequency and dominance of HIV-specific CD4+ T cells over time include the shorter life span of these cells, resulting in the maintenance of stable levels due to a higher cell turnover rate (Brenchley et al., 2006, Schieffer et al., 2014). In addition, an interesting observation was the varied responses in the dominance and magnitude of HIV-specific CD4+ T cell responses among the 15 individuals. While 14/15 individuals displayed responses targeting more than one region of the proteome, some individuals showed increased responses while others showed decline in responses. The average response increased over time from 2 months post infection to approximately 11 months post infection, decreasing once again 1 year post infection. These results are possibly linked to overall progression of the disease. Furthermore, they are consistent with previous studies of primary infection, where a slight decrease, although not significant, was observed in the breadth and magnitude of HIV-specific CD4+ T cells over time (Oxenius et al., 2001, Lubong Sabado et al., 2009, Schieffer et al., 2014). The magnitude of HIV-specific CD4+ T

cell responses was observed to vary at different time points within each individual. This could be resultant of fluctuations in viremia as well as the increased destruction and impairment of HIV-specific CD4+ T cells as disease progresses.

Gag-specific responses were shown to contribute largely to the overall total HIV-specific response. Given that previous studies in chronic infection indicated that increased Gag-specific responses lead to better control of viremia , the results point to the importance of Gag-specific CD4+ T cell responses and a potential role in contributing to the control of HIV replication, with possible cytotoxic mechanisms (Ranasinghe et al., 2012, Soghoian et al., 2012).

An interesting observation in our study was the significant positive correlation we found with the combined magnitude of Gag and Pol-specific CD4+ T cell responses with viral load set point. The data suggests that the higher the Gag/Pol specific response, the higher viral load set point is. The higher viral load set point would indicate faster progression to disease, suggesting a potentially detrimental effect of the induction of Gag/Pol-specific CD4+ T cell responses in acute infection. Intriguingly, analysis of Gag and Pol individually, rather than combined showed no significant association with viral load for Gag responses and a significant positive correlation for Pol-specific responses. There was no association of Env-specific CD4+ T cell responses and clinical outcome. Small sample size coupled with the fact that these responses were not examined at single epitope level may have biased our results. Overall, detailed analysis needs to be conducted to address the fluctuations of HIV-specific CD4+ T-cell responses during primary infection and the impact of these changes on disease progression.

Despite the surprising positive associations between CD4+ T cell responses with viral load set point, the results do re-iterate that HIV-specific CD4+ T cells responding to various

antigens may have differing functional roles. In a matched cohort study characterizing protein-specific CD8+ T-cell immunodominance patterns during the first year of HIV-1 infection, Gag-specific CD8+ T-cell responses were shown to be associated with viral control, where broad and persistent Gag-specific effector and central memory type CD8+ T-cells were required for the maintenance of low viral load levels in primary infection(Radebe et al., 2014). Here, we show that Gag-specific CD4+ T cell responses are the most dominant and persist over time, thus suggesting an active role of Gag-specific CD4+ T-cell responses in acute infection.

Overall, observations of CD4+ T cell responses during the acute phase of HIV infection in a clade C cohort indicate a role of Gag- and Pol-specific CD4+ T cell responses, having direct effects on viremia and on subsequent viral load set point. Furthermore, HIV-specific CD4+ T cell responses remain relatively stable in frequency and magnitude through which they are likely to mediate immune control of HIV viremia.

Having examined the role of HIV-specific CD4+ T cells in acute clade C HIV infection, we next looked at chronic infection in order to obtain a better overall view of these responses.

Therefore, chapter 3 further detailed the role of HIV-specific CD4+ T cell responses in chronic clade C HIV infection. The chapter defined the individual peptide sequences that are targeted by HIV-specific CD4+T cell responses in chronic HIV-1 infection, thus allowing a detailed identification of HIV-specific CD4+ T cells that are induced in natural HIV infection. Here, overlapping peptide pools that span every encoded HIV protein was used, allowing for a broader view of the overall immune response to HIV as well as its association to the various parameters associated with viral infection. Furthermore, immunodominance hierarchies of HIV-specific CD4+ T cell responses were elucidated.

We initially conducted a screening megamatrix ELISPOT analysis of 55 individuals from our chronic infection cohort. The results from this analysis indicated similar results as the previous analysis in the acute infection cohort, with Gag and Pol-specific CD4+ T cell responses being the most dominant. Gag-specific responses were observed in all individuals, with all demonstrating more than one detectable HIV-specific CD4+ T cell response. Once again, these results highlight the important role of Gag responses in immune mediated control of HIV-1 infection.

We wanted to confirm these results and therefore analysed HIV-specific CD4+ T cell responses in 35 responders from the megamatrix screening, at the single peptide level. Here, we found that HIV-specific CD4+ T cell responses in chronic clade C infection dominantly target the Gag region, particularly the Gag p24 region (15/40 peptides). This region of Gag epitopes has been defined in immunodominant HIV-specific CD8+ T cell responses and has been previously been associated with viral control (Starr et al., 2003). The next most immunodominant regions targeted by HIV-specific CD4+ T cell responses were the Gag p17 (9/40), Nef (7/40) and Gag p15 regions respectively (5/40). The frequent targeting of Gag and the evident lack of responses to Env, Rev, Tat, Vif and Vpu are concordant with observations from studies of clade B CD4+ T cell epitope mapping (Addo et al., 2003). The reason for the more frequent targeting of the Gag region by CD4+ T cells, together with evidence of a role in controlling viremia demonstrated by other studies could be because of the high degree of conservancy of the protein (Freed, 1998), as well as the increased number of Gag molecules (1500) and the precursor Gag-Pol molecules (100) found in a single immature virus particle (Kurle et al., 2004).

The epitope most targeted among the Gag epitopes, found in the Gag p24 region, was OLP 41 (YVDRFFKTLRAEQATQDV). This particular epitope was observed to be frequently targeted in a matched cohort study in clade C infection (Ramduth et al., 2009), as well as in

clade B (Kaufmann et al., 2004, Ranasinghe et al., 2012), clade A/G HIV-1 infection and displayed a high degree of cross recognition between HIV-1 and HIV-2 infected individuals (Ondondo et al., 2008). Ramduth et al. (2009) showed that particular epitopes were able to mount equal magnitudeIFN-γCD4+ T cell responses against both clade B and C viruses. OLP 41 in particular partially overlaps with the Major Homology Region (MHR), which is located at the C terminal dimerization domain of p24, displaying significant homology amongst various retrovirus genre (Freed, 1998). Virus particles become defective in viral assembly, maturation and infectivity if mutations to MHR occur (Freed, 1998), with OLP 41 thus presenting as a potential valuable constituent in an HIV vaccine, and needs to be further characterized.

Ramduth et al. (2012) alludes to the point of using consensus clade C sequences to test for HIV-specific CD4+ T cell responses, stating that these sequences could possibly limit detection of antigen specific CD4+ T cell epitopes to more variable proteins. Therefore, the relative lack of responses in various proteins other than Gag may be due to use of autologous HIV-1 peptides as opposed to consensus sequences previously shown to significantly increase in detection of CD8+ T cell epitopes in clade B infection (Altfeld et al., 2003). Env-specific responses were detectable in chronic clade B infection and even shown to have a negative impact on control of viremia and disease progression (Ranasinghe et al., 2012). However, our study together with a previous study in clade C infection (Ramduth et al., 2009), demonstrated the lack of Env-specific CD4+ T cell responses in chronic HIV-1 infection, thus the lack of detectable CD4+ T cell responses targeting Env need to be explored further.

Despite demonstrating that Gag was the dominant target of HIV-1 specific CD4+ T cells, we could not detect an association between total HIV-specific responses as well as Gag-specific responses (both breadth and magnitude) and viral load in chronic HIV-1 infection. However,

reasoning for our results could be sample number, where only 20 of the individuals were studied compared to 373 and 93 subjects in previous studies. Furthermore these immunodominant responses are captured at a certain time point, whereas they could fluctuate in response to continued viral replication and possibly disappear at later time points. In addition, assay sensitivity and lack of use of autologous HIV peptides could also be contributing factors. Longitudinal analysis tracking these responses would assist in understanding the kinetics of HIV-specific CD4+ T cell responses in chronic infection.

We undertook additional analysis to determine if differences exist between breadth of HIV-specific CD4+ T cell responses in controllers and progressors. We observed no significant differences in CD4+ T cell responses between the two groups. These results are contrary to studies conducted in clade B infection where remarkable differences were observed between groups varying in their ability to control viral replication (Ranasinghe et al., 2012) . Clear differences observed in the study between controllers and progressors include a noticeable dominance of in the targeting of HIV-specific responses to the Env (gp120) region by progressors and the Gag (p24) region by controllers. This discrepancy may be due to the small number of controllers assesses in our study (N=5 controllers vs. N=15 progressors).

We next wanted to assess further functional characteristics of CD4+ T cell responses in our chronic infection cohort using intracellular cytokine staining. These results, provides additional insight into the cytokine secretion profiles of CD4+ T cells in HIV infection. IFN-γ, IL-2 and TNF α were secreted by most of the individuals tested. IL-2 and IL-2/IFN-γ CD4+ T cell responses have been shown to be associated with the control of viremia (Younes et al., 2003, Boaz et al., 2002, Harari et al., 2005).This cytokine combination was rarely observed in our cohort, however this could be because of a skewing of the immune response in chronic infection to the dominant IFN-γ only producing effector memory phenotype (Day and Walker, 2003). In the ICS analysis as with previous ELISPOT data described above,

most of the responses were in the Gag region. The IFN-γ responses in some cases mirrored results obtained during the IFN-γ ELISPOT assay. However, some individuals that did not exhibit any HIV-specific CD4+ T cell responses during the ELISPOT assay displayed responses when tested by flow cytometry. An example would be that responses were seen in the Env region during ICS as opposed to no responses observed through ELISPOT. This could suggest that these responses were maintained at a lower frequency and could not be detected by the regular ELISPOT assay. In addition peptide pools were used during the ICS analysis as compared to single peptides in the ELISPOT, therefore small, undetectable responses by ELISPOT could be detected during ICS. Furthermore, there are certain co-stimulatory peptides required in order for a response to be exhibited, and this is possible when peptide pools are made use of. High levels of TNFα were observed in most individuals. TNFα is one of the greatly studied pro-inflammatory cytokines because of the key role it plays in the origin and progression of HIV-1 (Bahia and Silakari, 2010). Higher levels of TNFα would be expected, as the host's immune-regulatory response influences HIV-1 pathogenesis, triggering macrophages, monocytes and NK cells to produce TNFα (Alfano and Poli, 2005), resulting in a positive correlation between HIV-1 viremia and TNFα levels in the serum of HIV infected individuals. Further analysis into the polyfunctional capacity and memory phenotype of epitope specific CD4+ T cells need to be conducted to have a better overall view of the functional capacity and characteristics of HIV-specific CD4+ T cells.

Lastly, we wanted to assess the impact of host genetic factors on HIV-1 specificCD4+ T cell responses in clade C HIV-1 infection. HLA class I molecules have been shown to significantly influence CD8+ T cell responses, yet only more recently has the influence of HLA class II molecules on CD4+ T cell responses been described (Julg et al., 2011, Ranasinghe et al., 2013), it is important to further characterize the role of host genetics in

viral control, with findings possibly being beneficial in vaccine design as these epitopes can induce responses amongst a wide array of HLA class II types.

We assessed our chronic clade C HIV infected cohort to better understand the interaction between HIV-specific CD4+ T cell responses (previously elucidated in analysis mentioned above), HIV disease outcome and HLA class II alleles. The results obtained thus far are preliminary and provide a basis for subsequent analysis. We have found that the HLA-DRB1*13 allele is the most frequent in our cohort, which is consistent with previous analysis conducted in clade C infection(Ndung'u et al., 2005). The DRB1*13 has been shown to confer protection in various other infections, including hepatitis B, where the allele is associated with better clearance and clinical disease outcome (Ramezani et al., 2008, Hohler et al., 1997, Kummee et al., 2007). Further studies in human papillomavirus (HPV) have indicated the DRB1*13 allele with conferring protection against the HPV-16 serotype, as well as being negatively correlated with the incidence of cervical carcinoma (Apple et al., 1994, SastreGarau et al., 1996). In addition, DRB1*13 enrichment has been reported in individuals suffering from autoimmune diseases e.g. anti–citrullinated protein antibody–negative rheumatoid arthritis (Lundstrom et al., 2009). Fainboim et al., (2001) proposes that the effective antigen presentation capacity of DRB1*13 bestows protection against viral infections and thus the observed effect on disease pathogenesis. It is unknown whether a more effective T cell response is needed to exhibit the observed associations or they are mediated through various other mechanisms.

Our analysis of HLA-DRB1 restriction characteristics indicated high levels of promiscuity, with OLP-41, our most immunodominant peptide as indicated in results above, restricted by 4 different HLA-DRB1 variants. The mechanism behind the higher levels of peptide-binding promiscuity could be the open conformation of HLA class II, allowing for long peptides recognized by CD4+ T cells to extend beyond the HLA binding groove (Ranasinghe et al.,

2013). Our results indicate most of the HIV-specific CD4+ T cell responses from our ELISPOT analysis were made by individuals that possessed the DRB1*13 allele. However, this is most probably a causal effect of the allele frequency in our cohort. Our data provides further insight into the promiscuity and specificity of HLA-DR variants.

Despite, the limited data shown on HLA-DRB1 restriction, this study is one of the first in clade C infection to assess the impact of HLA class II alleles on HIV disease progression. We hope to provide detailed evidence linking HLA class II genetic associations with functional CD4+ T cell responses, further emphasizing the important role HIV-specific CD4+ T cells play in the control of HIV infection.

Study limitations:

Having detailed the findings of the research undertaken for this research, one of the most important limitations was sample availability, where additional samples would allow for identification of immunodominance patterns of HIV-specific CD4+ T cell responses in acute infection at the single peptide level. Furthermore, more samples are needed to further identify responses in chronic infection and conclusively elucidate associations between viral load and disease outcome. In addition, to convincingly make conclusions regarding change of HIV-specific CD4+ T cells over time, it would be important to follow the same individual from the acute to chronic phase of infection.

Future directions of this study will include:

- Further characterization of peptide sequences that induce HIV-specific T cell responses in longitudinal acute and chronic HIV infection.
- More detailed definition of HLA class II restriction characteristics to identify genetic association with HIV control.

- Evaluation of HIV-specific CD4+ T cell activity as a direct contributor to the control of viremia or as a consequence of viral control.
- Evaluation of the difference between cryopreserved and freshly isolated PBMC's to determine if long-term cryopreservation has detrimental effects on T cell IFN-γ responses.
- Assessing the lack of Envelope responses in clade C HIV infection as studies have suggested that Env-specific T cells may be more critical to support neutralizing antibody production.
- Further characterization of Pol versus Gag specific responses to examine if they differ in HIV susceptibility or other properties.
- Use of MHC class II tetramers to quantify numbers of antigen-specific T cells and characterize functional differences of CD4+ T cells.

In conclusion, Gag and Pol regions of the HIV proteome are most frequently targeted in acute HIV-1 infection, with CD4+ T cell targeting across the proteome remaining relatively stable over time. Furthermore, dominant HIV-1-specific CD4+ T cell responses are detectable against a limited number of epitopes in chronic HIV-1clade C infection. Epitopes in the Gag region are the most targeted by CD4+ T cell responses, with OLP 41 in the Gag p24 region being the most dominant epitope targeted. There were no significant associations observed between total and Gag-specific CD4+ T cell responses and contemporaneous viral load. Interestingly, responses in the envelope region were rarely targeted in chronic clade C infection as compared to multiple epitope targeting observed in this region in previous clade B studies. Functional analysis demonstrated that IFN-γ, IL-2 and TNFα were the most expressed cytokines by HIV-specific CD4+ T cells. The HLA class II DRB1 restriction in clade C HIV infected individuals showed epitope promiscuity, consistent with previous

studies in clade B infection. The HLA-DRB1*13:01 allele variant was seen in the highest frequencies in our cohort as well as being shown to restrict the highest number of HIV-specific peptides. Together, the identification of immunodominant regions and the characterization of HIV-specific CD4+ T cell responses present during an effective immune response are likely to be important in characterizing clade C immunogenicity. Furthermore, our findings will inform studies aimed at elucidating the underlying mechanism by which CD4+ T cells modulate effective CD8+ T cell and B cell responses. This information may be critical to vaccine efforts designed to induce these responses, as well as potential therapeutic manipulation of immunity in persons with acute and chronic HIV infection.

APPENDIX

R10 Medium

To 500 ml RPMI (Sigma) add:

50 ml heat inactivated and filter sterilized fetal calf serum

5 ml L-glutamine

5 ml Penstrep fungizone (100x)

5 ml HEPES

The medium was stored at 4ºC with an expiry time of 30 days.

Phosphate Buffered Saline (PBS)

One PBS tablet was dissolved in 100 ml distilled water. The solution was adjusted to a pH of 7.2. Thereafter, autoclaved for 10 minutes at 115ºC.

Blocking buffer

10 ml heat inactivated and filter sterilized fetal calf serum was added to 1 liter of sterile PBS solution. The solution was stored at 4ºC with an expiry time of 30 days.

Tris buffer

12.1 g of powdered Tris was dissolved in distilled water. The pH was adjusted to 9.3 with concentrated HCl, bringing the volume to 1 liter. The solution was autoclaved for 10 minutes at 115ºC and thereafter stored at room temperature.

NBT (Nitro-Blue Tetrazolium Chloride)

30 mg/l NBT powder was added to a 2 ml tube. 700 µl dimethylformamide (DMF) was added to the powder. Once completely dissolved, 300 µl sterile distilled water was added to the solution. The solution was stable at 4ºC for 7 days.

BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt)

1 ml of DMF was added to 15 mg/ml of BCIP powder in a 2 ml tube to dissolve the powder. The solution was stable at 4ºC for 7 days.

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