The Use of HIV-1 envelope epitopes for characterizing humoral immune responses

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Plagiarism and originality statement

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PREFACE

Acknowledgements

I would like to take this opportunity to acknowledge the following people, without whom, this work would not have been possible.

First and foremost, I would like to express my deepest gratitude to my supervisor, Professor Debra Meyer. Her patience, encouragement and immense knowledge were key inspirations throughout my Masters degree. Prof Meyer has been my supervisor and motivator throughout my three years in the biochemistry department, in my honours and masters research. I am truly thankful for her unwavering integrity, and am fortunate to have had a supervisor with tolerance, dedication to both my personal and academic development. I cannot think of a better supervisor to have. I am also grateful to her for offering thorough and indispensable feedback on my research and for finding the time to propose consistently excellent improvements to my work. As her student, I have learnt the vital skill of disciplined critical thinking and have had the opportunity to hone my interpersonal and scientific communication skills. She has always conveyed an interest in my work and professional development, and I am thankful for my inclusion in the peptide project as a member of the HIV research group, under her supervision.

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Presentations and awards

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Women in Science fellowship award in 2012 from the Department of Science and Technology (award conferred by Minister Naledi Pandor), at a ceremony held on 30 August 2012 at the Royal Elephant Hotel in Pretoria, South Africa.

Preliminary results were accepted for poster presentation (following peer review) at the 6th SA AIDS Conference held on 18-21 June 2013 at the International Convention Centre in Durban, South Africa. The poster was entitled Assessing Immune Responses To HIV-1 Using Antigens Based On Host-derived Beta-2 Microglobulin (authors Sindisiwe Nondaba and Debra Meyer).

SUMMARY

Supervisor: Prof Debra Meyer

Department: Biochemistry

Degree: MSc Biochemistry

Background: HIV envelope (env) proteins are highly antigenic making them useful for detecting the immune response to natural infection. Envelope protein epitopes are not just virus derived but can be acquired from the host during budding. Some epitopes of host protein beta 2 microglobulin (β2M) become exposed only during incorporation by the virus. These epitopes are defined as cryptic (newly exposed) and are being investigated here, alongside virus-derived envelope protein epitopes for the ability to detect HIV-induced immune responses *in vitro*. Data obtained in this manner provides information on the potential use of these epitopes as vaccine components or antigens in prognostic/diagnostic assays.

Methods: Synthetic peptides based on epitopes of β2M (designated as R7V, F7E, S7K, β2Mp) or env (designated as 2F5, DC1, DV3, and MPER) were synthesized and used in an indirect ELISA to detect antibodies in the serum of HIV infected patients who were treatment-naïve or receiving highly active antiretroviral treatment (HAART). The effect of the peptides on peripheral blood momonuclear cells (PBMCs) was assessed through measuring cytokine secretion (using Cytometric beads) and proliferation (tetrazolium dye uptake, real time cell electronic sensing (RTCES) and flow cytometry using carboxyfluorescein diacetate succimidyl ester (CFSE)). New Zealand white rabbits were immunized with the peptides and affinity purified antibodies prepared and tested for the ability to neutralize viral constructs (pseudo-virus designed to undergo one replication cycle).

Results and Discussion: In individuals infected with HIV-1 subtype C the seroprevalence of antibodies against epitopes of β2m was variable (between and within experimental groups). β2M antibodies were prominent (up to 2 fold) in newly infected individuals who were not on

treatment or had recently started HAART. One β2M epitope (R7V) sparked interest in the literature when it repeatedly served as an indicator of non-progression to disease.

In the current study the strongest response was observed for antibodies against V3 loop peptides while antibodies against gp41 epitopes were generally low. At times a notable response was seen against a peptide based on the membrane proximal external region (MPER) of gp41. Proliferation data confirmed that the peptide concentrations used were non-toxic to cells. However, PBMC proliferation was minimal (stimulation index ±1) on all accounts. Tetrazolium dyes are notoriously weak detectors of peptide-induced proliferation, which is why RTCES and flow cytometry were employed as methods with improved levels of sensitivity. Flow cytometry and a fluorescent dye, CFSE, were better detectors of proliferation, allowing the calculation of stimulation indices between 4 and 7. DV3, B2Mp and R7V stimulated proliferation of infected cells. IL-6 ($P<0.05$) was significantly secreted following stimulation of PBMCs with env antigens. The peptides also stimulated TNFα, IL-17 and IL-10 production, but to a lesser extent. Although the study was designed for the assessment of peptides for characterizing natural infection, the ability of these antigens to elicit an immune response in New Zealand white rabbits, was also evaluated. Titers of >1:6400 and up to 1:152000 were observed. These Polyclonal antibodies (raised against DV3 and MPER) neutralized HIV-1 pseudo-virus ZM53 at >50%.

Conclusion: Antibodies against host-derived beta-2 microglobulin epitopes were reportedly ideal prognostic indicators (prominent in long term non-progressors (LTNP) according to Galea *et al*, 1996) in infection with HIV-1 subtypes A and B. Margolick *et al.* (2010) qualified these findings by retrospectively demonstrating that the presence of β2M epitope antibodies in early infection indicated a tendency toward LTNP. In the current study, using sera collected from HIV-1 subtype C infected individuals; peptides based on β2M were able to detect antibodies in recently infected individuals and also stimulated cytokine production *in vitro*. When compared to the viral-derived antigens, the responses detected using host-derived peptides were lower. Although immunogenic, host-derived epitopes do not appear to have value in diagnostic use. The responses to host-derived peptides distinguished recent from later infection, suggesting prognostic potential. The antigenicity of the viral peptides confirmed, what is well reported in the

literature, potential use in vaccine development, while the peptides based on host derived protein epitopes showed less ability in this regard.

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LIST OF IMPORTANT ABBREVIATIONS

AMINO ACIDS

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arganine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine

W	Trp	Tryptophan
Y	Tyr	Tyrosine

OTHER ABBREVIATIONS

Ab	antibody
Ag	antigen
Abs	absorbance
ACK	Ammonium chloride potassium
β2M	Beta- 2 Microglobulin
AIDS	Acquired Immunodeficiency syndrome
ARV	antiretroviral
CDC	Centre for disease control
cDNA	complementary DNA
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CTL	cytotoxic T lymphocyte
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
Fab	fragment antigen binding
Fmoc	Fluorenylmethyloxycarbonyl
HAART	Highly active antiretroviral therapy
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HRP	horse radish peroxidase
LTNP	long term non-progressor
LTR	long terminal repeats
MHC	major histocompatibility complex

MW	molecular weight
NK	natural killer
PBS	phosphate buffered saline
RT-CES	Real Time Cell Electronic Sensing
scFV	single-chain variable fragment
tBoc	Tert-Butoxycarbonyl
PBMC	peripheral blood mononuclear cell
Env	envelope
HERV	endogenous retrovirus
WHO	World Health Organisation
CCR5	Chemokine coreceptor R5
DNA	deoxyribose nucleic acid
TCR	T cell receptor
APC	antigen presenting cells
Th1	T helper 1
TNF	tumour necrosis factor
INF	interferon gamma
IL	interleukin

CHAPTER 1 – GENERAL INTRODUCTION

The destruction of the immune system is a hallmark of progressive HIV infection and is considered to predict disease outcome (Hazenberg *et al*., 2003). Although highly active antiretroviral therapy (HAART) is available as a means of managing HIV-1 infection, a final resolution or 'cure' to the disease is yet to be discovered (Iwaela 2004, Koff *et al*., 2012). HIV diagnosis is achieved through the detection of core (p24) and surface (env) proteins, provided the sample is collected following seroconversion. A sample collected within the window period or one that is obtained from an infant can lead to false negative or false positive test results (Iwaela, 2004). Therefore, subsequent confirmatory tests (Western blot) are usually performed in addition to the diagnostic ELISA. These tests are primarily used to detect HIV-1 infection and are usually not relied upon for information on disease status or progression.

With emerging technologies allowing for integrative biology and the expansion of 'omics'; following over 30 years of HIV research, it is time to consider expanding diagnostic technology to provide more information on disease status. Moreover, the fact that HIV incorporates host proteins not subject to the variability associated with viral proteins provides new potential targets for incorporation in novel diagnostic/prognostic tests or for use as vaccine components (Bremnaes and Meyer, 2009).

Several viral protein epitopes are presently being investigated as potential vaccine components or as part of prospective prognostic/diagnostic tools to characterize natural HIV infection. Some epitopes are based on envelope glycoproteins gp120 and gp41 (produced following proteolytic cleavage of gp160) which facilitate attachment and entry of HIV-1 into target cells (Kowalski *et al.,* 1987). Whereas the others are based on cryptic epitopes of a host-derived protein known as beta-2 microglobulin (β2M), which is incorporated by HIV-1 during budding and is presented, exposed on the viral envelope. Previous studies have revealed that these cryptic epitopes did not induce autoimmune responses (Haslin and Cherman, 2002; Bremnaes *et al*., 2009) when used as immunogens but showed an ability to detect antibodies in HIV-1 subtypes A and B infected individuals. Galea *et al.* stirred interest and controversy when suggesting antibodies to these epitopes (especially R7V) as indicators of long term non progression to AIDS. This was

controversial because given how an immune response is raised and the fact that HIV is latent in LTNPs (not producing progeny nor therefore antigens) the observation was not supported by fact. Margolick *et al* revised the Galea observations by doing a retrospective study which demonstrated that R7V antibodies detected in samples collected in early infection, suggested the patients would eventually become LTNPs. These studies were performed in subtype A/B infections, primarily.

This current study was designed to determine to what extent synthetic peptides which mimicked the abovementioned epitopes (env or cryptic epitopes of β-2m) would characterize immune responses induced during natural HIV-1 subtype C infection in order to recommend further investigation of those as vaccine components or the antibodies detected by these antigens as diagnostic or prognostic indicators. The viral env peptides based on V3 were expected to recognize HIV-1 humoral immune responses strongly while the anticipation of responses for the β2M peptides, were less certain.

Synthetic peptides (produced by solid-phase methods, Merrifield, 1964 were chosen because of their many benefits. They carry no risk of infection making them safer than peptides produced by recombinant protocols and can be made with great purity without such contaminants as DNA or cellular debris (Koff *et al.*, 2012). It is also worth noting that existing vaccine candidates that have made it to clinical trials, have included peptide-based techniques that rely on chemically synthesized fragments of HIV proteins known to stimulate specific immune functions (Koff *et al.*, 2012).

Serum antibodies and PBMCs were screened against the peptides as antigens and the peptides were also used as immunogens in New Zealand white rabbits. Anticipated and unexpected observations were made but all were substantiated by immunological and biochemical principles.

The next chapter provides the literature review which contains information pertaining to HIV/AIDS, a brief description of the immune system and HIV parthenogenesis, that the viral envelope contains host proteins as well as other relevant background content. The hypothesis and aims of the study are highlighted at the end of Chapter 2, which is then followed by a description

of the methods and protocols (Chapter 3) used to investigate the hypothesis. Chapter 4 describes the results while the discussion and concluding remarks are located in chapters 5 and 6 respectively. All the references utilized, precede the appendix where all additional data is explained.

CHAPTER 2 – LITERATURE REVIEW

2.1 A Brief overview of HIV/AIDS

With more than 34 million people currently living with HIV/AIDS worldwide, Sub-Saharan Africa remains the region most heavily affected (Joint United Nations program on HIV and AIDS (UNAIDS) 2013, World Health Organization (WHO)). This region accounted for 67% of HIV infection and 72% of the world's AIDS-related deaths (figure 2.1).

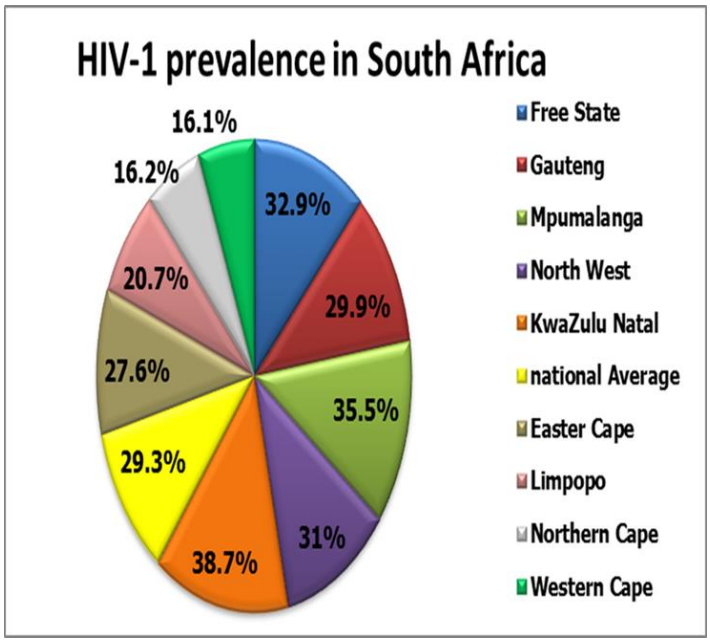


Figure 2.1: Variation in HIV antenatal prevalence between provinces in South Africa

Pie chart showing variation in HIV antenatal prevalence between provinces in South Africa. (Adapted from Department of Health, South Africa, report 2010)

Globally, the greatest mortality impact is in people between the ages of 20 and 40 years of age. The UNAIDS estimates that there are 5.7 million people living with HIV in South Africa (Sub-Saharan Africa: AIDS epidemic update 2007, regional summary), making the country one of the world's largest populations of people living with HIV. There is significant variation in HIV antenatal prevalence between the 9 provinces in South Africa, ranging from a high measure of 38% in Kwa Zulu Natal to the lowest prevalence of 16.1% in the Western Cape with the national average being 29.3% (Department of Health, South Africa, report 2010).

Owing to efforts in the laboratories of Luc Montagnier and Robert Gallo, the Human immunodeficiency virus (HIV) was identified as the causative agent of the Acquired Immunodeficiency Syndrome (AIDS) (A Vahlne Retrovirology 2009, S B Prusiner Science 298 2002). HIV is a retrovirus and a member of the lentivirus genus, of the Retroviridae family; members of the lentivirus genus are characterized by a long incubation period (Gallo *et al*., 1984).

The origin of HIV is thought to be cross-species transmission (zoonosis) involving Simian immunodeficiency virus (SIV) from African primates transferred to humans. Due to multiple cross-species transmissions brought about by predation or coital acts among different primate species, different SIV strains have been identified and are referred to based on the identity of the natural host. Natural SIV reservoirs were identified through the development and use of screening methods including the detection of faecal antibodies in suspected primate populations (Sharp *et al*., 2005). There are two known types of HIV, type 1 (HIV-1) and type 2 (HIV-2) which are distinguished based on genomic organization and geographic location. HIV-2 is primarily found in West Africa and is derived from the primate lentivirus naturally infecting sooty mangabeys known as SIVsm. Although the HIV types are morphologically similar, individuals infected with HIV-2 present with lower viral load, leading to a lower transmission rate compared to those infected with HIV-1 (Sharp and Hahn, 2011). HIV-1 is the more common of the two human retroviruses and is divided into 4 lineages called groups; a major group (group M) and 3 minor groups (N, O and P), each representing independent cross-species transmission events. A transmission event involving common chimpanzees infected with SIVcpz that gave rise to HIV-1. Group M accounts for 90% of HIV/AIDS cases and is subdivided further into clades or subtypes. These subtypes are differentiated based on geographical origin and are denoted by a letter (A – D, F - H, J, and K). Another important aspect is the existence of circulating recombinant forms (CRFs) of HIV, which occur as a result of multiple subtypes infecting the same population. HIV-1 subtype C is the dominant form in southern Africa, India, Nepal as well as parts of China. (Sharp and Hahn, 2011). Studies revealing virion structure and replication have contributed immensely to improving our understanding of the transmission and pathogenesis of HIV.

2.2 HIV Structure

HIV is sphere-shaped and has an outer coat known as the viral envelope which is derived from the membrane of the host cell (figure 2.2). This membrane envelope is composed of a lipid bilayer that has, along with several host-cell proteins, exposed surface glycoprotein-120 (gp 120) units which are anchored to the virus by interactions with transmembrane spanning glycoprotein-41 (gp 41).

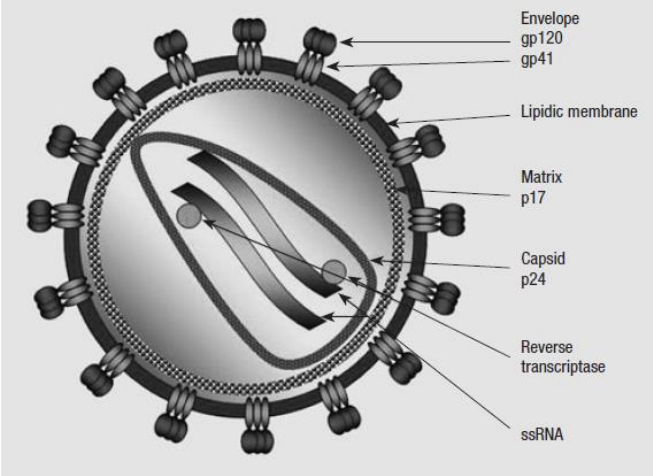


Figure 2.2: The structure of an HIV particle

Structure of an HIV particle: The matrix protein (p17) is anchored to the inside of the viral lipoprotein membrane. The virus membrane and the matrix protein include the capsid composed of polymers of the core antigen (p24). The capsid contains two copies of HIV RNA combined with a nucleoprotein and the enzymes reverse transcriptase, integrase and protease and become enriched with phospholipids and cholesterol. "Reproduced with kind permission from: Fanales-Belasio E *et al.*, HIV virology and pathogenetic mechanisms of infection: a brief overview. Ann. Ist. Super. Sanità, 46: 5-14, 2010".

Within the envelope is a conical core capsid particle comprised of capsid protein (p24) which is located at the center of the virion. This capsid encapsulates two copies of unspliced viral genome stabilised as a ribonucleotide complex. Along with this single-stranded viral RNA (ssRNA), the capsid also contains three virally encoded enzymes, namely, protease (p10), integrase (p32) and two copies of reverse transcriptase (p64).

The HIV genome contains three major genes: group-antigen (gag), polymerase (pol), and envelope (env) (Rubbert, 2006). These genes produce classical structural and enzymatic factors (figure 2.3) that are common to all retroviruses. In addition, there are a number of accessory genes which encode regulatory proteins and play a role in expression (tat, rev, nef). Other genes

such as vif and vpu, encode proteins required for virion maturation and vpr encodes a weak transcriptional activator.

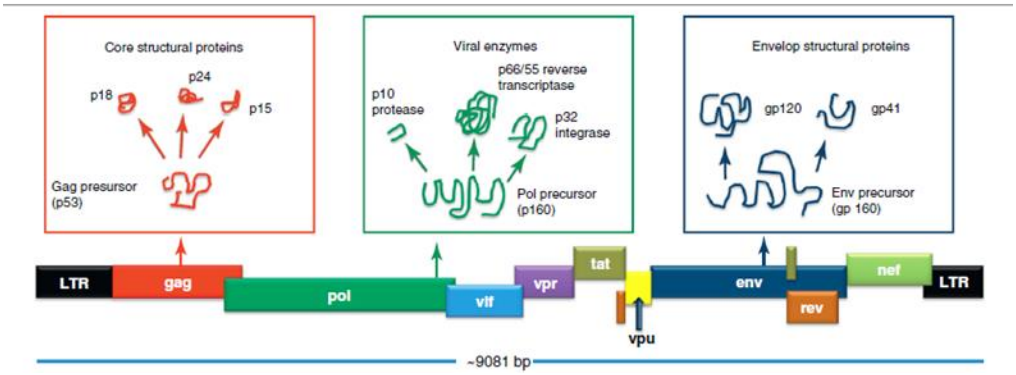


Figure 2.3: Genomic organization of HIV-1

Genomic organization of HIV-1. The *gag* gene (red) encodes the structural proteins of the core (such as p24) and matrix (p17). Viral envelope glycoproteins gp120 and gp41, which recognize cell surface receptors, are encoded by the env gene. The *pol* gene encodes for reverse transcriptase that converts viral RNA into DNA, integrase that incorporates the viral DNA into host chromosomal DNA (the provirus) and the protease that cleaves large Gag and Pol protein precursors into their component parts. These are enzymes which are vital for viral replication. (Permission granted by Chhatbare C *et al.*, 2011 (review Elsevier)

2.3 Viral Cycle

Not only is HIV transmitted through sexual contact (via semen or vaginal secretion) or through blood (drug addiction, blood transfusions, accidents), it is also transferred from mother to child during pregnancy, birth and/or breast feeding. HIV primarily infects cells that express $CD4^+$, a surface receptor protein that functions in immune recognition and is found predominantly on T helper cells but is also present, although to a lesser extent, on microgial cells and macrophages.

(i) Entry

For HIV-1 to infect a target cell, the target cell membrane must fuse with the viral membrane thereby allowing viral cell content to be released inside the target cell. Gp120, a component of the Env protein, serves as the viral receptor and binds with high affinity to $CD4^+$ on target cells (Wyatt and Sydroski, 1998). The formation of gp 120/$CD4^+$ complex induces conformational changes in gp120 that allow for interaction of the gp120/ $CD4^+$ with coreceptor CXCR4 or CCR5 depending on the viral tropism(Wyatt and Sydroski, 1998; Moore *et al*., 1993).

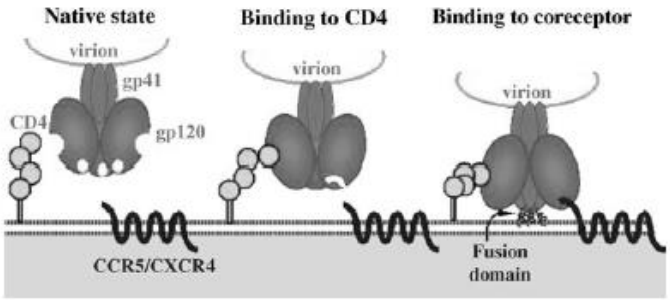


Figure 2.4: Model for virion attachment and entry

A major determinant HIV-1 tropism is the variation in expression of chemokine receptors on cell targets (Broder and Burger, 1995). To study the role of the V3 loop and its amino acid sequence in viral coreceptor usage (CXCR4 versus CCR5) researchers have made use of fusion assay systems that have led to evidence supporting that certain substitutions of basic amino acids in the V3 loop can influence or change virus isolate phenotype from nonsynctium inducing (NSI) to syncytium inducing (SI) thereby decreasing the ability of the virus to replicate in macrophages (verrier *et al.*, 1999). Subsequent engagement of the coreceptor promotes conformational changes in gp41 that mediates membrane fusion, which involves the merging of cell and virus membranes thereby allowing HIV proteins and nucleic acids to enter the host cell (Salzwedel *et al*., 1999).

Viruses that use CXCR4 (X4 viruses) or both coreceptors (X4/R5 viruses) are frequently associated with CD4 T-cell depletion and disease progression *in vivo*, while viruses that use CCR5 (R5 viruses) usually predominate during transmission and the asymptomatic stages of HIV-1 infection (reviewed by Berger *et al.*, 1995). In 1998, Baza *et al.* were able to demonstrate that the expression of CD4 in combination with either CCR5 or CXCR4 is necessary and sufficient for primary HIV-1 entry. Thus, despite the number of chemokine receptors implicated in viral entry, CCR5 and CXCR4 are likely to be the physiologically relevant chemokine receptors used as entry cofactors *in vivo* by diverse strains of primary viruses isolated from blood.

(ii) Replication

After membrane fusion, the viral nucleocapsid enters the cell and the viral genome and enzymes are released following removal of the core proteins (figure 2.4). A viral enzyme, reverse transcriptase, catalyzes the reverse transcription of the viral RNA template into its complementary DNA strand (cDNA). The cDNA copy is translocated to the host cell nucleus and integrated into the host chromosomal genome by integrase. Once the cDNA is transferred to the host genome, the virus is known as a provirus. Thereafter, HIV may either stay in a latent, proviral state for many years or the proviral DNA is transcribed into ssRNA by transcription factors and various mRNAs are processed to yield proteins. The mRNA is made by RNA polymerase and encodes different viral proteins.

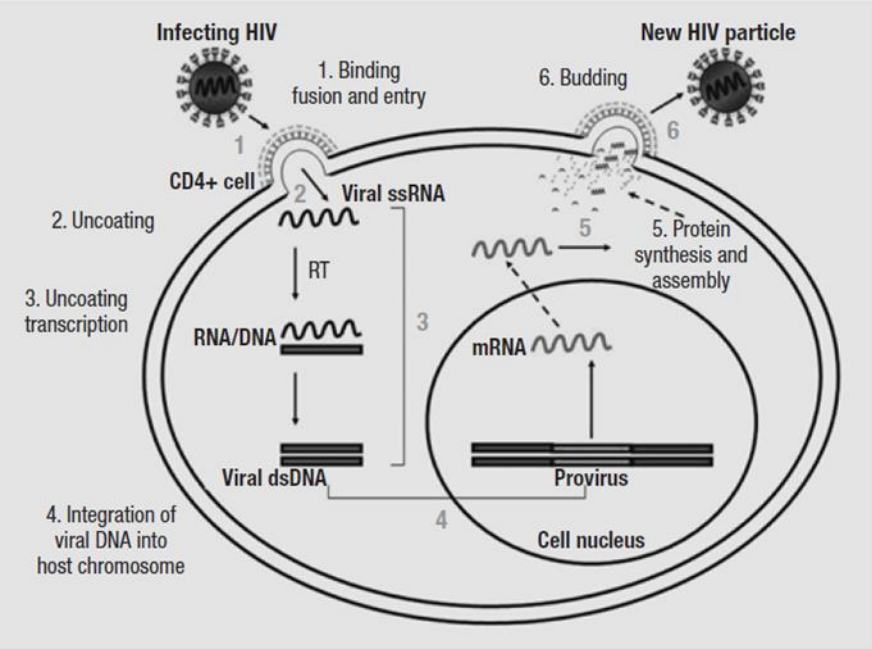


Figure 2.5: The HIV life cycle

HIV life cycle "Reproduced with kind permission from: Fanales-Belasio E *et al*., HIV virology and pathogenetic mechanisms of infection: a brief overview. Ann. Ist. Super. Sanità, 46: 5-14, 2010"

Transcription of proviral DNA into a messenger RNA results in the early synthesis of regulatory HIV-1 proteins such as Tat and Rev. Viral messenger RNA coding for long fragments migrates

into the cytoplasm, here structural proteins (gag, pol, env precursor) of new virions are synthesized. Two viral RNA strands associate together with replication enzymes, and together with core precursor proteins assemble, forming the virus capsid. This immature particle migrates towards the cell surface. Retroviral gag proteins facilitate assembly into virus particles as well as budding from the host cell membrane through interactions of short motifs within the gag precursor protein with host endosomal sorting machinery (Dimiter *et al*., 2004).

(iii) Budding and maturation

Retroviruses exploit the mechanisms and pathways (endocytic or biosynthetic) used to sort host proteins into multivesicular bodies, to promote their release (Dimiter *et al*., 2004). During assembly, the nucleoclaspid domain of newly synthesized Gag mediates the binding of Gag molecules to unspliced viral genomic RNA for packaging into virions (Harrison *et al*., 1992; Lever *et al*., 1989). Gag molecules are directed to the plasma membrane through palmitoylation and myristoylation (Bryant and Ratner, 1990) signals of the matrix domain. The plasma membrane is the principle site for viral assembly. The capsid core and nucleocapsid domains drive a process called multimerization that involves oligomerizatio of Gag molecules around Gag-RNA complex at the plasma membrane resulting in viral core formation (Ganser-Pollinos *et al*., 2008; Mateu, 2009). This is followed by recruitment of cellular components including host endosomal sorting machinery (Dimiter *et al*., 2004), clathrin adaptor molecules (Boge *et al*., 1998) as well as lipid second messenger phosphatidylinositol 4,5 bisphosphate which has an impact on the site of virus budding.

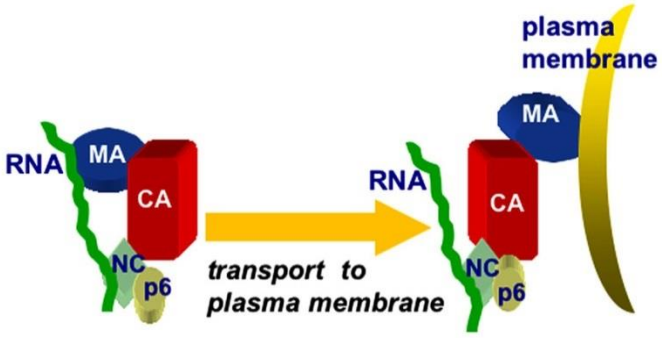


Figure 2.6: Chaperone model for MA–RNA binding.

Budding results in the acquisition of a new envelope provided by the infected host cell, the virus also incorporates into its membrane various proteins from the host cell membrane, such as ICAM-1 adhesion proteins that may facilitate adhesion to other target cells. Also MHC class I and II proteins and β-2Microglobulin because of its association with MHC I (Ott, 2008). *Some of these host proteins may have a role to play in vaccine, improved diagnostic or prognostic tool development because unique epitopes from these proteins elicit an immune response especially during early infection. This is the issue that was explained in this dissertation.*

Following release from the host cell, the non-infectious virus-like particles undergo a process known as maturation. Maturation is characterised by the cleavage of the large precursor molecules by the HIV-1 protease, resulting in new infectious viral particles. The nucleus of the maturing HIV particle is formed by proteins coded for by pol and gag genes; the gene products coded by the *env* gene form gp160 precursor molecules that are cleaved by HIV-1 protease into gp120 and gp41. The Gag and Pol proteins are also derived from a large 160 kD precursor molecule, from which the HIV protease cleaves the p24, p17, p9 and p7 Gag final products and the Pol proteins**.** The cleavage of the precursor molecules by the HIV-1 Protease is necessary for the generation of infectious viral particles (Fanales-Belasio *et al*., 2010). Other cell membrane proteins that are constituents of the viral envelope are adhesion molecules (integrins, ICAM family, L-selectin) and complement control proteins (CD55, CD59). The underlying mechanism responsible for HIV-1 acquisition of host encoded proteins remains a matter of speculation.

2.4 HIV env proteins and epitopes considered for vaccine development or use for prognostic purposes

Several HIV env proteins have been tested and are still being investigated as possible vaccine components. Early work focused on epitopes of virus-derived proteins (gp120, gp41) only and recently some host incorporated proteins (β2M) are being considered.

2.4.1 Beta-2 microglobulin

Beta-2-microglobulin is an invariant membrane protein associated with MHC molecules. It is 12 kDa in size and is composed of 96 amino acids. It is essential for the expression of MHC class 1 which is in almost all nucleated cells and binds peptide antigens for presentation to CD8+ T-cells during initiation of a cellular immune response. During its replication cycle, HIV-1 incorporates host cell proteins into its envelope during budding, either on its surface or inside the viral envelope (Ott, 2008), according to Ott (2008), most of the cellular proteins incorporated by the virus are taken up as spectators as a result of their proximity during the budding process. The significance of these non-specifically incorporated proteins is that they can indicate the local environment where HIV-1 assembles and the site of budding. B2m is expressed on all cells, and is therefore on all potential virus target cells (Arthur *et al.*, 1992). Consequently, each virus independent of tropism, genotype or serotype will incorporate β2m on its surface.

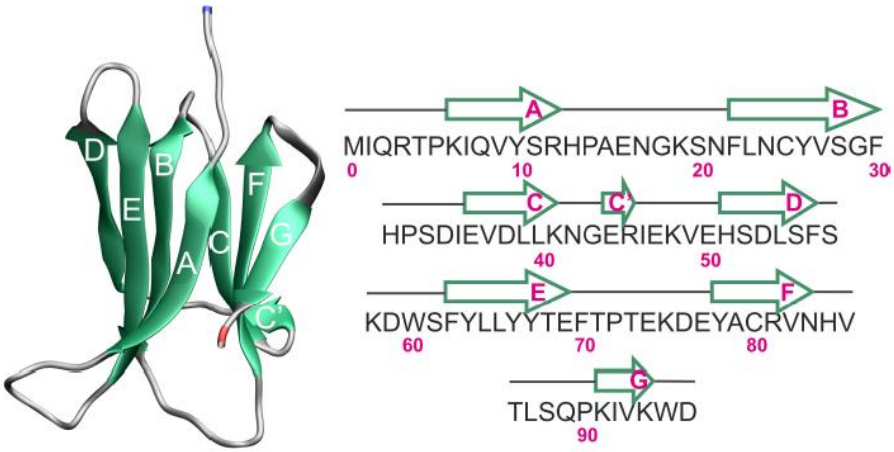


Figure 2.7: The primary and secondary structure assignment of human β2M is shown. Disulfide bonds between cys25 and cys80 stabalise the protein as well as the β strand is displayed.

2.4.2 Epitopes of β2M

A seven amino acid sequence (Arg-Thr-Pro-Lys-Ile-Gln-Val) derived from ß2m, has been identified as an epitope that is present at the surface of divergent HIV isolates (Le Contel *et al*., 1996). This R7V peptide, designated as RTPKIQV, is hidden and unclear on human ß2m but is revealed by HIV following incorporation into the envelope, and could be targeted by monoclonal antibodies (Galea *et al*., 1996). In the same way that viral glycoproteins exposed at the viral surface are targeted by neutralizing antibodies, anti-R7V monoclonal Ab were shown to have the capacity to neutralize infection *in vitro* by HIV from different genotypes (primary isolates of strains A–F from an international panel) as well as from different tropisms (lymphotropic or macrophage tropic laboratory strains) (Le Contel *et al*., 1996). Of the other short overlapping peptides derived from B2m studied by these authors, the heptamers S7K (Ser-Gln-Pro-Lys-Ile-Val-Lys) and F7E (Phe-His-Pro-Ser-Asp-Ile-Glu) were found to be capable of reversing the neutralising action of monoclonal antibodies directed to B2m.

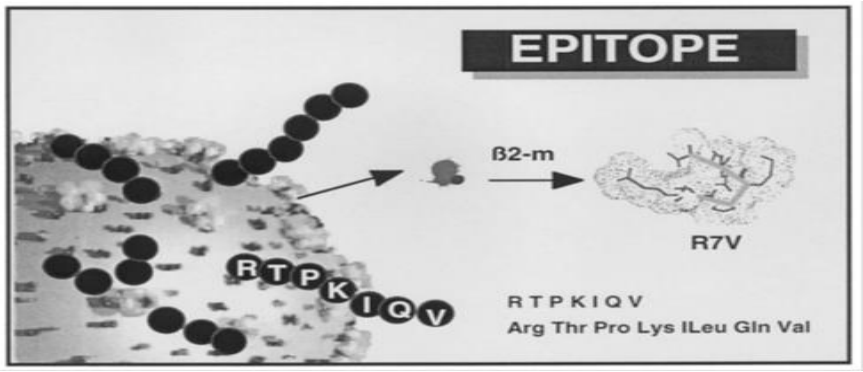


Figure 2.8: R7V epitope of β2m is presented at the HIV surface following incorporation of β2m into the viral envelope (Galea *et al.*, 1999*)*.

Earlier work (mentioned above) highlighted R7V as the most immunodominant of the short, overlapping peptides derived from B2M. This epitope was able to block HIV neutralization (Haslin and Cherman, 2002). R7V was suggested as a vaccine target (Le Contel *et al*., 1996; Galea, 1999; Chermann, 2001) because functional anti-R7V antibodies were detected in HIV-infected patients that were claimed by Galea *et al.* (1999) to be prognostic and indicative of slower progression of HIV-1 infection. These allegations were reviewed by Bremnaes and Meyer, 2009 and the potential of R7V epitope as a vaccine target was investigated in our lab. The study conducted by Bremnaes, 2010, made use of recombinant anti-R7V antibodies and

synthetic peptides based on this epitope along with a prospective study conducted by Margolick *et al*., 2010 provided strong evidence that the individuals who had detectable antibodies against R7V early in infection had progressed further in HIV-1 disease than those without R7V antibodies. These authors found no association between the incidences of R7V antibodies with nonprogression of HIV-1 infection, furthermore, in our lab, recombinant anti-R7V antibodies could not neutralize HIV-1 subtype C virus.

2.4.3 Gp120 and gp41 epitopes

Gp120 is coded by the HIV env gene, which is around 2.5 kb long and codes for around 850 amino acids (Kuiken *et al*., 2008). The primary env product is the protein gp160, which gets cleaved to gp120 (~480 amino acids) and gp41 (~345 amino acids) in the endoplasmatic reticulum by the cellular protease furin (Hallenberger *et al*., 1995). The crystal structure of core gp120 shows an organization with an outer domain, an inner domain with respect to its termini and a bridging sheet (as is illustrated by figure 2.6). Gp120 has 5 variable regions (V1-V5) combined with 5 conserved regions. The first 4 variable regions are incorporated into loop-like structures through intermolecular disulphide bonds (Wyatt and Sodroski *et al*., 1998).

Since CD4 receptor binding is the most evident step in HIV infection, gp120 was among the first targets of HIV vaccine research. This glycoprotein anchored to the viral membrane, or envelope, via non-covalent bonds with the transmembrane glycoprotein, gp41. Gp120 is considered to be immunogenic but efforts to develop HIV vaccines targeting gp120 have been hampered by the chemical and structural properties of the glycoprotein, which make it difficult for antibodies to bind to it (Carrow *et al*., 1991). Gp 120 can also easily be shed from the surface of the virus and captured by T cells due to its loose binding with gp41. The V3 loop is considered to be quite variable between different strains of HIV-1, a region was identified that forms a binding site for antibodies that function as entry and fusion inhibitors. This region forms a conserved sequence within the V3 loop, Gly-Pro-Gly-Arg (GPGR) and is called the principal neutralizing determinant (PND) (Freed *et al*., 1990). The PND is considered as the primary site for type

specific antibodies that were reported to be broadly neutralizing making it a region of interest in vaccine design.

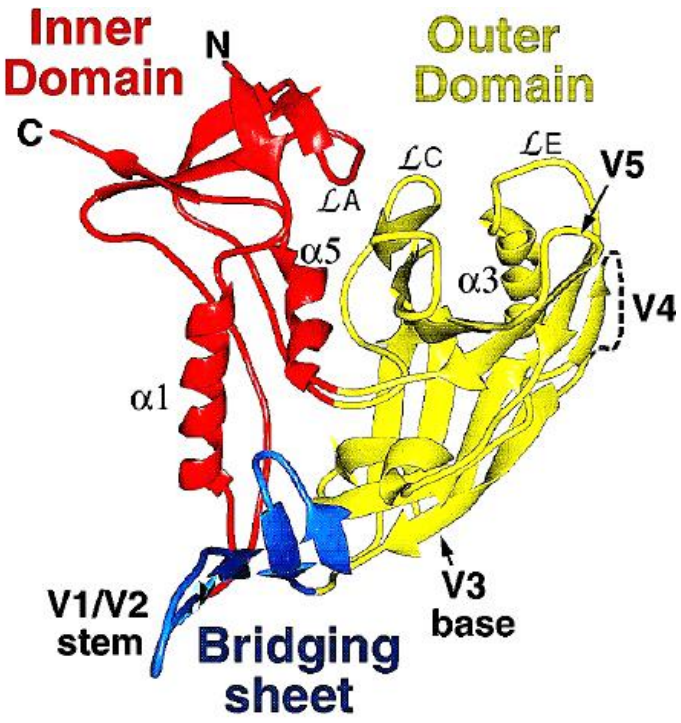


Figure 2.8: The above figure illustrates the arrangement of the 5 variable regions and the bridging sheet within the outer domain and inner core region of gp120. Figure obtained from wyatt *et al.*, 1998

The conformational changes that gp120 and gp41 undergo during viral entry lead to exposure of critical epitopes that could have potential as antigenic targets for neutralizing of HIV-1 (Peachman *et al*., 2010). It was studies investigating the exposure of gp41 epitopes (Crooks *et al*., 2005; Moore *et al*., 2006) during viral entries that lead to the discovery of the membrane proximal external region (MPER) of gp41. This region was identified after broadly neutralizing monoclonal antibodies (MAb) were described that were specific for this region (Hartono *et al*., 2013). The conformational changes that gp41 undergoes prior to fusion render MPER accessible to 2F5, 4E10 and Z13 that have led to this site being proposed as a potential neutralizing determinant.

2.5 A brief introduction to the immune system as described by Campbell and Reece

Due to the effect of HIV on the immune system, background on this virus must include reference to the immune system. The principle function of the immune system is to protect the host from pathogens present in the environment and from potentially dangerous self-constituents. The development and maintenance of immunity is dependent on the body's ability to distinguish self from non-self, with subsequent elimination of foreign invaders and development of immunologic memory. This complex and highly sophisticated defense system is functionally divided into the innate and adaptive immune systems (Campbell and Reece, 2003). The first line of defense is the innate immune response. Innate defenses are mainly nonspecific and can quickly recognize and respond to a broad range of microbes regardless of their precise identity. Most innate immunity components are present before the onset of infection and create a set of disease-resistance mechanisms that are not specific to a particular pathogen. These are external barriers formed by skin and mucous membranes, a set of internal-cellular as well as chemical defenses that combat infectious agents that breach the external barriers. Key players in the internal defenses are phagocytic cells such as macrophages, which ingest and then destroy pathogens (Campbell and Reece, 2003). Many of the molecules involved in innate immunity recognize highly conserved pathogen-associated molecular patterns or structures to detect broad classes of invading pathogens. This type of response is amplified through lysis of pathogens by complement proteins, phagocytosis by natural killer cells and macrophages along with acute phase inflammatory proteins leading to an inflammatory response which triggers elements of adaptive immunity through the production of cytokines.

Cytokines are low molecular weight regulatory proteins/glycoproteins that assist in regulating the development of immune effector cells and are secreted by lymphocytes and various other immune cells in response to stimuli. These proteins have control over the intensity and duration of an immune response by regulating inhibition, activation, and proliferation of various cells as well as antibody secretion or the secretion of other cytokines.

Lymphocytes, a type of white blood cell produced in the bone marrow that display antigen binding cell surface receptors and are key players in adaptive immunity. These receptors mediate

the immunologic attributes of specificity, diversity, and memory. Distinguishing between "self" and non-self is an important feature of immunity; lymphocytes undergo a process called negative and positive selection that instils tolerance of "self" thereby minimising the chance of autoimmunity. The two major populations of lymphocytes are B lymphocytes (B cells) which mature in the bone marrow and T lymphocytes (T cells) which mature in the thymus.

Not only does this adaptive immunity display specificity, it is also capable of immunologic memory and extraordinary diversity. Antigenic challenge occurs when T lymphocytes recognize short peptide sequences of a protein antigen through the T cell receptor (TCR) in context with MHC I/II, and B lymphocytes recognize antigenic determinants through B cell receptors (antibodies). An immunogen is a substance that is capable of inducing an immune response. Immunologically active regions of an immunogen that bind to antigen-specific membrane receptors on lymphocytes or secreted antibodies are called antigens. Antigenicity is the ability to combine with the final product of the response (antibodies or T cell receptor). The region of an antigen that binds to a T cell receptor or an antibody is called an epitope and is the part that determines antigenicity, thus the term antigenic determinant. Antigen recognition causes lymphocyte activation which leads to a process called clonal expansion. Differentiation results in the production of specific antibodies by plasma cells.

Once the immune system has recognised and responded to a foreign antigen, exposure to that same antigen in future results in an immunologic memory response; that is, heightened, quicker and is often more effective than the first challenge. The basis for the use of vaccines comes from exploiting the characteristic of immunologic memory through preparations of altered infectious or toxic agents so as to mount an effective immune response without causing disease. The modification should not, however, be so drastic as to destroy all the epitopes. The aim is to produce antibodies (and/or T cells) in response to the vaccine that also protect against the unaltered disease-producing agent. An effective immune response involves antigen presenting cells (APC), lymphocytes, antibodies as well as other accessory molecules they produce (such as cytokines) which facilitate neutralisation of pathogens by removal of infected target cells and elimination of inflammation.

2.6 How HIV infection affects the immune system

After transmission, HIV-1 is initially localized to the point of entry. Antigen-presenting cells (such as macrophages and dendritic cells) have been implicated in allowing the virus to spread throughout the body. Dendritic cells can bind HIV-1 and deliver viral antigen to the lymph system. Lymph nodes are fortified with white blood cells, once dendritic cells have matured and are expressing co-stimulatory signals in the lymph node they can stimulate T cells. Macrophages are also able to process viral antigens and present antigen to T helper cells thereby stimulating them. The stimulation of T cells activates them and results in their proliferation, and secretion of cytokines which alert other parts of the immune system including B cells and cytotoxic T lymphocytes (Blancheta *et al.,* 2011)**.** B cells recognize the antigen using their soluble receptors and are activated to produce millions of antibodies specific to the antigen. The antibodies are released and attack the antigen through opsonisation, cytotoxic T lymphocytes. To prevent prolonged activation of the immune system, once the number of invaders has dropped and the infection has resolved; suppressor T cells signal other immune cells to rest.

During HIV infection cytotoxic CD8+ T lymphocytes* (CTL) that may limit the spread of infectious agents by recognizing and killing infected cells or secreting specific antiviral cytokines are generated. The generation and maintenance of both B and CD8+ T cell responses is supported by growth factors and signals provided by CD4+ T helper (Th) lymphocytes, which are commonly subdivided into T helper 1 (Th1) and T helper 2 (Th2) subtypes (Blancheta *et al.,* 2011)**..** Th1 cells primarily secrete IL-2, INFϒ, TNFα and B-chemokines and are thought to provide protective responses against intracellular pathogens. Production of, INFϒ activates CTLs (initiating killing of infected cells) and restricts Th2 responses. Th2 cells lead to humoral responses chiefly through the production of Il-4, which results in B-cell activation and antibody production. Other cytokines produced during Th2 response are Il-5, IL-6, IL-10 and IL-13. HIV infection is associated with increases in Th2 cytokine production and a loss of IL-2 in chronic individuals (Morgan *et al.*). This massive immune activation caused by HIV/AIDS requires control by regulatory T cells (Treg) that are involved in maintaining immune tolerance. This is important in maintaining the stability of the immune system because progression to AIDS leads to immune exhaustion.

2.7 Vaccine Development for HIV/AIDS

The extensive genetic variability of the HIV envelope glycoprotein due to a lack of proof reading mechanism by reverse transcriptase, as well as the glycosylation these proteins go through are a huge obstacle for vaccine design. To circumvent these factors the use of synthetic peptides that mimic conformational epitopes (especially of the viral envelope) were introduced. These were made in many modified forms in an attempt to maintain immunogenicity *in vitro*. Approaches included the use of multiple antigen peptide, cyclic branched varieties as well as hypervariable epitope constructs (Meyer, 2004). Another problem is the ability of HIV to establish latent infection integrating its genome. Several approaches have been taken to design novel vaccines against HIV including RV144 prime-boost combination of two vaccines: ALVAC® HIV vaccine (the prime), and AIDSVAX®B/E vaccine (the boost). The vaccine regimen included two vaccine candidates, ALVAC® HIV (vCP1521) and AIDSVAX® B/E (gp120), and involved a total of six immunizations over six-months: four immunizations with ALVAC-HIV and two with AIDSVAX B/E given at the same time as the last two ALVAC-HIV injections. This combination of two different vaccines is called a prime-boost approach. In this approach, two vaccines are given in sequence with the goal of inducing the strongest and most comprehensive immune response possible (Rerks-Ngarm *et al.*, 2009)**.** SAV001-H is an example of a phase 1 vaccine attempt based on a genetically modified killed whole-virus.

An HIV vaccine may be totally successful in preventing any infection in vaccinated people, providing what is known as "sterilizing immunity." Sterilizing immunity may be possible in 100% of the population, or perhaps only in certain groups. In another scenario, a preventive vaccine may not prevent primary infection, but may decrease the possibility of HIV transmission from an infected person who has been vaccinated to another person. Yet another possibility is that a vaccine may not prevent infection entirely, but may slow the course of infection when it occurs. In this case, even if a vaccinated person becomes HIV infected, the vaccine helps that person remain healthier longer.

The nature of the interaction between HIV and the immune system is complex, and the relevance of different immune responses to the control of infection is only partially understood. Other challenges in HIV vaccine design include the lack of correlates of protective immunity; the

capacity of the virus to target and integrate its genome into cells of the immune system; and most importantly, the unprecedented hyper-variability of HIV (Meyer,2004).

Thus, safe and effective HIV vaccines will likely require induction of broadly protective neutralizing antibodies to prevent HIV infection, and broad cellular immune responses to control HIV infection (Koff, 2012). While the product development pipeline includes some approaches for induction of broad cellular immune responses, including the use of conserved epitopes across the genome, and mosaic antigens there currently are no candidates in the pipeline that elicit broadly neutralizing antibodies (bNAbs) against HIV.

2.8 HIV disease progression

The challenge of controlling HIV infection involves an understanding of the heterogeneity of the virus, its wide cellular host range, its primary routes of transmission, and the immunologic and intrinsic cellular factors that can prevent its transmission and replication. Identification of HIV-infected individuals who have survived more than 10 years without signs of the infection and without therapy encourages studies examining the natural mechanisms for resistance to infection and disease.

HIV disease, however, is not uniformly expressed in all individuals. Some HIV-1 positive people develop AIDS and may die within months following primary infection, however, there exists a small subset of individuals; approximately 5 percent of HIV-infected individuals that exhibit no signs of disease progression and have stable CD4 T cell counts despite years of living with HIV; these people are called long-term non progressors (Pantaleo *et al.*, 1995a; Cao *et al.*, 1995). This is in contrast to the typical HIV-infected individuals in whom the number of CD4+ T cells progressively declines over time (see the clinical stages of disease in figure 2.4). Host factors such as age or genetic differences among individuals, the level of virulence of the individual strain of virus, as well as influences such as co-infection with other microbes may determine the rate and severity of HIV disease expression in different people (Fauci, 1993a; Pantaleo *et al.*, 1993a). The duration of clinical latency varies but the progression to acquired immunodeficiency syndrome usually occurs after an average of 10 years.

The fact that some of these asymptomatic patients have shown a lack of progression for more than 10 years raises the question of possible natural AIDS resistance mechanisms. Studies have indicated that host as well as viral factors are involved in non-progression (Haynes *et al*., 1996; Hogan and Hammer 2001a, b; Lopalco, 2004).

The mechanism of late progression to AIDS is complex and multifactorial; nevertheless the identification of a predictive marker of this phenomenon would have a very important impact on the treatment and management of HIV-infected patients. Research has indicated an association between non-progression status and the presence of anti-R7V antibodies (Ab) (Le Contel *et al*., 1996; Galea *et al*., 1996). These antibodies are directed against R7V peptide, which is an epitope of beta-2 microglobulin.

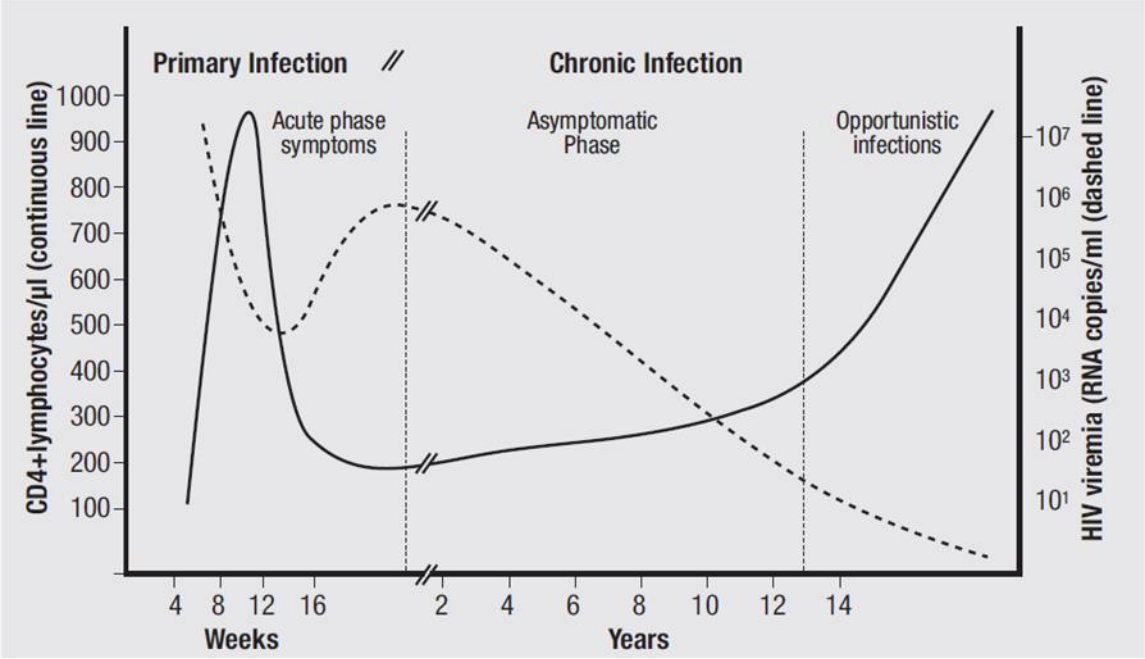


Figure 2.9: Clinical stages of disease progression during HIV infection

"Reproduced with kind permission from: Fanales-Belasio E *et al*., HIV virology and pathogenetic mechanisms of infection: a brief overview. Ann. Ist. Super. Sanità, 46: 5-14, 2010".

2.9 Monitoring infection and disease

ELISA is the most widely used assay for detection of antibody or antigen, owing to the fact that it is extremely sensitive, highly economical on reagents and can test large sample numbers in a short time. ELISA also allows quantitative measurement or qualitative detection of antibody. The Western blot assay is used to verify the presence of HIV-1 as a confirmatory test (Lange *et al.*, 1986). Antigens are separated by Poly Acrylomide Gel Electrophoresis (PAGE) and trans-blotted onto nitrocellulose/nylon membranes and are revealed as characteristic bands of different molecular weight in the presence of HIV-1 antibodies.

Determination of CD4 T-cell count and plasma viral load: Monitoring CD4+ T cell count and keeping records of viral load are important aspects of HIV disease management. Although several alternative methods have been described such as The ELISA TRAx CD4+ test kit (T Cell Diagnostics, Cambridge, MA, USA) and enumeration by way of dried blood specimens (Mwaba, Cassol *et al.*, 2003), flow cytometry remains the gold standard for CD4+ T lymphocyte count measurement. This method makes use of fresh whole blood from which CD4+ cells are enumerated. The viral load represents the amount of HIV RNA in the blood and is measured from plasma using either real-time polymerase chain reaction (RT-PCR), isothermal nucleic acid based amplification (NASBA) assay or by the branched DNA signal amplification assay.

Continuous updating of knowledge on structure, variability and replication of HIV, as well as the characteristics of the host immune response, are essential to refine virological and immunological mechanisms associated with the viral infection and allow us to identify key molecules in the virus life cycle that can be important for the design of new diagnostic assays and specific antiviral drugs and vaccines. The characteristics of molecular structure, replication and pathogenesis of HIV, as well as the important aspects for the design of diagnostic assays, are reviewed by Fanales-Belasio *et al.*, 2010.

2.10 Principles of methodologies used in HIV/AIDS research

The principles of the methods used in this study are provided in the methods section of the dissertation immediately followed by the actual protocol used.

2.11 HYPOTHESIS

Synthetic peptides based on epitopes of HIV envelope proteins (host-derived & viral antigens) can be used to characterize the immune response during HIV infection.

2.12 AIM

In order to investigate the hypothesis, synthetic peptides that mimicked epitopes of envelope proteins (host and virus-derived) were synthesized and used to measure humoral immune responses. Characterization in the context of this study, refers to the ability of the epitopes to measure immune responses *in vitro* following natural infection. To this end, answers to the following questions were investigated:

(i) Were the peptides antigenic? Antigenicity is the ability of a molecule to react with products of the immune response such as antibodies or T cell receptors. The epitopes these peptides were based on, were part of proteins found, exposed on the virion surface and the accessibility of these epitopes to the immune system is important if they are to have therapeutic potential.

(ii) **Were these peptides able to stimulate the proliferation of PBMCs and TZM-bl cells *in vitro*?** When the immune system is presented with a recurring encounter of a pathogen or antigen (component of the pathogen), an immunologic memory response leads to the proliferation of lymphocytes in response to the challenge.

(iii) **Did the synthetic peptides have an influence on cytokine production of PBMCs?** The profile of secreted cytokines *in vitro* was taken as indicative of T-lymphocyte function *in vivo*. Antigens are expected to influence cytokine production and depending on the response, conclusions can be made regarding the influence of the peptides on immune functions.

(iv) **Were the peptides able to elicit an immune response?** As immunogens, these peptides would have to induce antibody and or cell-mediated responses in test subjects (e.g mice or rabbits) following injection. Immunization often involves conjugation of the immunogen to carriers and the use of adjuvants to enhance responses.

This work focused on the humoral immune response i.e. the presence of antibodies following immunization with the peptides.

(v) **In the event that the peptides were immunogenic, were the antibodies functional?** Functional antibodies are able to neutralize virus, potentially more than one strain.

(vi) **Could the peptides distinguish stages of HIV-1 infection?** Diagnostic peptides detect the presence or absence of specific antibodies. If the detected antibodies are found primarily in a specific stage of disease (e.g early/late infection) then the antibodies (and by extention, the epitope) have prognostic potential

CHAPTER 3 – METHODS

3.1 Introduction to experimental work

Synthetic peptides based on the epitopes of the HIV envelope proteins were synthesized (Genscript and Lifetein) and used to assess immune responses following natural HIV infection. These peptides were also used to elicit polyclonal antibodies in rabbits. Affinity purified rabbit polyclonal antibodies were screened for neutralising antibody activity at NICD using the pseudo virus assay (Montefori, 2004). Antibodies from HIV positive individuals which responded to the peptide antigens in ELISAs were also screened for neutralizing abilities. An in-house indirect peptide ELISA was performed to detect antibodies directed against these antigens in the serum of; HIV negative, HIV infected on HAART and HIV infected treatment-naïve individuals. The Cytometric bead array as well as XTT viability/proliferation assays was carried out to measure the secreted cytokines and the viability of peripheral mononuclear cells (PBMCs) respectively following peptide stimulation. Flow Cytometry and CFSE was used to measure the effect of the peptides on proliferation of PBMCs of infected and uninfected individuals. The workflow is explained in figure 3.1.

WORK FLOW DIAGRAM

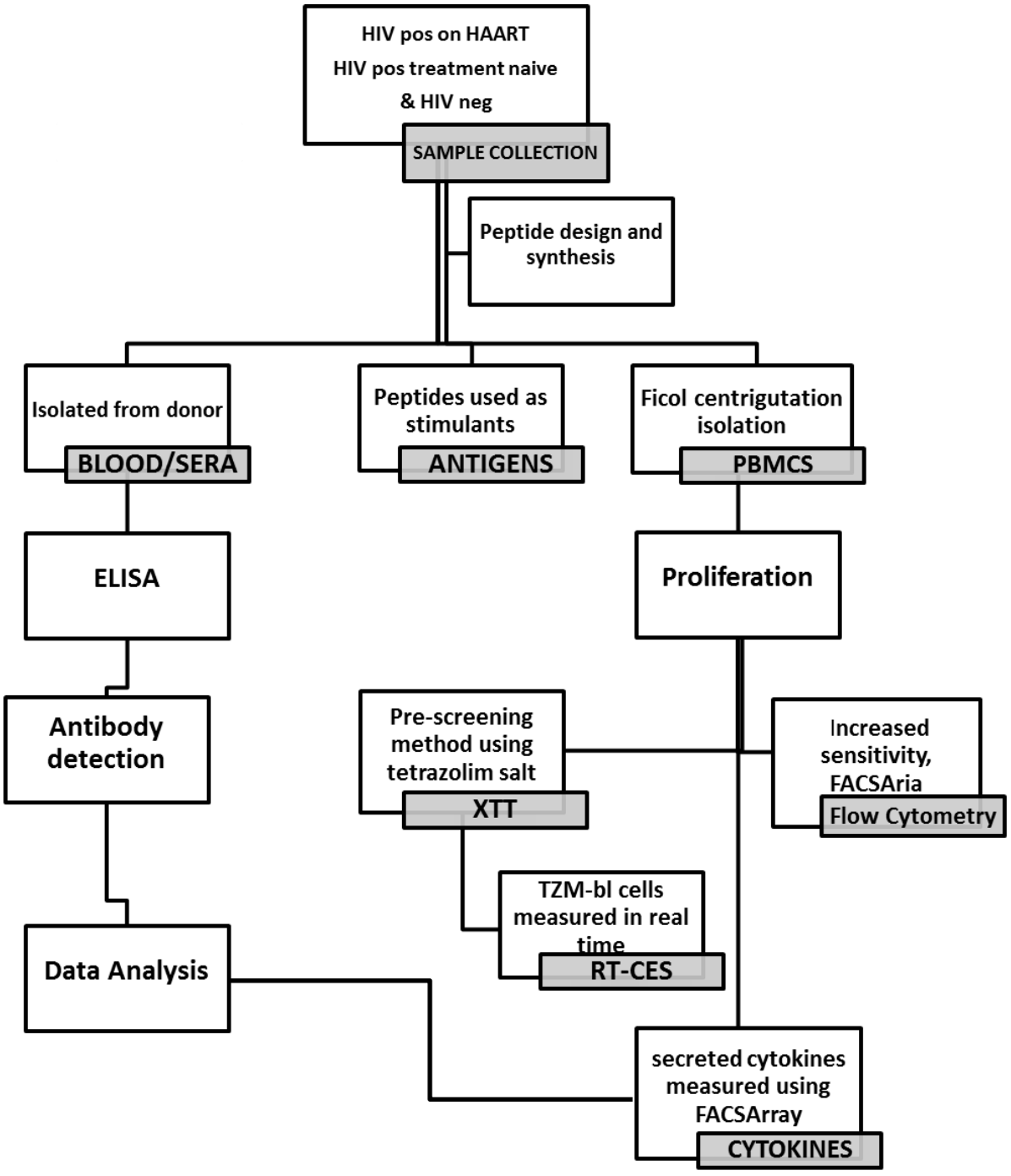


Figure 3.1: Workflow diagram

The workflow of study including designing peptides, having them synthesized then screening the peptides in several biological assays. Minimal sensitivity assays (XTT) were compared to advanced methods (FACS and RTCES) and the ELISA was used for antibody detection.

3.2 Peptide synthesis

Synthetic peptides play numerous roles in HIV/AIDS; mimicking functionality of important protein regions, as diagnostic tools or vaccine components and drugs e.g. Enfuvirtide (Fuzeon®), a 36 amino acid derived from the HIV-1 glycoprotein-41. Peptides capable of mimicking functionally important regions of HIV proteins can function as tools for exploring structure and function and is investigated in the development of new treatments for AIDS. Synthetic peptides have inherent benefits such as their relatively inexpensive production, ease of use, that they can be produced in large quantities in highly pure form and can be easily adapted to high-throughput and automated platforms. Furthermore only immunodominant regions or epitopes can be selected thus increasing specificity and decreasing possible cross-reactivity with non-specific antibodies.

With the aim of designing peptides that mimic epitopes on proteins that play a role in HIV pathogenesis; databases and literature was consulted over several years. The SwissProt, GenBank, Protein data bank (PDB), Entrez as well as the Los Alamos HIV sequence database were among the databases used as sources from which HIV protein sequences can be obtained. Another valuable tool was the HIV Molecular Immunology Database which is an annotated, searchable collection of HIV-1 cytotoxic and helper T-cell epitopes and antibody binding sites. These databases along with literature provided a comprehensive listing of defined HIV epitopes. The chosen peptide sequences were based on epitopes of HIV envelope glycoproteins; gp41 (MSc C Philippeos 2007), gp120 (Hewer and Meyer 2002) as well as host-derived beta 2 microglobulin (Braemnes and Meyer 2009), that have been reported to be presented on HIV-1 envelope and believed to be immunodominant. A peptide based on human endogenous retrovirus (HERV-K) was also included, HERVs are considered inert but have been reported to be activated during HIV infection resulting in T cell responses in infected individuals (Garrison and Jones *et al.*, 2007, Sengupta *et al.*, 2011).

Considerations for peptide design included primary structure, size of the peptide and properties that define critical epitopes for HIV vaccine design. Some of the peptides have unique features such as the addition of dibasic motifs that flank antigenic peptides and are recognised by

endoproteases. These endopeptidase cleavage sites were included in an effort to enhance T cell stimulation for the given epitope (Schneider *et al.*, 2000).

Method: Peptides used for this study were synthesized by Lifetein cc, USA and Genscript. Peptides were synthesized from the N to the C terminus using peptide syntechnology (Lifetein cc, USA) which makes use of solid phase peptide synthesis (SPPS) based on Fmoc or tBoc chemistry to protect the alpha group. Briefly, Fmoc or tBoc deprotection agents are used to free the alpha amino group in preparation for coupling with the next amino acid in the sequence. A new N-terminal amine to which the next amino acid may be attached to form a peptide bond is then revealed, following peptide synthesis, peptides are cleaved from the polymeric resin to which they were anchored, and deprotected. Peptides are precipitated, washed and then lyophilized. A detailed account is provided below (Merrifield, 1963).

The SPPS method is based on the formation of a peptide bond by coupling two amino acids through an activated carboxyl and a protected amino group of the N-terminal amino acid. Coupling the C-terminal amino acid to a functionalized insoluble resin provides a protected carboxyl group of the C-terminal amino acid. Another type of protection group than the one used for the *C*-amino group is used for protecting functional groups of the side chains of the amino acids. Fmoc or tBoc deprotection agents are used to free the alpha amino group in preparation for coupling with the next amino acid in the sequence. Following deprotection of the amino function, the next amino acid is coupled; all reactants are then washed away, leaving a protected dipeptide on the resin. This cycle continues for the required number of times and the peptide is detached from the resin. Cleavage of the peptide from the resin results in the simultaneous removal of the side chain protecting groups. Deprotection and cleavage are achieved by treatment with HF **or** trifluoromethanesulphonic acid. Growth of the peptide chain thus occurs from the C-terminus to the N-terminus. The a-fluoreneylmethyloxycarbonyl group (Fmoc), a base labile protecting group (20 % piperidine is necessary for deprotection) for the amino group. This enables a change to resulting in a combination with mild acid labile side chain protection cleavable by TFA. The Fmoc strategy allows for real time spectrophotometric monitoring of the progress of coupling and deprotection. The need for repetitive TFA acidolysis of t-Boc

deprotection carries the risk of leading to the alteration of sensitive peptide bonds as well as acid catalyzed side reactions.

Fmoc is preferred over t-Boc because the Fmoc strategy has the advantage that is that the growing peptide chain is subjected to mild base treatment using piperidine during Fmoc group deprotection and TFA is only required for the final cleavage and deprotection of peptide resin. Unlike cleavage and deprotection in t-Boc strategy which requires the use of dangerous HF and expensive laboratory apparatus, which is not always readily available.

The concentration of the desired peptide can vary from roughly a few per cent to 90% in a crude preparation depending on the nature of the peptide and sequence length.

Gel filtration, HPLC and anion-exchange chromatography can be used to purify the product. HPLC is used most frequently for purification today. Following deprotection and cleavage from the resin the crude product is subsequently lyophilized and after ether precipitation, the peptide mixture is dissolved in water with 0.1 % TFA. The peptide mixture is separated by Reversed phase HPLC using a C^{18} stationary phase is used to separate the peptide mixture which is eluted with a gradient of acetonitrile and 0.1 % TFA as counter-ion. Peptides can then be dissolved in water, failing which, diluted acetic acid, formic acid or other organic solvents can be tried. For an acidic peptide, ammonia may be necessary for dissolving it. Amino acid analysis can be performed on the hydrolysed peptide or the mass spectrum can be verified to confirm peptide composition.

3.2.1 Peptide Properties

In general, hydrophobicity or hydrophilicity plots are designed to find out the polar and apolar residues of a given protein sequence. Residues that span through the membrane are highly hydrophobic in nature while residues exposed on the surface of proteins are hydrophilic. (Welling *et al.*, 1985).

The Hopp-Woods scale, an essential hydrophilic index was developed to predict potential antigenic sites of globular proteins rich in charged and apolar residues. A negative value is

assigned to apolar residues. The program calculates each residue value from the input fasta file and a moving window size of 6 was employed by default. Values greater than zero represent hydropathic regions whereas negative values represent hydrophobic regions (Hopp *et al*., 1981).

3.3 Sample collection and Serum isolation

Due to the fact that it is not possible to obtain pre-infection blood samples of HIV infected individuals, negative controls were collected from different individuals but matched to the infected individual as close as possible with regards to specific potentially confounding factors. When making comparisons between responses of infected and uninfected individuals these potentially confounding factors (for example age and life style differences (potential for exposure), traditional medicine use of especially infected individuals etc.) need to be kept in mind as they can influence data interpretation (as is shown in figure of age matched samples in ELISA results section). Blood samples from HIV-1 infected (on and off treatment) as well as uninfected patients (negative control) were collected over the period of the study. Before blood was drawn, informed consent was obtained from both HIV negative donors and HIV positive patients and samples stored anonymously. The blood was collected by way of venepuncture.

The blood used for the isolation of serum was collected in red -capped vacutainers without anticoagulant. The blood was allowed to clot (at room temperature for 30-60 minutes), then centrifuged to separate the serum supernatant from the rest of the blood debris. The serum was then aliquoted and frozen at -70 $^{\circ}$C until use. Before use in the ELISA assay, the frozen serum aliquots were thawed on ice and then the serum was heat inactivated (56°C) for 30min to inactivate complement proteins and remove heat labile anticomplementary activity (Soltis *et al.*, 1979).

3.3.1 Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

The indirect ELISA is one in which antigens (represented by different synthetic peptides in this case) are immobilised by way of passive adsorption onto a solid phase (ELISA plate). All

unbound antigen is removed using a blocking buffer before the plate is washed and incubated with the test sera (serum samples from different patients). Any antibodies are detected in the test sera following binding to the antigen. Patients with previous exposure to the specific antigen (due to natural HIV infection) are expected to have produced antibodies. A secondary antibody that binds the primary antibody (in sera) and has an enzyme conjugate which reacts with a substrate is used for calorimetric detection. The colour change is proportional to the amount of antibody bound (produced) and is analysed using a spectrophotometer set at the appropriate wavelength defined by the substrate used (voller *et al.*, 1978). This assay is described in detail in the University of Pretoria, Biochemistry Department HIV laboratory Rules, Standard Operating Procedures and Protocol book, 2013 in section VII: ELISA Protocols (pgs. 7-10), a brief description is provided below.

Method: Each well in the ELISA plate (96-well Nunc^{TM} plate, Denmark) was coated with 100 µl of coating buffer (0.05M $NaCO_3$ at pH5) containing 250 ng/ml synthetic peptide and left at 4°C overnight to allow attachment of the antigen to the plate. Following incubation, 300 µl of blocking buffer (5%FCS (v/v), 0.1% Tween20 (v/v), 5% skim milk (w/v) in 10mM PBS at a pH of 7.4) was added to each well to minimize non-specific binding. The plates were incubated for 60 minutes at 37°C. After incubation, the wells were washed 5 times (using a well wash4 system^{TM}) with wash buffer (0.1% Tween20 v/v in PBS at a pH of 7.4). Test sera from HIV positive as well as negative individuals were diluted (100X) in blocking buffer then loaded (100 µl /well) in triplicate. Next, 100 µl of a 5000x dilution of horse radish peroxidase-conjugated goat anti-human immunoglobulin (IgG H+L, Jackson Immunoresearch laboratories, West Grove, USA) was added. After incubation at 37°C for one hour the plates were washed as previously described. Finally, 100 µl of the substrate consisting of 10 mg O-phenylenediamine and 8 mg urea-hydrogen peroxide in 12 ml OPD substrate buffer (2.6 g citric acid and 6.9 g Na_2HPO4 dissolved in 500 ml distilled water to a pH 5) was added to each well. Absorbance readings were measured at 15 minute intervals over a 60 minute period starting at time zero and read at 450nm, using a Biotek EL808 microplate reader (Thermo Lab systems, Helsinki, Finland).

For the purpose of the above assay, positive controls in the form of single-chain variable fragments (scFv) were made, corresponding to host-derived antigens, using phage display technology. Phage display technology entails the expression of peptides or proteins on filamentous phage (Smith, 1985; Azzazy and Highsmith, 2002). These peptides or proteins are able to be displayed on the phage surface through the insertion of the gene encoding for the peptide into the phage genome (Nilsson *et al*., 2010). The various steps involved in selection and affinity maturation using this technique are described by Hoogenboom *et al*., 1998 and Thie *et al*., 2008. Phage display technology is able to produce scFv or antigen-binding fragment (Fab) (smith *et al*., 2004). The scFv fragment is composed of both the variable domain of heavy (VH) and the light (VL) chains (Brekke and Loslet, 2003). Although scFv fragments do not retain the parental whole antibody effector functions, they are able to exhibit the binding function like the parental antibody (Adams *et al*., 1998; Azzazy and Highsmith, 2002) through the complementary determining regions (Adams *et al*., 1998). The size and stability renders the scFv fragments suitable for phage display, allowing the scFv gene to be fused into the phage coat protein for expression of the scFv fragment on the phage surface (Davies *et al*., 2000).

This screening technique does not depend on an animal's immune system, therefore, antibodies to a wide variety of antigens, including the molecules that cannot stimulate immune system of the animals such as non-immunogenic, "self", cell surface or antibodies with unusual specificities not present in the natural repertoires, can be generated (Griffiths *et al*., 1994). The advantage of smaller antibody fragments is that they have high tissue penetrability, while maintaining their affinity and specificity. They are also easier and faster to produce in recombinant form.

3.3.2 Cytokine detection: CBA

The Cytokine Bead Array (CBA) kit (BD Biosciences, San Jose, California) uses multiplexed beads (varying in FL3/FL4 intensity) labeled with capture antibodies for specific analytes. The test sample (e.g., serum or cell culture supernatant) is added together with PE-labeled detector antibody and formation of sandwich complexes allow for FCAPArray^{TM} software (Soft Flow Hungary LTD.) to calculate and quantity the level of each analyte based on PE fluorescence of each bead population relative to a standard curve. Cytokine analysis was performed using multiplexing technology for the analysis of the human Th1/Th2/Th17 cytokinome through flow cytometric detection. Secreted cytokine levels were measured following the isolation and stimulation of PBMCs obtained from infected individuals on HAART as well as HIV negative donors.

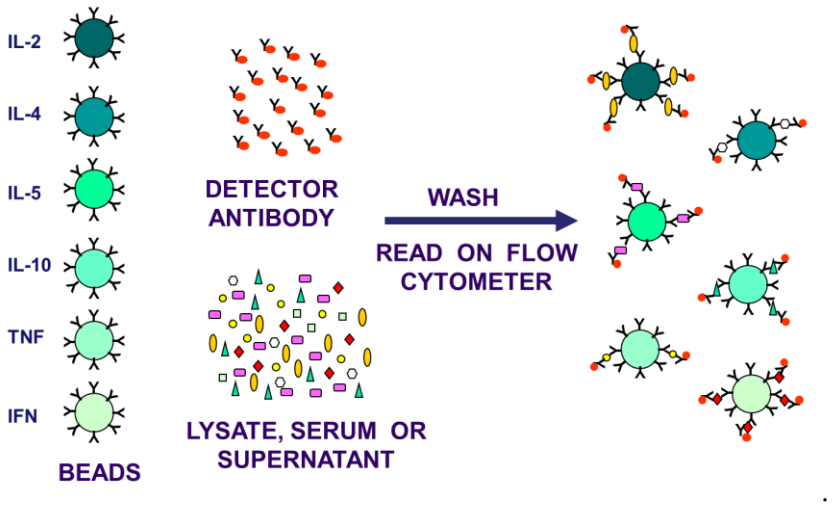


Figure 3.0.2: The principle of the CBA assay illustrated for Th1/Th2 cytokines. The CBA software automatically quantifies the amount of each cytokine per sample, based on a standard curve.

Method: The PBMCs from the abovementioned experimental groups were isolated fresh and treated with 10 µg/ml peptide and incubated for 7 days. Following incubation, secreted cytokines were quantified from the supernatant using the cytokine bead array (CBA) kit. This Human Th1/Th2/Th17 CBA kit (BD Biosciences, San Jose, California) allows for the simultaneous detection of cytokines using capture beads coated with antibodies specific to IL-2, IL-4, IL-6, IL-10, TNF-a, IFN-ϒ and IL-17A. Briefly, 50 µl of pooled capture bead mixture was added to 50 µl

of test samples and standards respectively. A PE-conjugated detection antibody (50 µl) was then added to these and the mixture was incubated in the dark for 3 hours to allow for sandwich complexes to form. Samples were subsequently washed with 1 ml wash buffer and the pellet was resuspended in 300 µl wash buffer following centrifugation (258 xg). A volume of 200 µl /well of each sample was added to a PRO-BINDTM 96 well assay plate and analysed on a specialized flow cytometer with plate sampler used for the detection of cell-associated, secreted or lysate protein known as a FACS Array Bioanalyzer. Following data acquisition, debris was filtered out from the data and the bead populations and mean fluorescent intensities (MFI) were automated using FCAP FCS Filter and FCAP Array Software (BD Biosciences, San Jose, CA, USA) respectively. The Concentration of the respective cytokines in the test samples was obtained by fitting the MFI of the test samples into a 4-parameter logistic curve-fitting equation. Five independent experiments were performed.

3.3.3 Viability: Tetrazolium salt XTT

Lymphocyte proliferation assays measure the ability of lymphocytes in short-term tissue culture to undergo proliferation when stimulated *in vitro* by a foreign antigen. Typically, CD4 lymphocytes proliferate in response to antigenic peptides in association with class II major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs). This proliferative response of lymphocytes to antigen *in vitro* occurs only if the patient has been immunized to that antigen, either by having recovered from an infection with the microorganism containing that antigen, or by having been vaccinated. Lymphocytes from normal healthy volunteers can be stimulated to proliferate non-specifically by stimulating them with mitogens e.g. phytohemagglutinin (PHA) or antibodies against T cell receptors CD3 and CD28. These reagents often serve as positive controls for such assays.

Viability dyes are not the most sensitive means for determining proliferation. The best assay uses bromodeoxyuridine (BrdU) or tritiated thymidine incorporation, these radioactive labels are incorporated into DNA during cell division and stimulation indices of 2 or more are considered positive. XTT measures viability of cells by assessing the metabolic state which relies on live cells, and is an indication of proliferation. Flow cytometry is a more sensitive method than XTT

and provides information about proliferation of cells of interest through the use of proliferation tracking dyes such as CFSE. The CSFE dye allows for discrimination of cell generations along with the enumeration of the total frequency of cells in division, this dye also reports the number of times cells divide and allows for the calculation of the frequency of the starting population that divided. The XCELLigence system, also called Real Time Electronic Sensing, is a real time cell analyzer. This label-free, cell-based assay was used to monitor and unlike the other two, is not an endpoint based method. The cell index reflects, among other biological processes, the viability of these cells.

The use of tetrazolium salts, including MTT (3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and XTT (2,3-Bis(2-methoxy-4-nitro-5- sulfophenyl)-2H-tetrazolium-5-carboxanilide), to assess cell proliferation, cell viability, and/or cytotoxicity is a widespread, established practice. The reduction of XTT is primarily due to glycolytic activity within the cell and is dependent upon the presence of NADH and NADPH. Cleavage of the tetrazolium salt to formazan occurs via the succinate tetrazolium reductase system in the mitochondria of metabolically active cells. The reaction is attributed mainly to mitochondrial enzymes and electron carriers, but a number of other non-mitochondrial enzymes have been implicated as well.

XTT, a yellow tetrazolium salt, is cleaved to a soluble orange formazan dye, which can be measured by absorbance at 490 (or 450) nm in a microplate reader. The sensitivity of an XTT assay is greatly improved by the usage of an intermediate electron carrier, PMS (N-methyl dibenzopyrazine methyl sulfate). PMS helps drive XTT reduction and the formation of its formazan derivative (figure 3.2). The second generation tetrazolium dye, XTT, offers the advantage that solubilzation of the reduced foramazin dye is not required as in MTT products. Due to the simplicity of the working principle and the high throughput microplate format, tetrazolium reagents are more popular as convenient non-radioactive alternatives for determining the number of viable cells in proliferation (Cory *et al*., 1991).

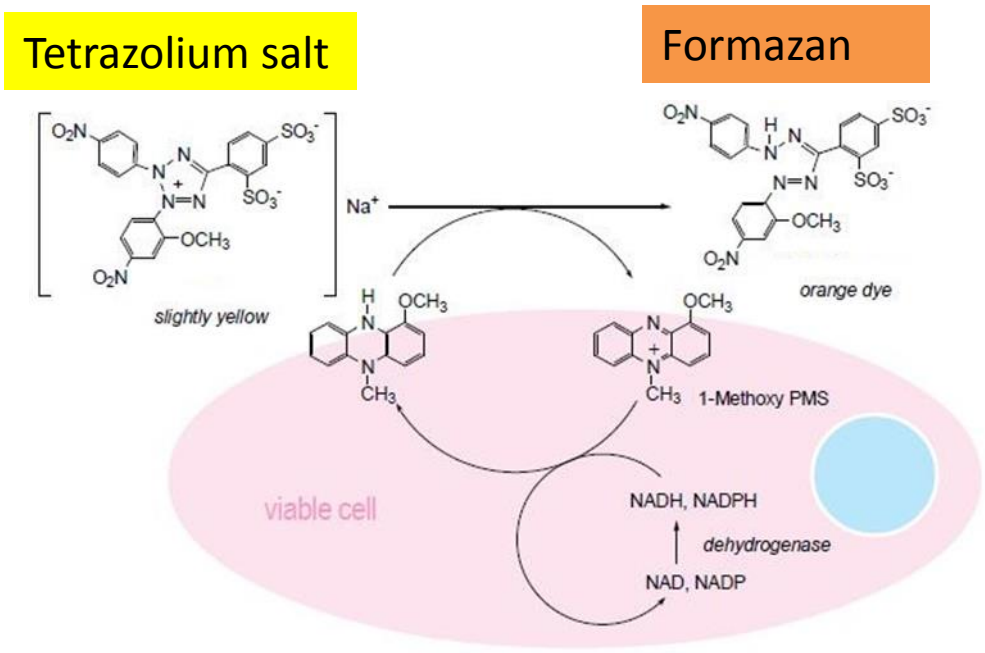


Figure 3.3: The reduction of XTT by mitochondrial enzymes

The reduction of XTT by mitochodrial enzymes, in the presence of PMS, to yield a water-soluble formazan product In the presence of PMS XTT is metabolically reduced to an aqueous-soluble formazan product by cellular dehydrogenases. PMS is an electron coupling agent that increases the XTT metabolism efficiency of cells, thereby enhancing the reduction of XTT to yield absorbance values for cell viability.

Method: Following PBMC isolation, cells were plated into 96-well tissue culture plates at a density of 1x 10^5 cells per well in 10% RPMI. Peptides at 10 µg/ml (diluted in 10% RPMI) were then added to the cells and incubated at 37 ºC, 5% CO_2, 95% humidity for a period of 7 days. Following incubation a XTT/PMS solution (20 µl PMS/ml XTT) was made, then 50 µl of the solution was added to each well. The plate incubated for a further 24 hours after which the absorbance was measured on a spectrophotometer (ELISA reader) at a wavelength of 450/620 nm.

3.3.4 Proliferation - Flow cytometry

Carboxyfluorescein diacetate, succinimidyl ester is a fluorescein derivative which is non-fluorescent. Flourescence is achieved when the cellular esterases cleave acetate groups, rendering the molecule fluorescent and cell impermeant. Succinimidyl ester binds to free amines, resulting in long lived fluorescent adducts. This technique is based on the serial dilution of a stably binding intracellular fluorochrome, CFSE, which allows for eight sequential divisions to be analysed by a flow cytometer.

When incubated with cells, the fluorescein-based CFSE crosses the cell membrane and attaches to free amine groups of cytoplasmic cell proteins. Following enzymatic removal of carboxyl groups by endogenous intracellular esterases, CSFE attains identical spectral characteristics to fluorescein, with optimal excitation by 488 nm argon laser light, emitting strongly at 519 nm, and as such is compatible with almost all single and multiple laser flow cytometers. During cell division, CFSE is distributed equally between progeny, allowing the division history of a cell population to be determined. This technique can be used for both *in vitro* studies and analysis of cell behaviour when transferred *in vivo*.

Other techniques for monitoring cell proliferation, such as the use of tritiated thymidine incorporation, can quantify overall division behaviour of a population but give no information on the division history of individual cells. Furthermore appropriately conjugated monoclonal antibodies can be employed to identify the cells undergoing division and whether their phenotypic properties change with division number. A major advantage of the CFSE based technique is that viable cells from defined division cycles can be recovered, allowing functional characteristics to be related to differentiation stages. For this assay whole blood samples were obtained, PBMCs isolated and treated with the peptides. These were then incubated and analysed by flow cytometry to monitor cell proliferation.

Method: PBMCs were isolated from EDTA anticoagulated peripheral blood. The blood was transferred to 50 ml falcon tubes and diluted in a 1:1 ratio with incomplete media RPMI 1640, Gentamycin sulfate (SIGMA Aldrich Midrand South Africa). Histopaque was then added to the

diluted blood in 1:2 ratio. This was followed by centrifugation and collection of PBMCs. After a washing step, the red blood cells were removed by the addition of 5 ml ammonium chloride potassium (ACK, 150 nM NH4Cl, 10 mM KHCO3, and 0.1mM EDTA) for 5 minutes at room temperature. The ACK was removed by centrifugation and the PBMCs were resuspended in complete RPMI 1640 supplemented with 10% (v/v) fetal calf serum. Trypan blue (Sigma Aldrich, Midrand, South Africa) was used to determine cell concentration as well as viability.

The isolated PBMCs were washed with staining buffer (5%, v/v, FCS in 1x PBS). The pellet was then labelled with 2 µl of 5mM CFSE (Invitrogen Corporation, Carlsbad, USA) for 15 minutes at 37^{o}C at 5% CO_2. The stain was quenched by the addition of 5 volumes of ice cold staining buffer then left on ice for 5 minutes. Three washes in staining buffer for the removal of unbound CFSE followed. The stained PBMCS were finally resuspended in complete media and incubated at a concentration of 1x 10^6 cells/ml with various amounts of antigen (peptides at different concentrations) in a 24-well plate (Nunc^{TM}, Denmark). All samples were analysed in triplicate with cells PBMCs killed with methanol used as a control for cell death. The positive control was made up of cells treated with 5 µg/ml Phytoheamaggluttinin-protein (PHA-P, Sigma Aldrich, South Africa) and unstimulated, untreated cells were used as representatives of naturally proliferating PBMCs. These PBMCs were incubated for seven days at 37^{o}C, 5% CO2. Following incubation, PBMCs were harvested, then resuspended in 1x PBS and analysed using a flow cytometer (FACSARIA, BD Biosciences). A 488 nm laser, 530/30 Bp filter (FITC channel) as well as 695/40 Bp filter (PerCP channel) situated in front of the detectors were used during the analysis. FLOWJO software version 7.6.1 was used to process all proliferation data.

3.3.5 RT-CES

In this study real time cell electronic sensing was used for the measurement of viability using an electronic sensing device called Xcelligence (Roche diagnostics, MA, Germany). This method uses microelectronic plates (E-plates) which are composed of interdigitated gold micro-electrode arrays incorporated at the bottom of the wells of specific tissue culture plates with an 80% electrode coverage per well. An electric field between electrodes is generated in the presence of

media and a low alternative current (AC) voltage of 10 mV is produced (Roche diagnostics, MA, Germany).

The impedance of this electric field caused by the presence of adherent cells is directly proportional to the number of cells attached per well. RTCA software is then used to convert impedance values to a cell index (CI) which is indicative of cell number, morphological parameters and degree of attachment to plates (Abassi *et al.*, 2009; Atienza *et al.*, 2006) thereby allowing for monitoring of proliferation of TZM-bl cells in the presence of synthetic peptides. A detailed account is provided by Fonteh *et al.,* 2011; a brief description is given below.

Method: A TZM-bl cell titration was performed prior to all Xcelligence experiments and a concentration giving an average of 1± 0.4 cell index following 24 hours was chosen. Following titration a number of 10 000 cells per well was seeded as was determined by the titration. Untreated cells were used as the control. A period of 24 h prior to treatment of TZM-bl cells with 10 µg/ml of each peptide, cells were allowed to adhere to the plates. The cell index was allowed to climb until it was between1-1.4 which is ideal for adding samples to cells. Following treatment, cells were monitored for 72 h and the cell index was recorded.

3.3.6 Immunogenicity tests – Rabbit ELISAs

Two rabbits were used per peptide. Pre-immune sera were collected before treatment with Peptide-KLH conjugate. The rabbits were immunised for 5-10 days., and blood collected following seroconversion ELISAs were performed using free peptide as antigen, with a coating Concentration of 4 µg/ml per well. The coating Buffer used was Phosphate Buffered Saline, pH 7.4, and goat, anti-Rabbit IgG (H&L) antibody Peroxidase Conjugated for detection. The findings are tabulated.

3.3.7 Neutralization assay

TZM-bl is a genetically engineered HeLa cell line that expresses CD4, CXCR4 and CCR5 and contains Tat-inducible Luc and β-Gal reporter genes.These modified HeLa cells, are susceptible to infection by HIV. In this work these cells will be used to test the capacity of the purified polyclonal antibodies raised against the peptides, in rabbit hosts, to neutralize HIV pseudo-virions *in vitro*.

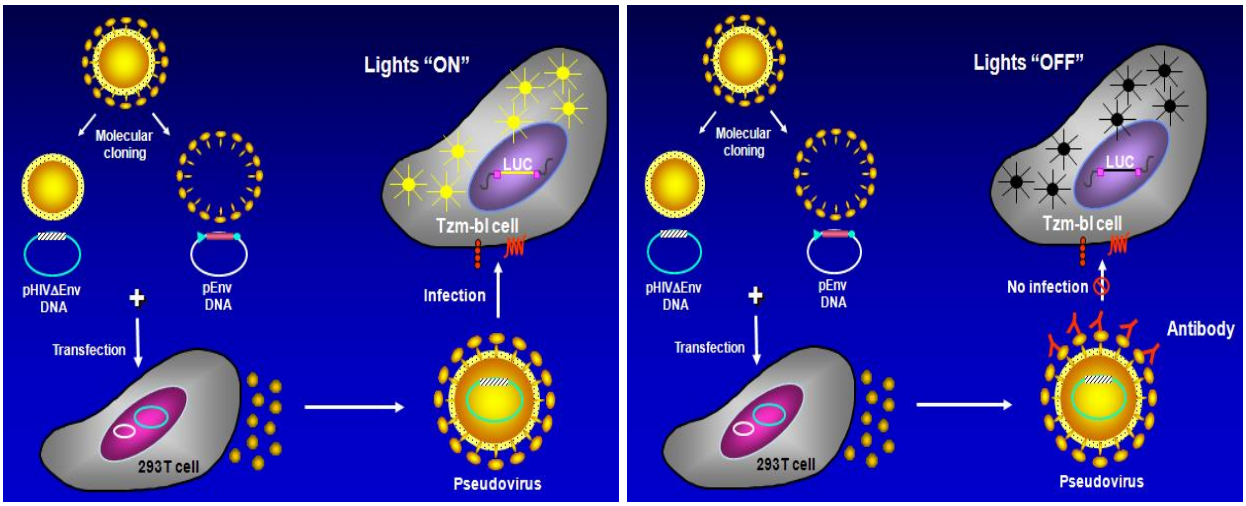
The efficacy of entry inhibitors can be measured with the Luciferase Reporter Gene Assay which detects the inhibition of HIV-1 envelope (env) pseudo virus infection in TZM-bl cells in-vitro. The pseudo-viral particles are generated in 293T cells through co-transfection of env-expressing plasmids with backbone plasmid DNA. These pseudo-virions can infect cells but are unable to reproduce due to an incomplete genome, termed single-round infection. The infections are detected in genetically engineered TZM-bl cells (HeLa cell clones) that express CD4, CCR5 and endogenous CXCR4 and contain a Tat-responsive firefly luciferase gene under the control of an HIV-LTR. The luciferase activity is detected and quantified by luminescence and is proportional to the infectious viral particles that have entered the cell. The specificity of the compound was also determined with this assay against a vesicular stomatitis virus glycoprotein (VSV-G) env pseudovirus.

Method: Sixty microliters of cell culture medium (DMEM) with 5 % v/v FBS was added to all the wells of gold plated Xcelligence E plates. To the experimental wells, 15 µl of each polyclonal affinity-purified antibody was added to a final concentration of 10 and 5 µg/ml according to the plate layout. Nevirapine was used as the positive control owing to its ability to inhibit virus, the positive control wells were treated with 100 µg/ml of the drug.

This was followed by the addition of 25 µl of ZM53 pseudo virus to all wells except the designated cell control (TZMbl cells in 5% DMEM). The pseudo virus used was obtained from the CSIR and prepared to a 1:1 dilution. The cell control wells contained untreated cells and 25 µl of 5% DMEM was added to them, to compensate for volume difference due to addition of

pseudovirus in experimental wells. Untreated cells were used to control viral activity in which the total number of cells was equivalent to that in the test wells.

The principle of this assay used to measures % neutralization in TZM-bl cells based on the reduction of *Tat*-induced luciferase gene expression. TZM-bl is a genetically engineered HeLa cell line that expresses CD4, CXCR4 and CCR5 and contains Tat-inducible Luc and β-Gal reporter genes. Protocol uses molecularly cloned pseudoviruses expressed in a different cell line (293T/17) Pseudovirus contains an Env expression plasmid and a second plasmid expressing the entire HIV genome except Env (Figure 8).



A: no neutralization B: neutralization

Figure 3.4: Diagram showing the principle of the neutralization assay
Diagram showing the principle of the neutralization assay. Pseudoviruses are made through the process of transfection in 293T cells. In the absence of a viral inhibitor or neutralizing antibodies, pseudoviruses are able to infect TZMBl cells, once, (A). Infection is prevented by the presence of neutralizing antibodies as seen in (B). (Montefori *et al.*, 2004).

Viral particles produced are not infectious but because recombination events could occur resulting to replication competent viruses, this assay should always be done with the assumption that infectious particles are present (safety procedures). Assay can also be used in determining effect of test compounds on viral infectivity (entry inhibition).

CHAPTER 4 – RESULTS

Although drug therapies (HAART) have been able to limit HIV virulence, no cure for HIV is currently available. The discovery of neutralizing antibodies suggested that stimulating the humoral immune response could present a means to control infection. The envelope proteins are among the most antigenic of HIV-1 proteins. In this study, synthetic peptides mimicking immunodominant regions in viral env proteins (gp120, gp41, B2M) were used to characterize humoral immunity in individuals who contracted HIV-1 infection naturally.

To this end, synthetic peptides comprising 7-22 amino acids, representing the sequences of previously determined immunodominant epitopes were synthesized. Peptide homogeneity was confirmed by reverse phase high-performance liquid chromatography (HPLC) and MS data confirmed purity and the molecular weight of each peptide. These data are provided in the Appendix, section 1.1: Peptide production. The table below (table 1) provides some of the antigenic properties of the peptides as were determined using *in silico* methods.

Table 1: Properties of the peptides used in this study

Peptide I.D	Originating protein	Sequence	# of amino-acids	Molecular Weight	Isoelectric point	Net Charge	Hydrophilicity	Subtype	Type of Env region	Reference
R7V	β2M	RTPKIQV	7	841	11.5	2	0.4	A-F	Conserved	Galea *et al.*, 1996, Le Contel *et al.*, 1996
S7K	β2M	SQPKIVK	7	799	10.6	2	0.5	A-F	Conserved	Le Contel *et al.*, 1996
F7E	β2M	FHPSDIE	7	843.9	4.1	-1.9	0.2	A-F	Conserved	Le Contel *et al.*, 1996
β-2Mp	β2M	SQPKIVKF HPSDIERTP KIQV	21	2447.9	10.3	2.1	0.2	A-F	Conserved	Le Contel *et al.*, 1996
CR7V	β2M	CRTPKIQV	8	944.2	10.1	2	0.2	A-F	Conserved	Galea *et al.*, 1996, Le

										Contel *et al.*, 1996
CS7K	β2M	CSQPKIVK	8	902.1	9.9	2	0.3	A-F	Conserved	Le Contel *et al.*, 1996
CF7E	β2M	CFHPSDIE	8	875	4.9	-1	-0.3	A-F	Conserved	Le Contel *et al.*, 1996
Cβ2Mp	β2M	CSQPKIVK FHPSDIERT PKIQV	22	2551	9.8	2	0.3	A-F	Conserved	Le Contel *et al.*, 1996
DC1	Gp160	LKPCVKLT	8	901.2	9.9	2	-0.1	B A	Conserved	Luchese *et al.*, 2007
DV3	ENV (V3)	KRIGPGQT FYATKRC	15	1726	10.7	4	0.2	C	(i)Tetrameric tip (ii)Dibasic endopeptidase cleavage site	Wyatt *et al.*, verrier *et al.*,1999
2F5	2F5 MAb	CKRALDS WND	10	1207.3	6.2	0	0.6	C	2f5 and endo-peptidase	Hartono, 2013

									cleavage site and 3 subsequent MPER amino acids	
MPER	MPER modified	ELDKWAS KRNWFDIT C	16	2012.3	6.4	0	0.2	A B C	2f5 (of subtypes A/B) and 4e10	Hartono,2013
GQ9	HERV-K GQ9	CGIPYNSQ GQ	10	1066.2	5.3	0	-0.4		HIV-1 Homology (B7)	Sengupta *et al.*, 2011

(i) Tetrameric tip: this is the GPGQ sequence found in the V3 loop principal neutralizing determinant, believed to be the binding site for neutralizing antibodies. (ii) Dibasic endopeptide cleavage site (KR) allows cleavage by proteases such as trypsin. The presence of these amino acids is believed to increase antigenicity.

A hydropathy plot is a quantitative analysis of the degree of hydrophobicity or hydrophilicity of the amino acids of a protein. It is generally used to assess the possibly exposed or buried domains of a protein and values are assigned from the Hopp-Woods scale. The plot has the amino acid sequence of a protein on its x-axis, and degree of hydrophobicity or hydrophilicity on its y-axis (Hopp and Woods, 1982). In this dissertation the hydropathy plots are accompanied by a curve showing net charge of each peptide as a function of pH. Figure 4.1 is a representative figure, the hydrophobicity plots and net charge curves for the rest of the peptides can be found in the appendix (A9 - A24).

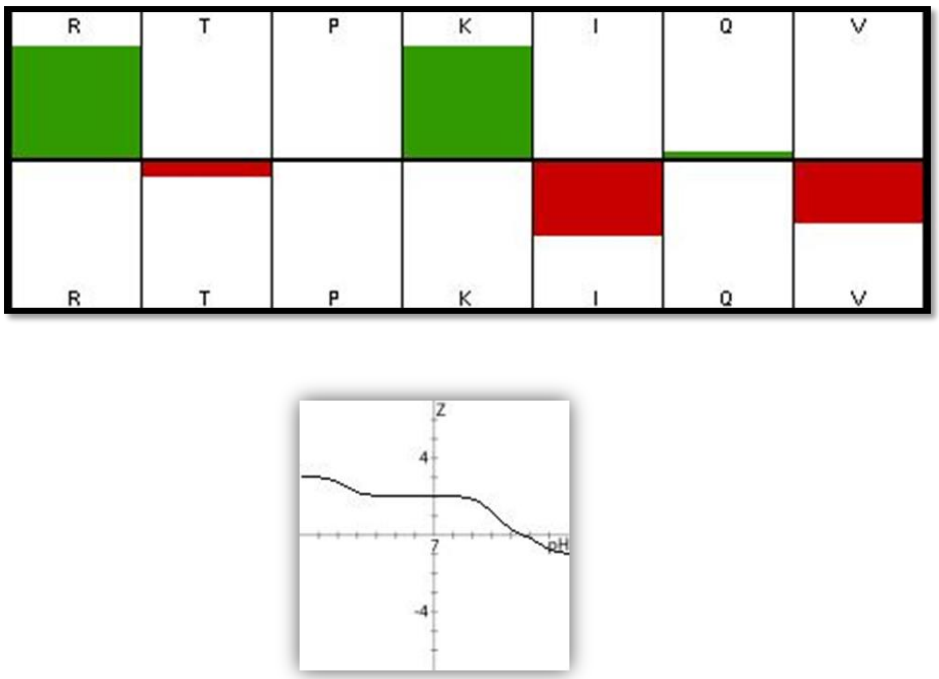
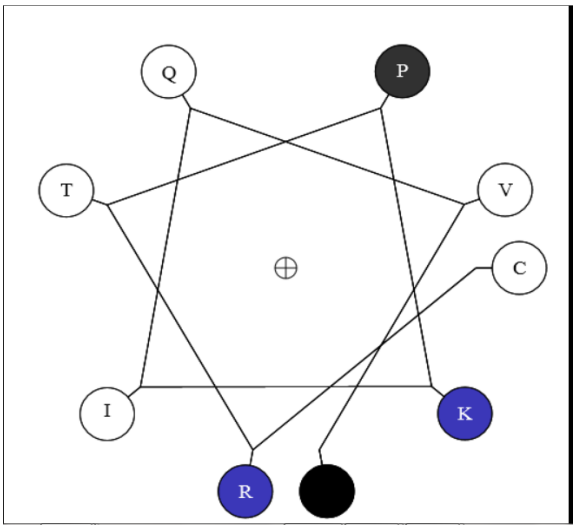


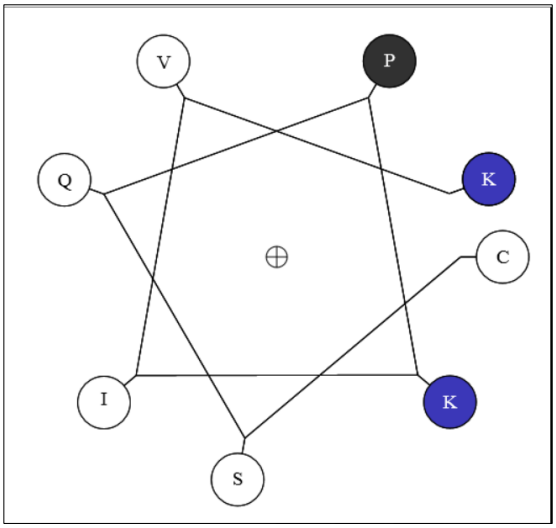
Figure 4.1: Hydrophobicity plots and Net Charge curve of R7V.

Visual representations used to illustrate the properties of alpha helices in proteins are called helical wheels (Figure 4.2 and 4.3). Helical wheels also present a means of assessing the antigenicity of a protein or peptide. The sequence of amino acids that make up a helical region of a protein's secondary structure are plotted in a rotating manner where the angle of rotation between consecutive amino acids is 100°, so that the final representation looks down the helical axis. The plot reveals whether hydrophobic amino acids are concentrated on one side of the helix, usually with polar or hydrophilic amino acids on the other. This arrangement is common in

alpha helices within globular proteins, where one face of the helix is oriented toward the hydrophobic core and one face is oriented toward the solvent-exposed surface (Kyte and Dooolittle, 1982). Specific patterns characteristic of protein folds and protein docking motifs are also revealed, as in the identification of leucine zipper dimerization regions and coiled coils.

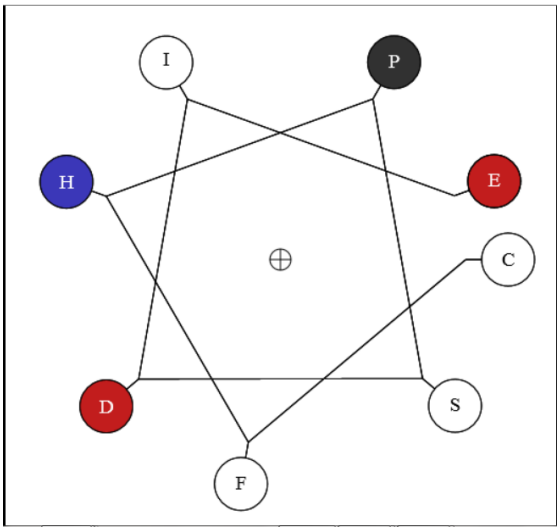
C-R7V

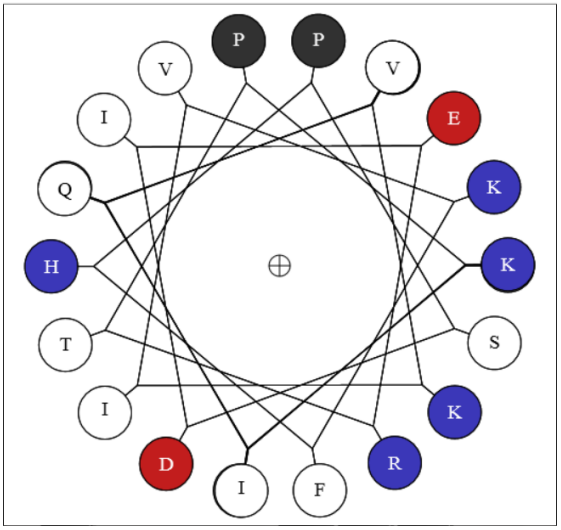




C-S7K

C-F7E

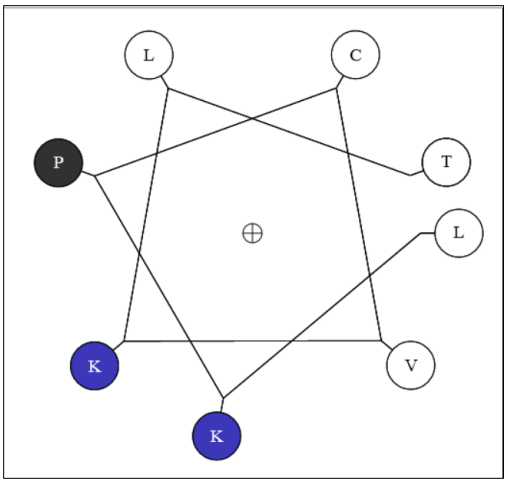


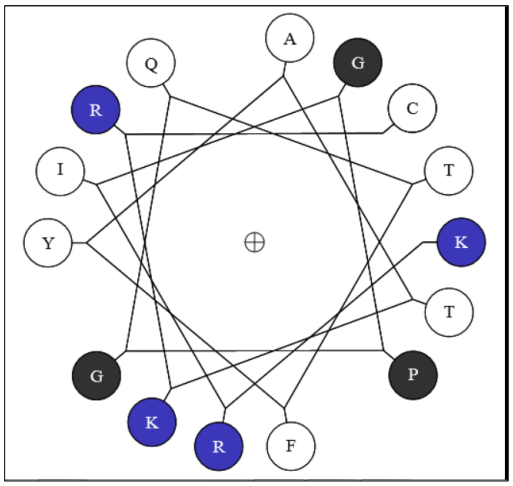


C-B2M

Figure 4.2: Helical wheels of host-derived peptides. Key: Positive amino acids – black; negative amino acids – red; Aliphatic amino acids – blue, the rest are neutral

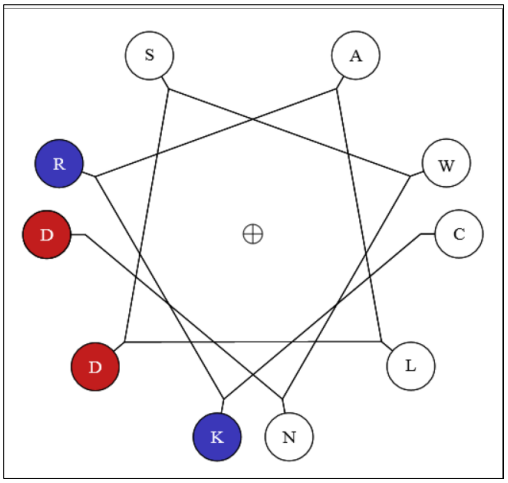
DC1

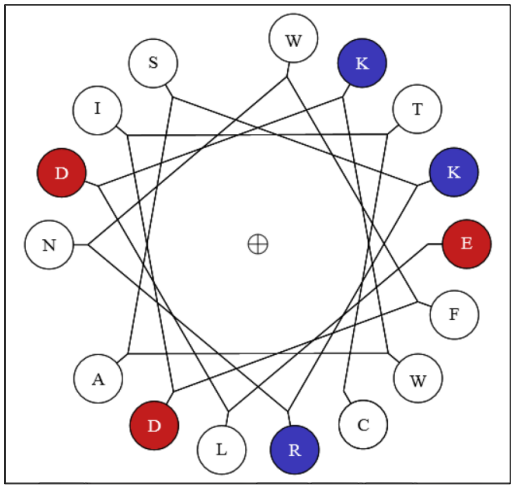




DV3

2F5





MPER

GQ9

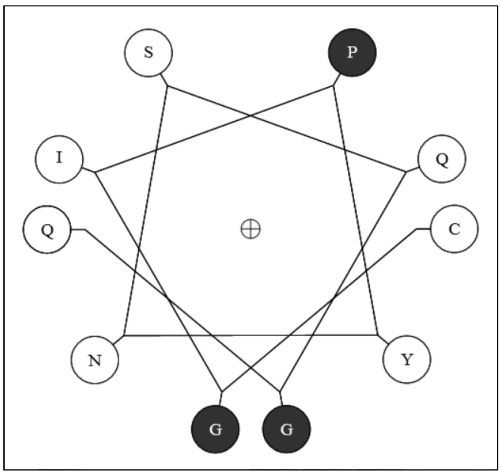


Figure 4.3: Viral peptide helical wheels. Key: Positive amino acids – black; Polar/negative amino acids – red; Aliphatic amino acids - blue

4.1 Antibody detection

For the purpose of this study an in-house peptide based indirect ELISA was used to determine the presence of antibodies against the synthetic peptides in serum from HIV positive individuals (on and naïve of treatment) as well as HIV negative individuals. This is an indirect ELISA in which the peptides based on the different epitopes are immobilized, and used as antigen to bind antibody in the sera of naturally infected individuals (as opposed to artificially infected systems) or HIV negative donors

During protocol development, the following parameters were optimized; incubation time (60 min), peptide concentration (250 ng – 1 µg/ml) and antibody dilution (the amount of active antibody in the donor sera; 100x, 500x, 1000x). The selected antibody dilution (100x) and peptide concentration shown in the figures were optimal. The assay accuracy was based on the OD values of the negative controls. Negative control OD values were 0.5±0.1, which was determined to be the cutoff and values below this cutoff were defined as negative, indicating an absence of the antibodies being detected against a particular antigen.

The figures below were obtained following an indirect ELISA where each peptide was used as antigen respectively; the data are representative of the general trends observed after each assay was performed at least 3 times. The viral derived antigens data are presented first, followed by the host derived peptides and then the endogenous retrovirus-based peptide.

The viral antigens had varying responses depending on the epitope on which the peptide antigen was based. Figure 4.1.1 shows heat-inactivated sera from individuals screened using 1 µg/ml of DV3 peptide. A total of 62 individuals were tested in the DV3 ELISA, 42 HIV positive and 17 HIV negative donors. Patients who exhibited low antibody responses against DV3 either had viral loads that were lower than detectable (LTD) or not determined (ND). The rest of the HIV positive sera were positive (up to 5 fold increase) as compared to the negative control samples (OD values all below 0.5).

4.1.1 Virus-derived antigens

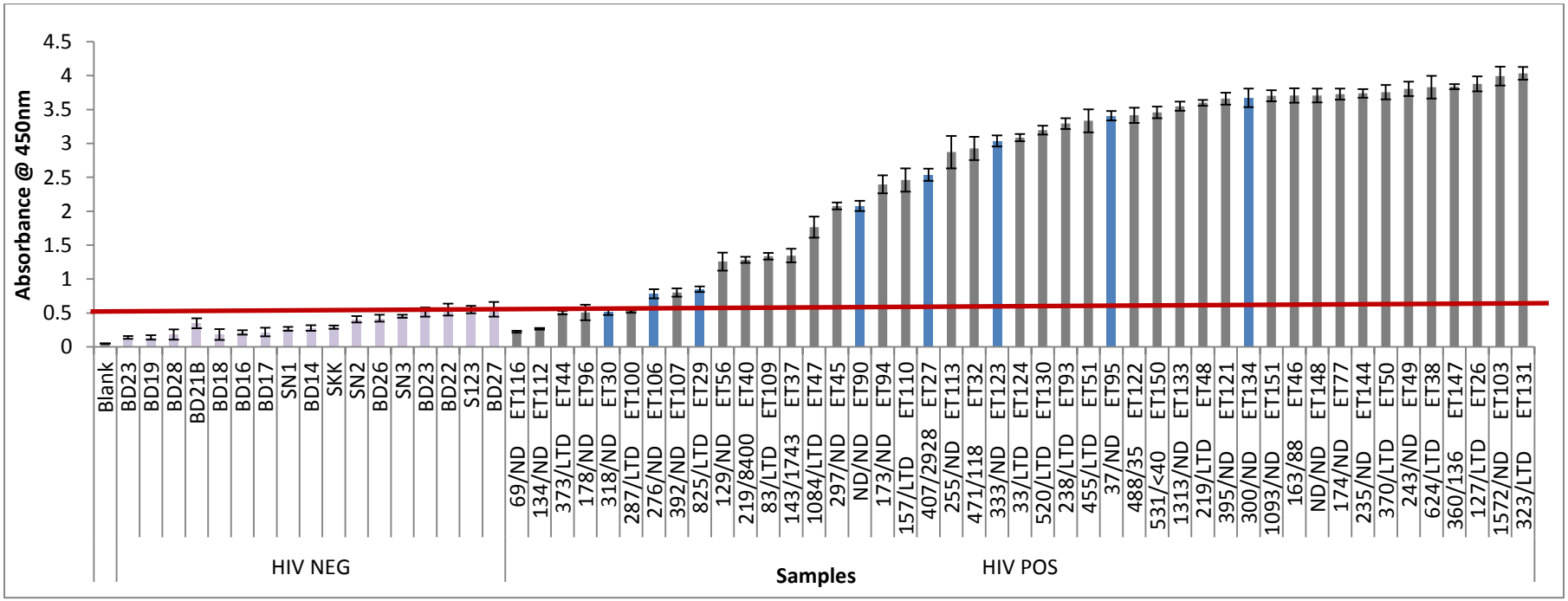


Figure 4.1.1: Seroprevalence of DV3 antibodies as indicated by an indirect anti-DV3 ELISA (using 1 µg/ml of DV3 peptide as antigen). The sera of both HIV negative as well as HIV positive donors was used and labelled accordingly above. For the HIV positive patients, the CD4 count and viral load are shown respectively. LTD was assigned to patients with a viral load that was lower than detectable; ND was used in instances where information was not determined and therefore unknown. Purple bars are used to show HIV negative donors and the HIV positive patients are represented by grey bars or blue bars in cases where patients were diagnosed within a year or less prior to their bleed date. The red line represents the threshold for antibody detection as determined by the highest HIV negative value, individuals who express OD values above the line are considered positive for DV3 antibodies. The signal was obtained at an absorbance of 450 nm after 60 min incubation period. n=3

HIV positive samples that responded positively to the MPER peptide had variable CD4 counts. The clinic records did not have viral load data available for a large number of the individuals tested for responses against this peptide.

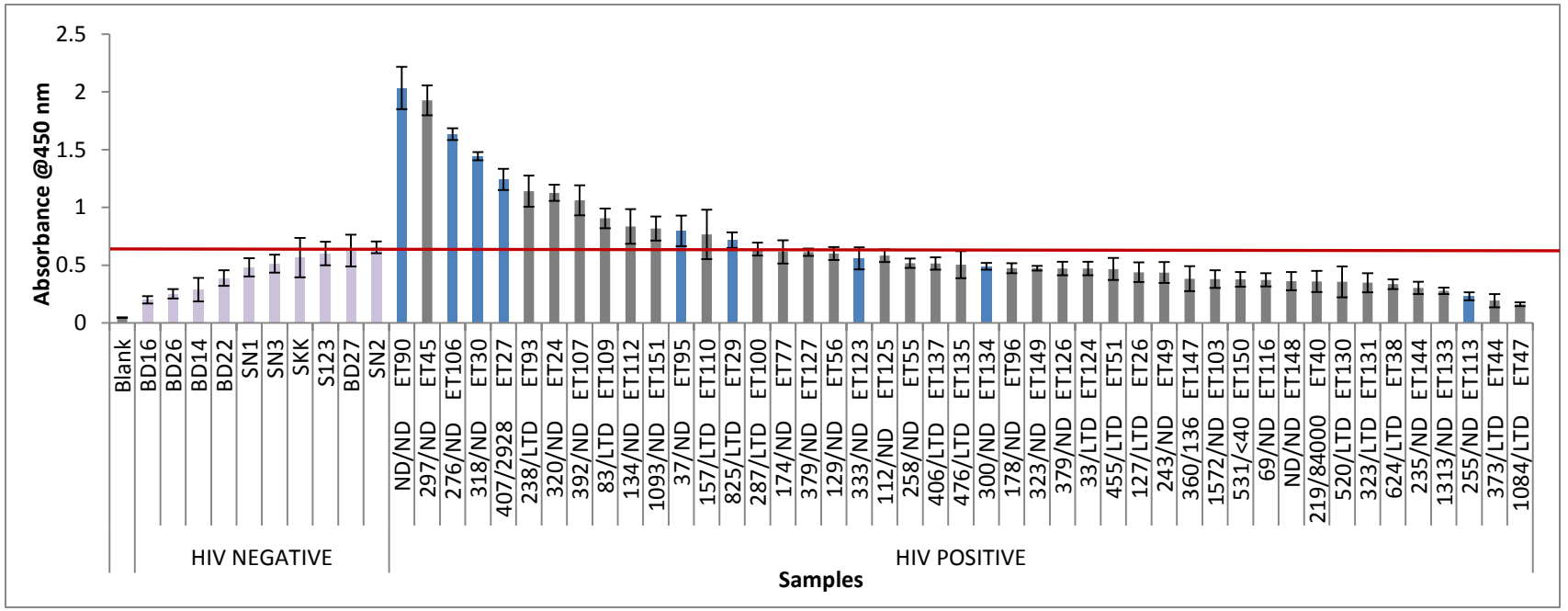


Figure 4.1.2: Seroprevalence of MPER antibodies as indicated by an indirect anti-MPER (using 1 µg/ml of MPER peptide as antigen). The sera of both HIV negative as well as HIV positive donors was used and labelled accordingly above. For the HIV positive patients, the CD4 count and viral load are shown respectively. LTD denotes a viral load that is lower than detectable and ND is used where information was not determined and therefore unknown. The red line represents the threshold for antibody detection as determined by the highest HIV negative value, individuals who express OD values above the line are considered positive for MPER antibodies. The signal was obtained at an absorbance of 450 nm after 60 min incubation period. n=3

Antibody levels detected against MPER were significantly higher in HIV positive patients with fold changes above 2 for the highest responders. The highest responses were observed for samples from both recently infected and some individuals on HAART (as shown in figure 4.1.2).

In contrast to these observations, the antibody responses detected against 2F5 (Figure 4.1.3) were low in HIV negative donors (as expected) as well as in most of the HIV positive samples tested. The highest responses were detected in the sera of ET27 and ET37, however there was no significant fold change observed. It is also noteworthy that all the antibody levels detected against this antigen had OD values below 0.7.

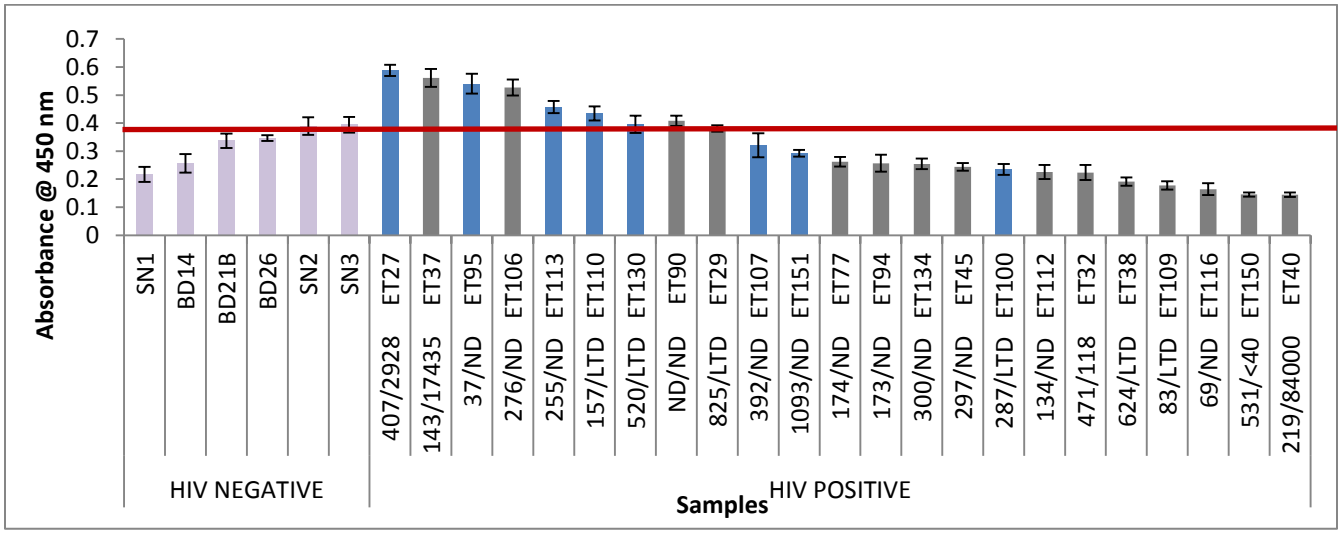


Figure 4.1.3: Seroprevalence of 2F5 antibodies as indicated by an indirect anti-2F5 (using 1 µg/ml of 2F5 peptide as antigen). The sera of both HIV negative as well as HIV positive donors was used and labelled accordingly above. For the HIV positive patients, the CD4 count and viral load are shown respectively. Viral loads that are lower than detectable are labelled LTD and cases where the information was not determined were labelled ND. The red line represents the threshold for antibody detection as determined by the highest HIV negative value, individuals who express OD values above the line are considered positive for 2F5 antibodies. The signal was obtained at an absorbance of 450 nm after 60 min incubation period. n=3

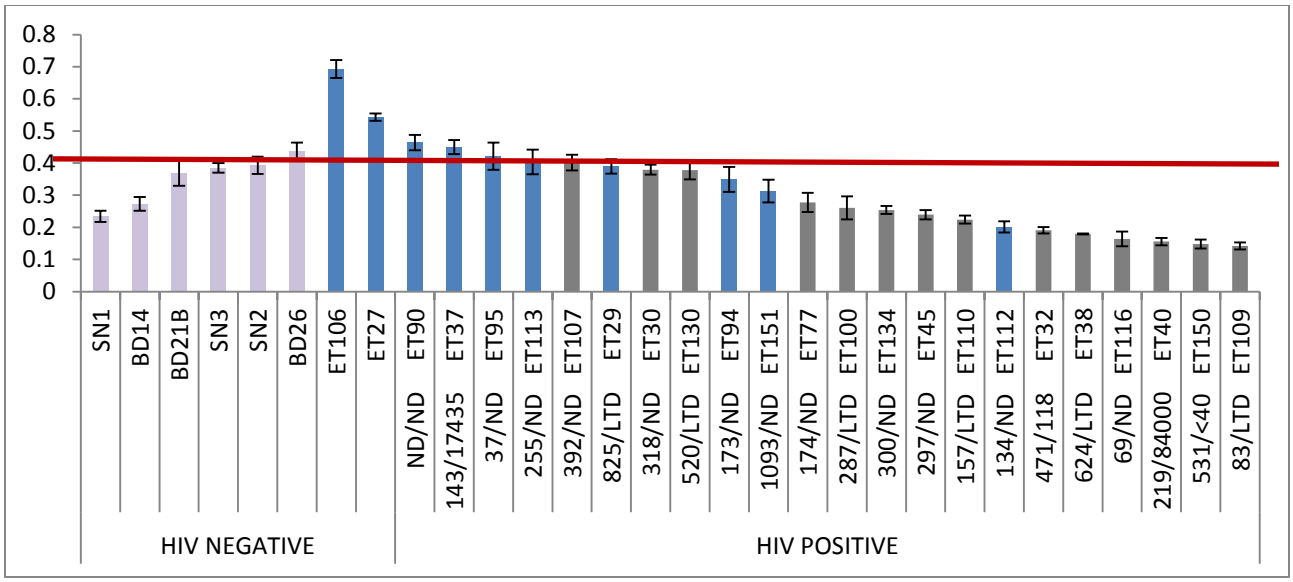


Figure 4.1.4: Seroprevalence of DC1 antibodies as indicated by an indirect anti-DC1 (using 1 µg/ml of DC1 peptide as antigen). The sera of both HIV negative as well as HIV positive donors was used and labelled accordingly above. For the HIV positive patients, the CD4 count and viral load are shown respectively. The red line represents the threshold for antibody detection as determined by the highest HIV negative value, individuals who express OD values above the line are considered positive for DC1 antibodies. The signal was obtained at an absorbance of 450 nm after 60 min incubation period. n=3

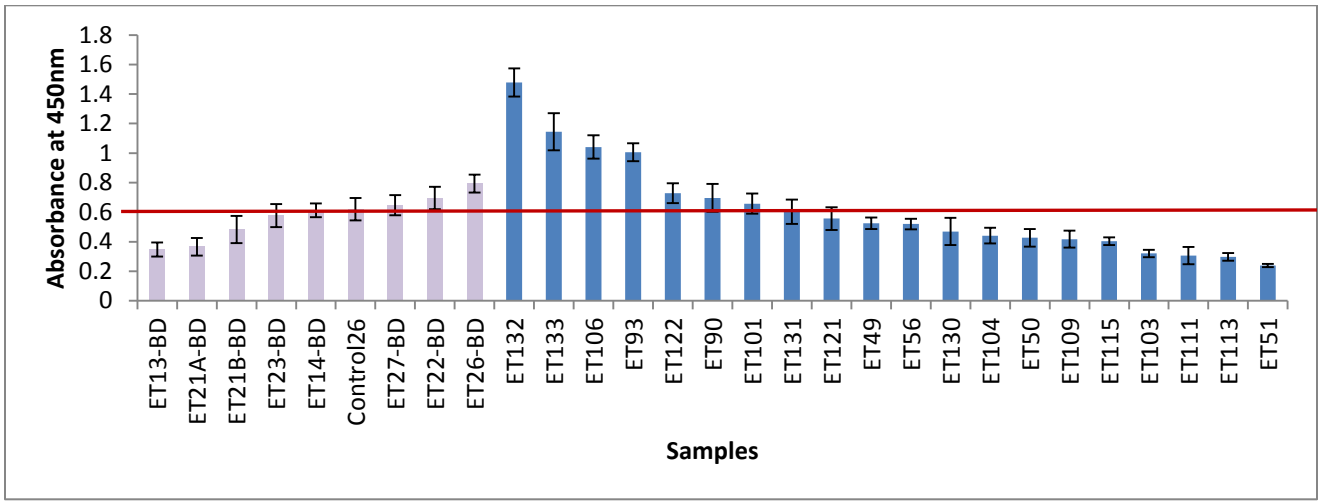


Figure 4.1.5: Seroprevalence of GQ9 antibodies as indicated by an indirect anti-GQ9 (using 1 µg/ml of GQ9 peptide as antigen). The sera of both HIV negative as well as HIV positive donors was used and labelled accordingly above. For the HIV positive patients, the CD4 count and viral load are shown respectively. The red line represents the threshold for antibody detection as determined by the highest HIV negative value, individuals who express OD values above the line are considered positive for GQ9 antibodies. The signal was obtained at an absorbance of 450 nm after 60 min incubation period. n=3

4.1.2 Host antigens containing additional c-terminal cysteine residue

A total of 56 individuals were tested in the C-R7V ELISA. Figure 4.1.6 shows heat-inactivated sera from 31 individuals screened using 1 µg/ml antigen. Thirty samples were HIV-1 positive (with only one individual naïve of treatment denoted with an asterix). The positive control (green) was a recombinant R7V antibody fragment that proved to be very responsive exhibiting OD values approaching 4 (with a 1:1 dilution)> 4 fold increase. HIV-positive sera were considered positive for R7V antibodies if OD values were above the 0.5 response exhibited by the HIV-negative sera representative sample (purple). HIV positive patients diagnosed within a year or less of the sample bleed date (blue) exhibited higher antibody levels (above 1.5 fold change observed) than the rest of the HIV positive patients (grey). Of the experimental samples ET106 had the highest detectable C-R7V antibodies (4 fold change). Individuals who were not diagnosed recently and were on treatment had antibody levels comparable to HIV negative controls. The same colour scheme will be used throughout the presentation of ELISA data. The other 27 samples were screened against 250 ng/ml antigen and the data is presented in the appendix. Both data sets show variability in the presence of antibodies against C-R7V during natural HIV infection.

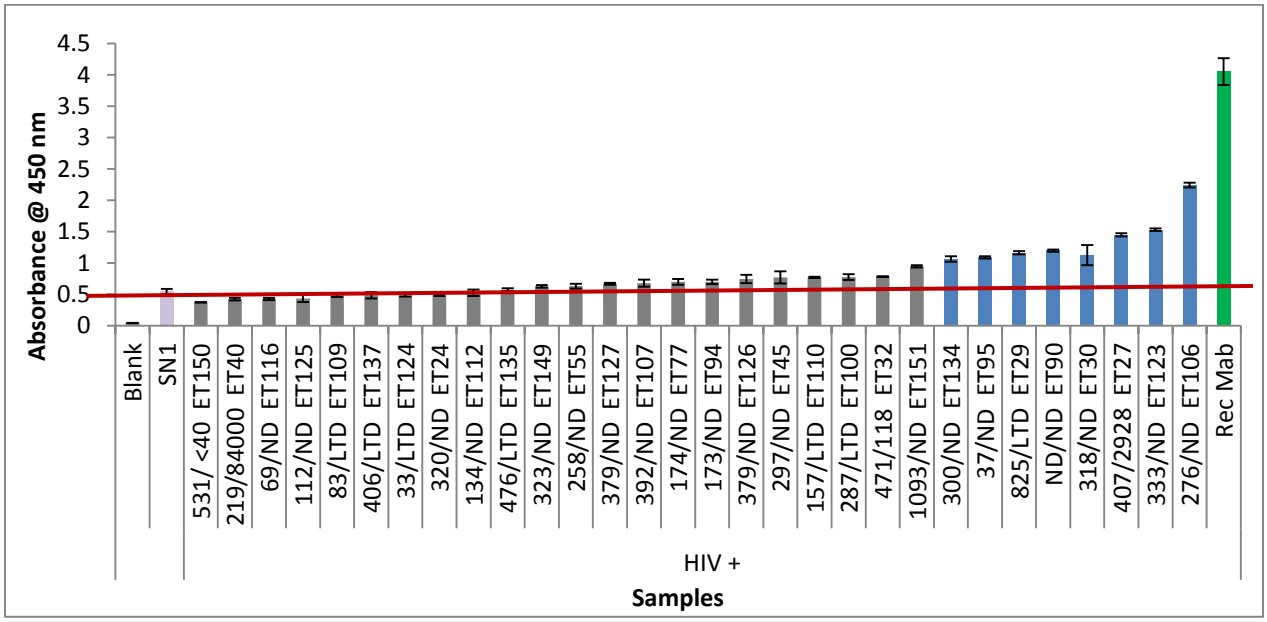


Figure 4.1.6: Seroprevalence of C-R7V antibodies as indicated by an indirect ELISA using 1 µg/ml of C-R7V peptide as antigen. The sera of both HIV negative as well as HIV positive donors were used, a blank is also shown. Here a representative sample (SN1- purple) is used to illustrate the antibody levels detected in

HIV negative patients. For the HIV positive patients, the CD4+ count and viral load are shown respectively. The blue bars represent patients diagnosed within a year or less prior to their bleed date. The rest of the HIV positive patients are grey. A Recombinant antibody fragment produced by phage display technology was used as the positive control and is denoted as "Rec mab" green on the graph. The red line represents the threshold for antibody detection as determined by the highest HIV negative value, individuals who express OD values above the line are considered positive for C-R7V antibodies. The signal was obtained at an absorbance of 450 nm after 60 min incubation period (n=3).

Antibodies against C-S7K in the sera of HIV negative and HIV infected donor patients were shown in figure 4.1.7. Although the response was relatively lower than that observed for C-R7V, it is still the individuals who have been diagnosed recently that exhibited higher antibody levels (1.5 fold) when compared to the mean HIV negative response. Sample ET111 had the highest antibody levels here and was obtained from an individual who was newly diagnosed and not yet on HAART. The positive control also responded very well. For the rest of the HIV positive patients, the amount of C-S7K antibodies was marginal and the responses were comparable to the HIV negative samples.

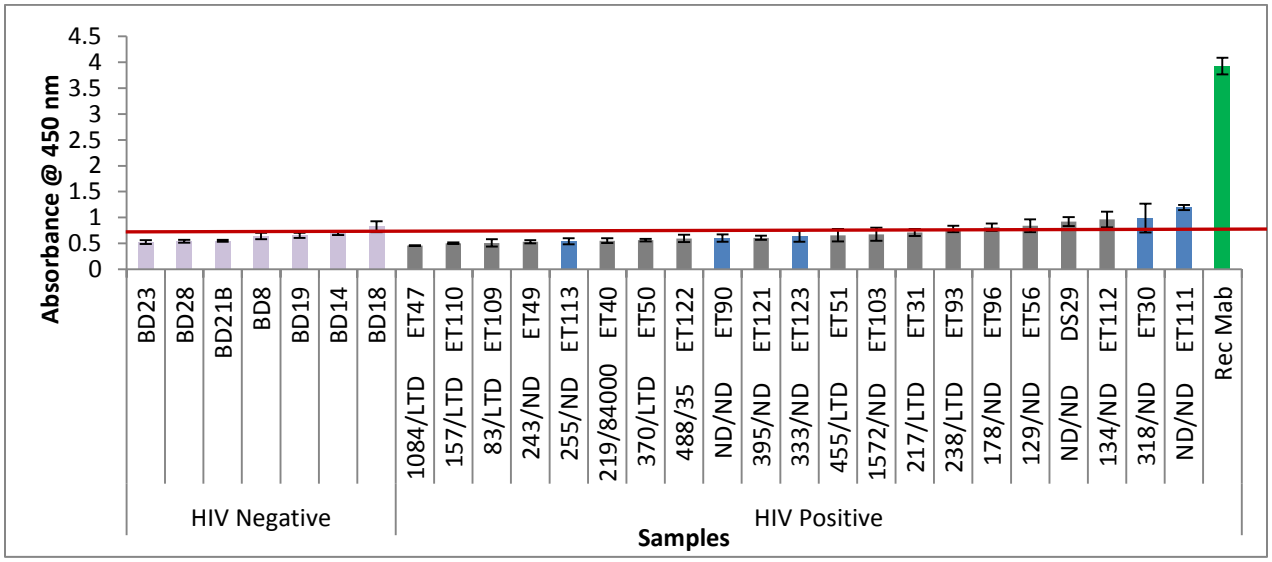


Figure 4.1.7: Seroprevalence of C-S7K antibodies as indicated by an indirect ELISA using 1 µg/ml of C-S7K peptide as antigen. The signal was obtained at an absorbance of 450 nm after 60 min incubation period (n=3).

The general antibody levels detected against C-F7E were lower than reported for the previously mentioned two host-derived peptide antigens. The mean OD for HIV negative patients was 0.54 with the patient in which the highest response detected being ET121 with a fold change above 2.

Of the two individuals not on treatment only ET 111 was positive for C-F7E antibodies. Individuals in which antibodies were detected were mainly recently infected. The positive control had a high response (OD above 2, fold change of above 6).

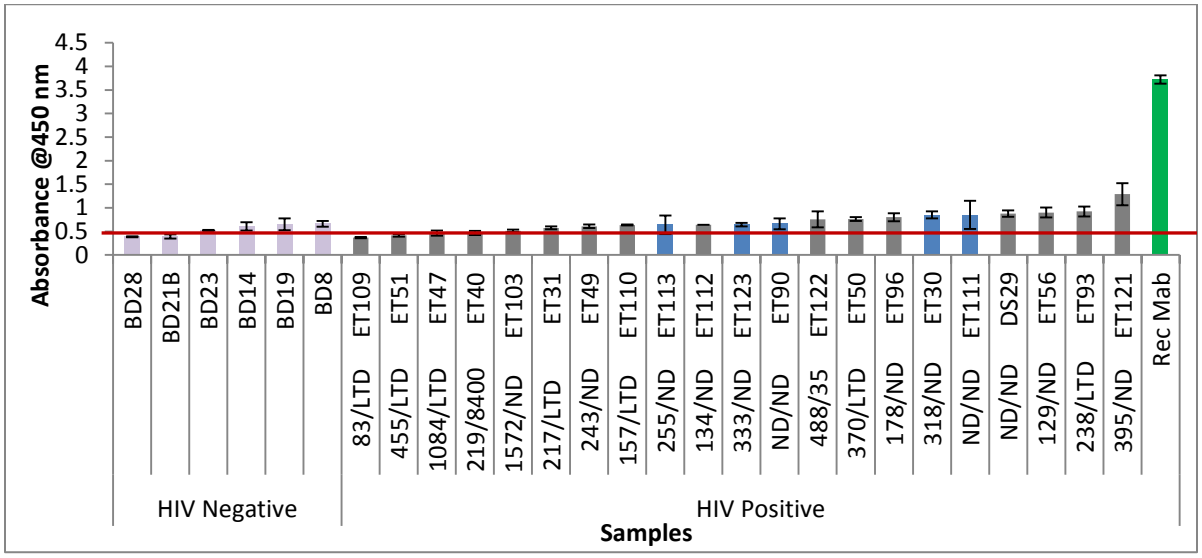


Figure 4.1.8: Seroprevalence of C-F7E antibodies as indicated by an indirect ELISA using 1 µg/ml of C-F7E peptide as antigen. The sera of both HIV negative as well as HIV positive donors were used and are labelled accordingly. For the HIV positive patients, the CD4+ count and viral load are shown respectively. A Recombinant antibody fragment produced by phage display technology was used as the positive control and is denoted as "Rec Mab" on the graph. The red line represents the threshold for antibody detection as determined by the highest HIV negative value, individuals who express OD values above the line are considered positive for C-F7E antibodies. The signal was obtained at an absorbance of 450 nm after 60 min incubation period (n=3).

When the host-derived peptides were used as a cocktail, the detected response was higher in all tested samples. HIV negative samples exhibited OD levels above 1; of the HIV positive samples, with ET 30 showing the highest response (fold change above 1.5), varying amounts of antibodies active against the Host mix cocktail (HM) were detected in the other HIV positive serum samples tested regardless of stage of HIV infection or HAART treatment. When peptides were combined and used as cocktails, FC values increased but the general trends did not change. Although there was variation in the antibodies detected against host-derived envelope antigens, it was in recently infected individuals that the highest antibody levels were detected. Individuals

not on treatment that had been infected for a period of up to 5 years at the sample bleed date (DS 29 – appendix) not on HAART exhibited lower levels of these antibodies in HIV subtype C.

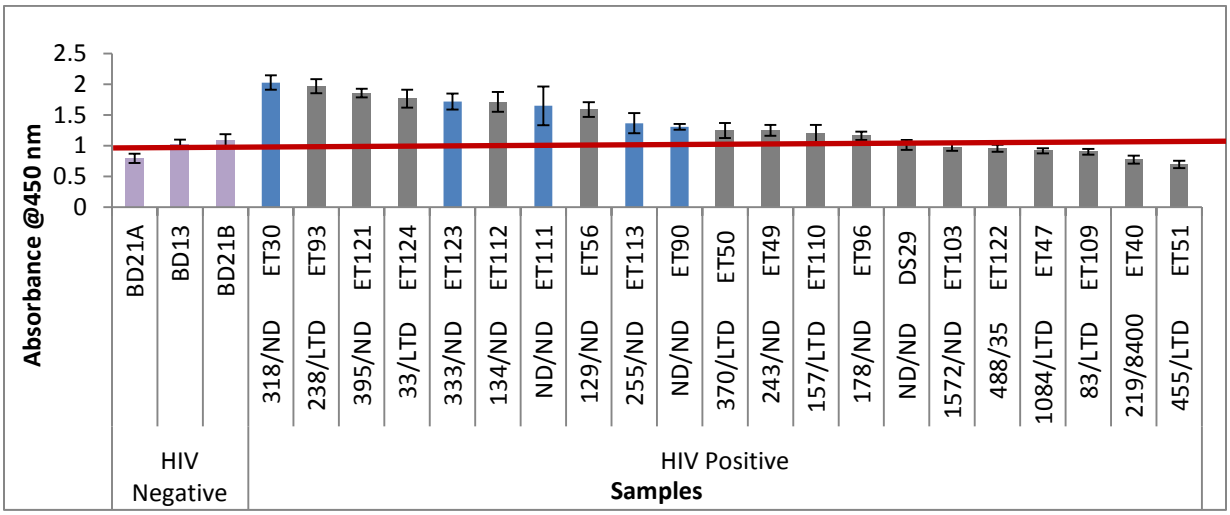


Figure 4.1.9: Seroprevalence of antibodies as indicated by an indirect ELISA using 1 µg/ml of a cocktail of host-derived peptides (C-R7V, C-S7K and C-F7E). The sera of both HIV negative as well as HIV positive donors were used and are labelled accordingly. For the HIV positive patients, the CD4+ count and viral load are shown respectively. A Recombinant antibody fragment produced by phage display technology was used as the positive control and is denoted as "Rec Mab" on the graph. The red line represents the threshold for antibody detection as determined by the highest HIV negative value, individuals who express OD values above the line are considered positive for Host Mix antibodies. The signal was obtained at an absorbance of 450 nm after 60 min incubation period (n=3).

Figure 4.1.10 illustrates that antibody responses against beta 2 microglobulin were variable and were higher in levels than the other host peptides. To illustrate the influence that compounding extrinsic factors have on antibody detection, the responses detected in samples of HIV positive patients were grouped according to age and the following graph was obtained (figure 4.1.11). This graph indicates that, although there is a level of variation between different people, in individuals who are younger, a higher antibody response was detected against host-derived antigens. It is for this reason that samples must be obtained from individuals who have been matched as closely as it is possible as this influences data interpretation.

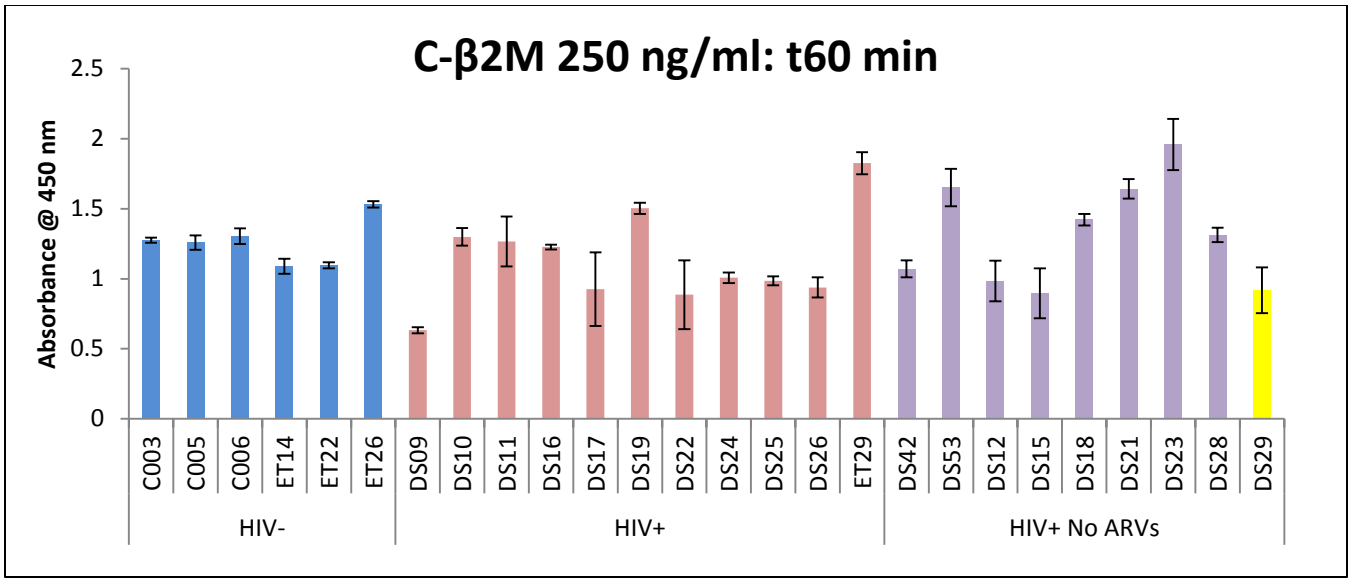


Fig 4.1.10: Seroprevalence of anti-C-B2M antibodies captured using 250 ng/ml of C-B2M. The blue bars represent antibodies detected in HIV negative serum, the pink represent HIV positive on HAART and the purple represent HIV positive not on treatment. Finally, the yellow bar represents a long term non progressor (infected and not on treatment for ≥ 5 years).

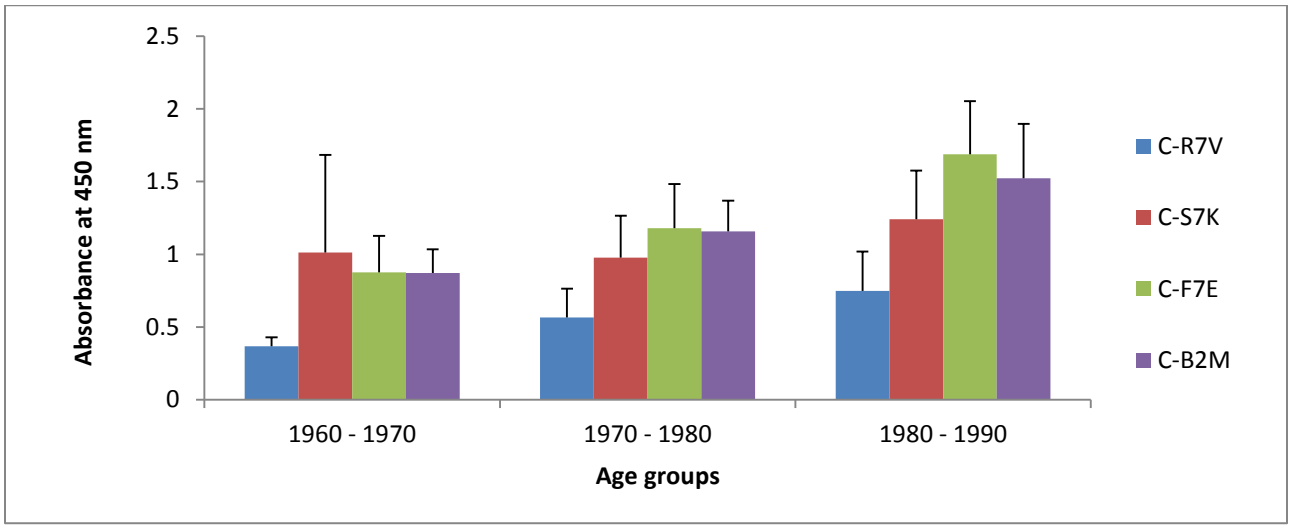


Figure 4.1.11: ELISA trends based on different age groups of HIV+ individuals in this study. HIV positive individuals were matched according to date of birth and the responses against C-R7V (blue), C-S7K (red), C-F7E C (green) and β2M (purple). All data was obtained from experiments where 250 ng/ml peptide antigens were used.

4.2 Cytokine quantification

The BD CBA Human Th1/Th2/Th17 Cytokine kit uses bead array technology for cytokine quantification, through the use of FCAP Array™ software (BD biosciences, San Diego). Individual standard curves were generated for each cytokine (ranging from 0 pg/ml – 5000 pg/ml), as shown in figure 4.2.1, the 4-parameter logistic curve fit option was used to extrapolate values based on sample intensities. The following are representative graphs from which cytokine quantification was conducted. These values were consolidated and are summarized as bar graphs (Figure 4.2.2 - 4.2.11) representing different experimental conditions.

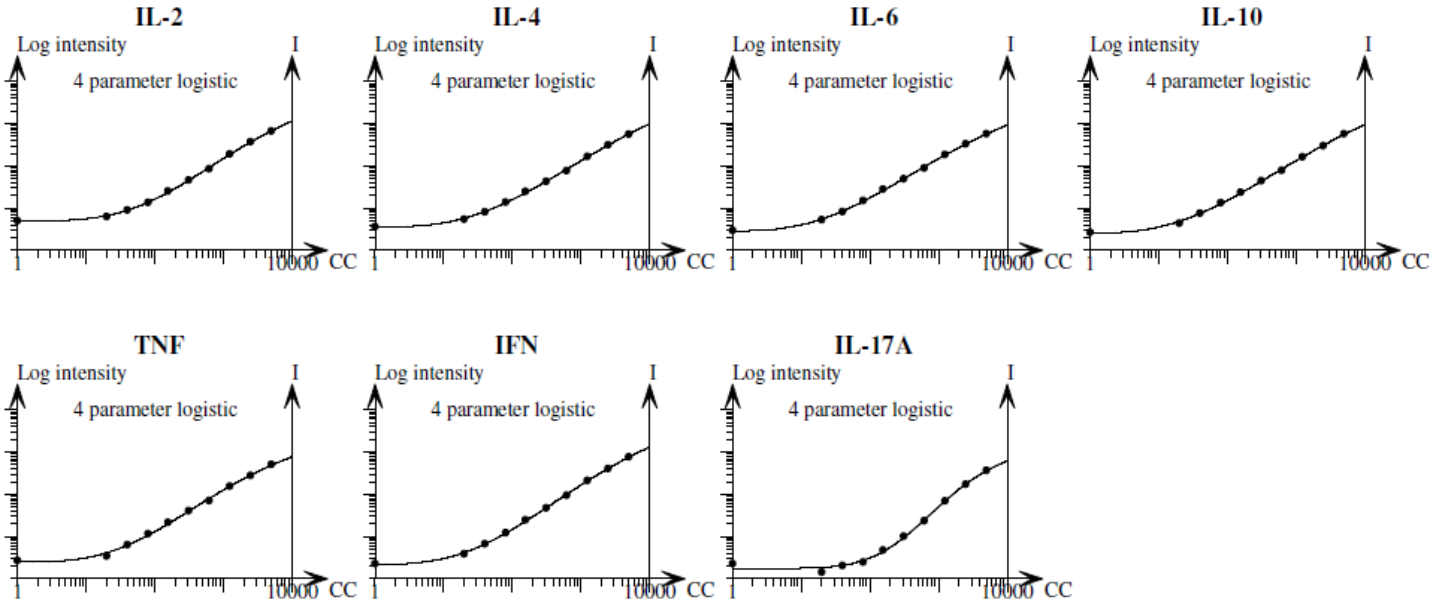
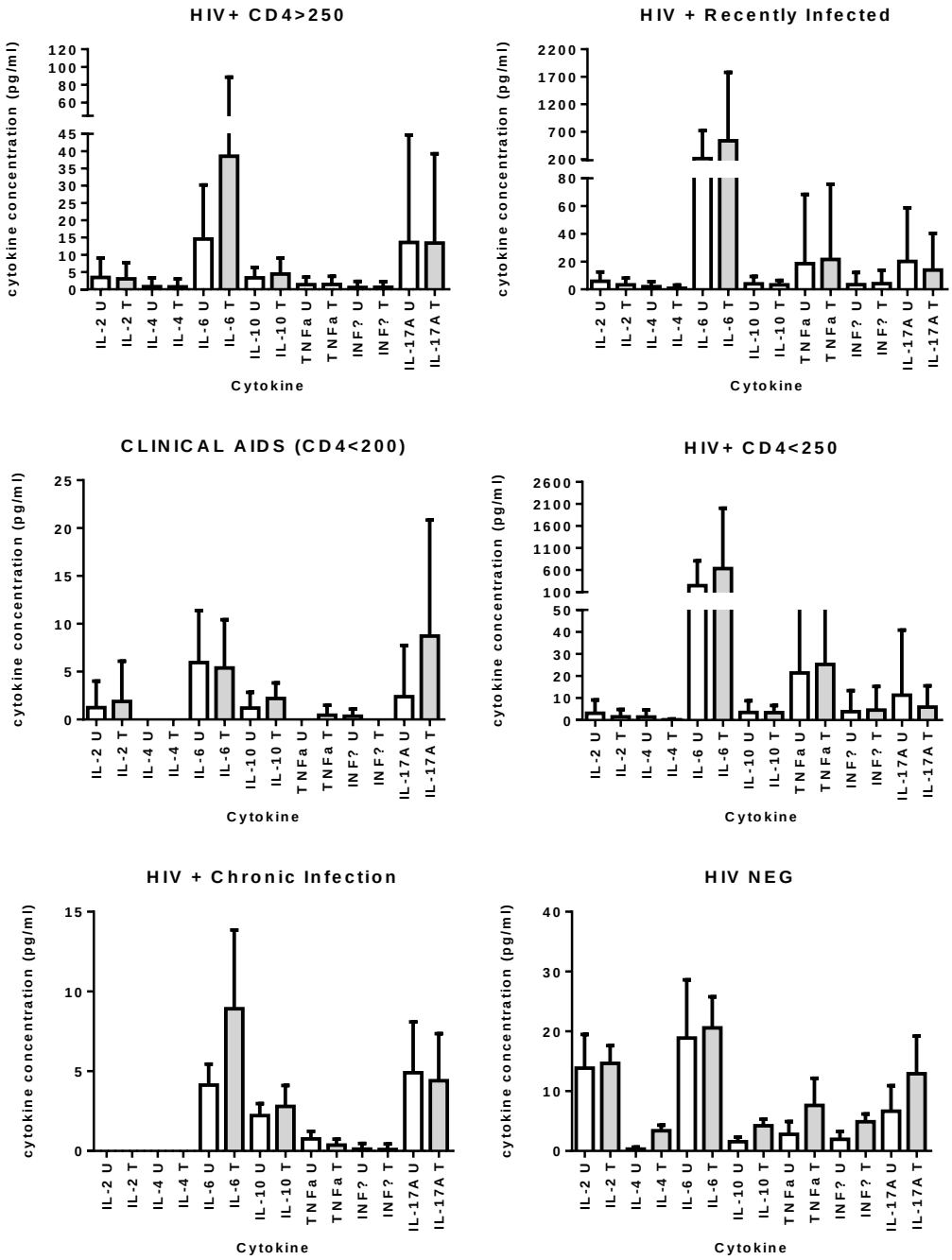


Figure 4.2.1: The standard curves obtained for different (Th1/Th2/Th17) cytokines. These range from 0 pg/ml – 5000 pg/ml and were calculated, the y-axis represents the log intensity. It is from these graphs that the cytokine concentration was determined.

4.2.1 Cytokine responses induced by host derived peptides

The diagrams that follow represent a summary of observations obtained following stimulation of PBMCs from HIV negative donors and HIV positive patients, with 10 µg/ml of each peptide in an effort to ascertain the influence of these antigens on the Th1/Th2/Th17 cytokine profiles. Following stimulation of PBMCs, the cytokines affected by exposure to host –derived antigens (F7E, S7K,R7V at 10 µg/ml) were IL-6 and TNFα which were decreased but not significantly ($p<0.05$). When comparing exposed (peptide treated) to unexposed (not peptide treated cells) IL-17A was also slightly decreased. In HIV positive patients in the acute stage of infection (which for the purpose of this study is defined as individuals who have had HIV for a period of two years or less at the time of the bleed date and were therefore in the acute stage of HIV disease progression. Newly infected patients exhibited an increase in IL-6 production. The level of secreted IL-6 decreased tremendously with duration of infection, with the lowest amounts observed in chronically infected individuals both prior to and at the point of clinical AIDS. Most cytokines were present in uninfected patients but not in infected individuals and peptide treatment upregulated cytokine secretion, sometimes significantly.



R7V

Figure 4.2.2: Cytokine profile from peripheral blood mononuclear cells (PBMCs) of HIV infected individuals (n=17) and HIV negative donors (n=6) stimulated with 10 µg/ml R7V antigen for a period of 7 days. Th1/Th2/Th17 cytokines were quantified using a multiplex cytokine bead array kit, which made use of FACS Array for detection. The amounts of cytokines (pg/ml) were grouped according to; CD4 count, duration of infection and HIV status; the means, medians and variation were compared. The letter U and T refer to untreated and treated PBMCs respectively.

S7K

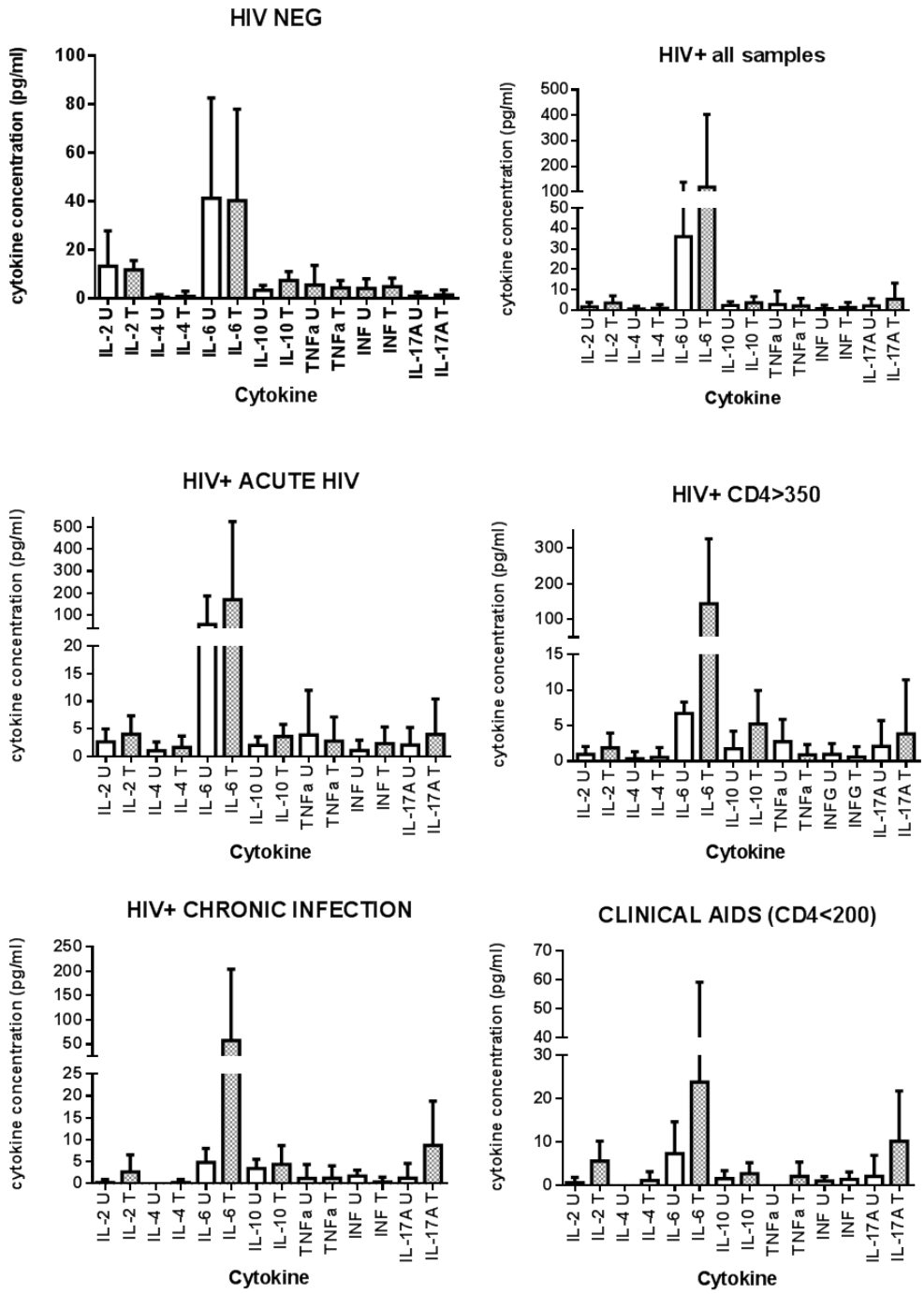


Figure 4.2.3: Cytokine profile from peripheral blood mononuclear cells of HIV infected individuals as well as HIV negative donors stimulated with 10 µg/ml S7K antigens (T) for a period of 7 days compared to untreated controls (U). A multiplex cytokine bead array kit, which made use of FACS Array for detection was used to quantify Th1/Th2/Th17 cytokines. The cytokine responses (pg/ml) were grouped according to: CD4 count, duration of infection and HIV status; and the means, medians and variation were compared.

F7E

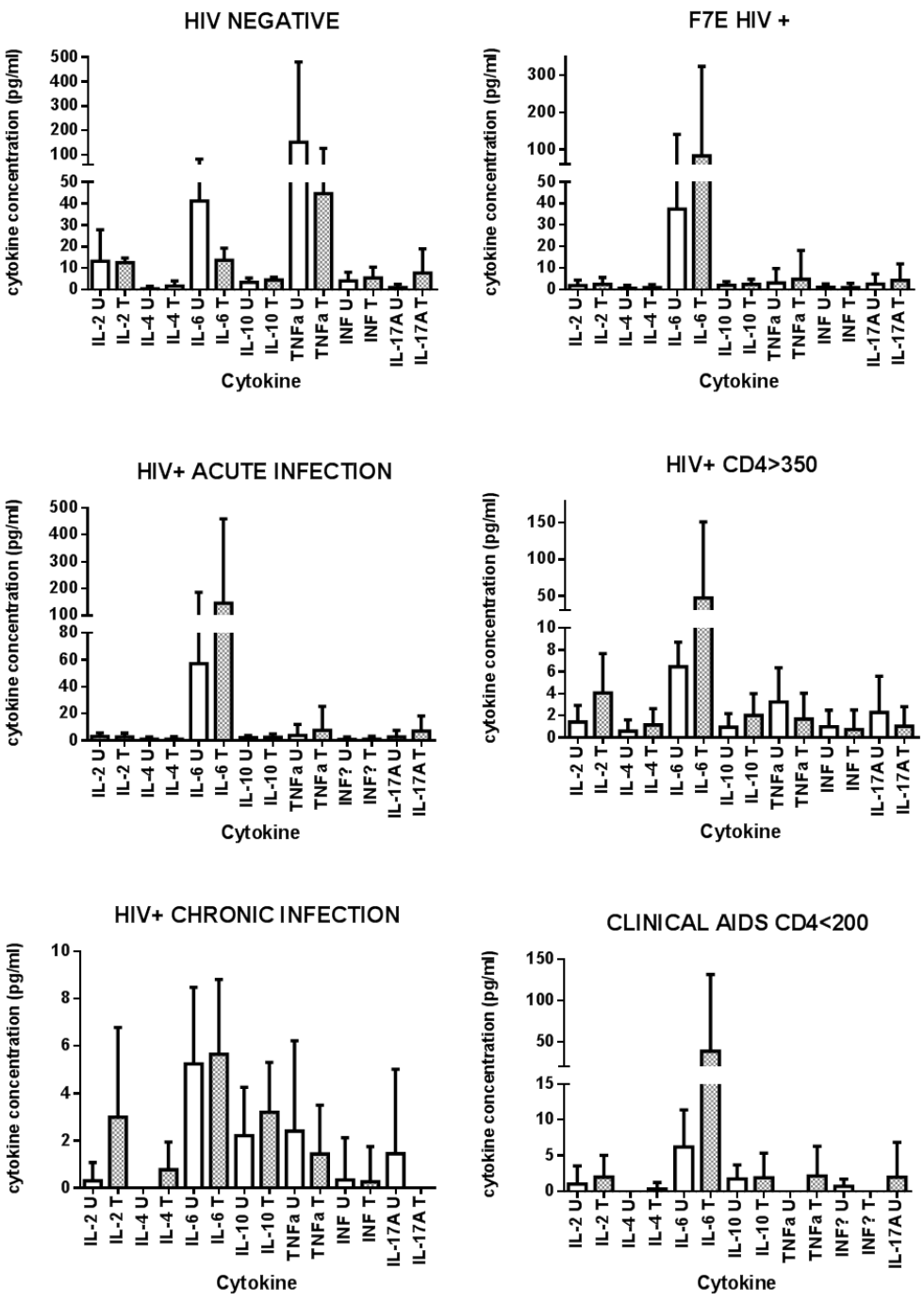


Figure 4.2.4: Cytokine profile of peripheral blood mononuclear cells (from HIV negative and HIV infected individuals) that were stimulated with 10 µg/ml F7E antigens (T) for a period of 7 days compared to untreated controls (U). Th1/Th2/Th17 cytokines were quantified using a multiplex cytokine bead array kit, which made use of FACS Array for detection. The obtained amounts of cytokines (pg/ml) were grouped according to the following; CD4 count, duration of infection and HIV status; and the means, medians and variation were compared.

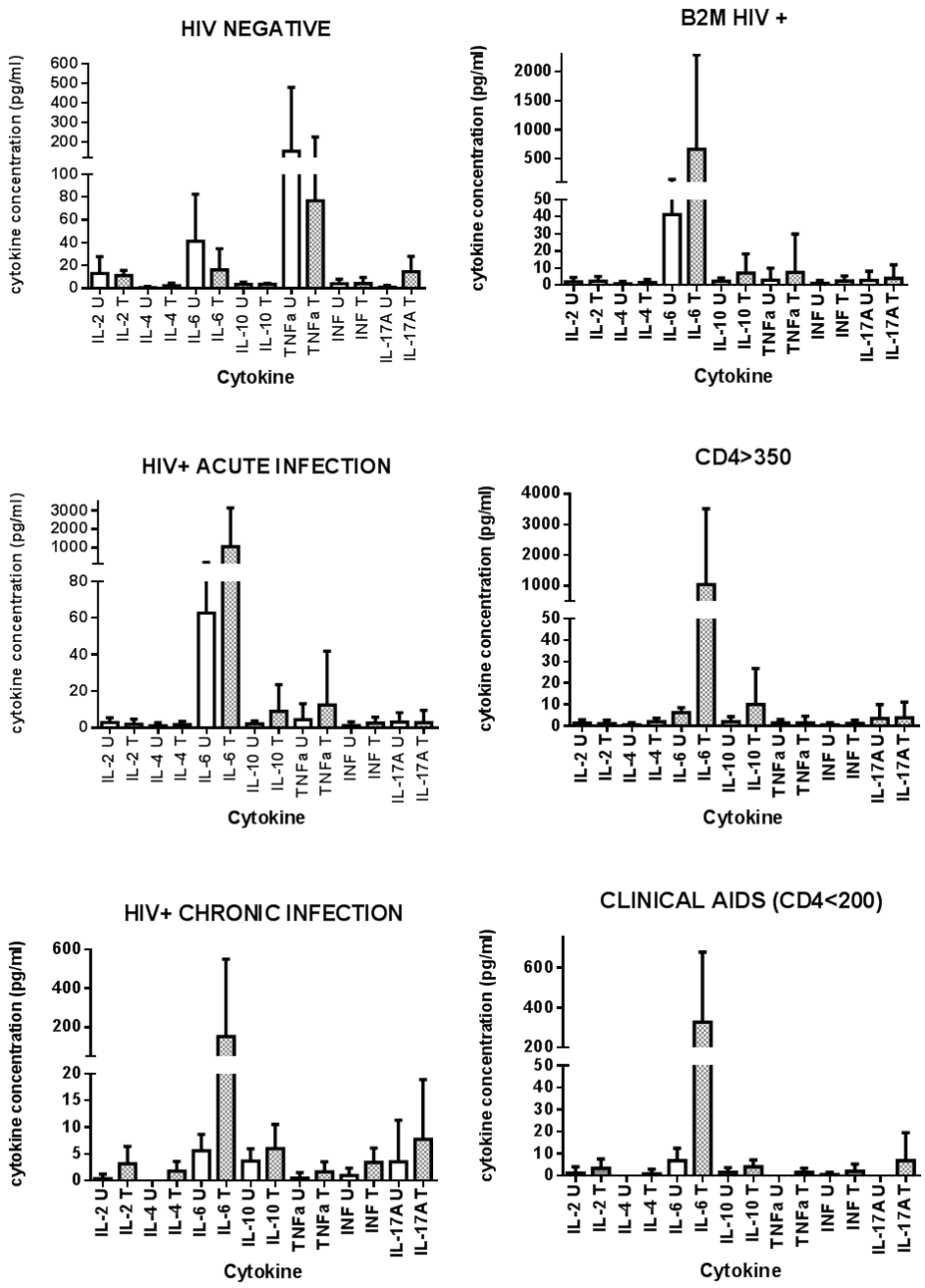


Figure 4.2.5: Cytokine profile from peripheral mononuclear cells of HIV infected individuals as well as HIV negative donors stimulated with 10 µg/ml B2M antigens for a period of 7 days. Th1/Th2/Th17 cytokines were quantified using a multiplex cytokine bead array kit, which makes use of FACS Array. The obtained amounts of cytokines (pg/ml) were grouped according to the following; CD4 count, duration of infection and HIV status; and the means, medians and variation were compared.

The cytokines influenced by β2M treatment in HIV negative individuals were IL-6, IL-10 and TNFα. Newly infected individuals demonstrated increased IL-6, IL-10 and TNFα. Individuals with CD4 counts greater than 350 cells/mm^3 exhibited IL-6 levels lower than acutely infected individuals but these were stimulated by β2M treatment. Individuals at the chronic stage of infection exhibited an increase although not significant (except in the case of IL-6). At the stage of clinical AIDS the cytokines production diminished but B2M was still able to stimulate IL-6 production significantly ($p<0.05$).

A cocktail of R7V/S7K/F7E (which is abbreviated in the figure as HM, which stands for "host mix') increased IL-6 in HIV positive As well as HIV negative samples. In HIV negative individuals, TNFα production was decreased whereas IL-6 was increased following treatment with the peptides. These two cytokines were the most affected by peptide treatment, due to the variation between different donors, the standard deviations were high.

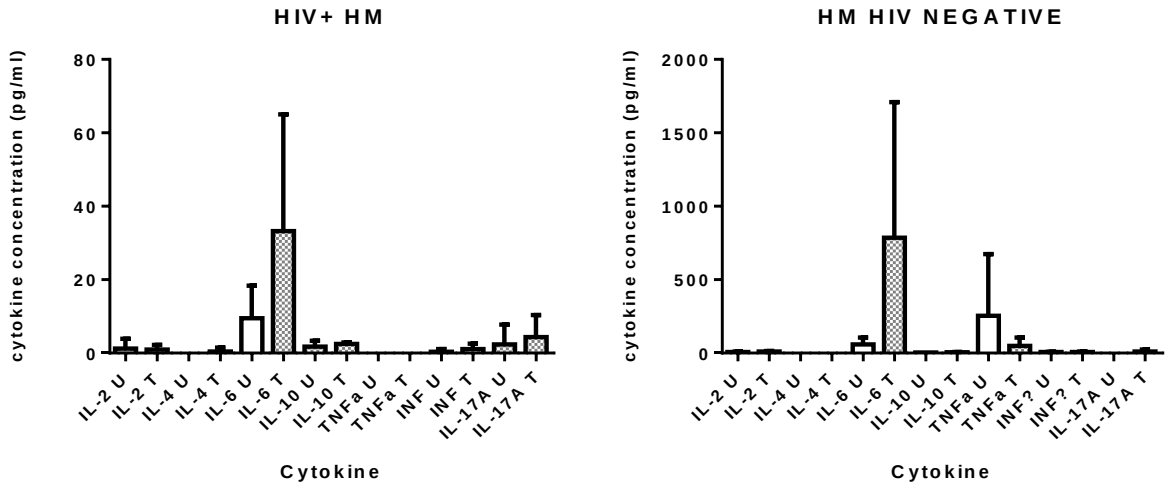


Figure 4.2.6: Cytokine profile from peripheral blood mononuclear cells of HIV infected individuals as well as HIV negative donors stimulated with 10 µg/ml of a cocktail of host-derived peptides for a period of 7 days. Th1/Th2/Th17 cytokines were quantified using a multiplex cytokine bead array kit, which made use of FACS Array for detection. The amounts of cytokines (pg/ml) were grouped according to the following; CD4 count, duration of infection and HIV status; and the means, medians and variation were compare

4.2.2 Viral Peptides

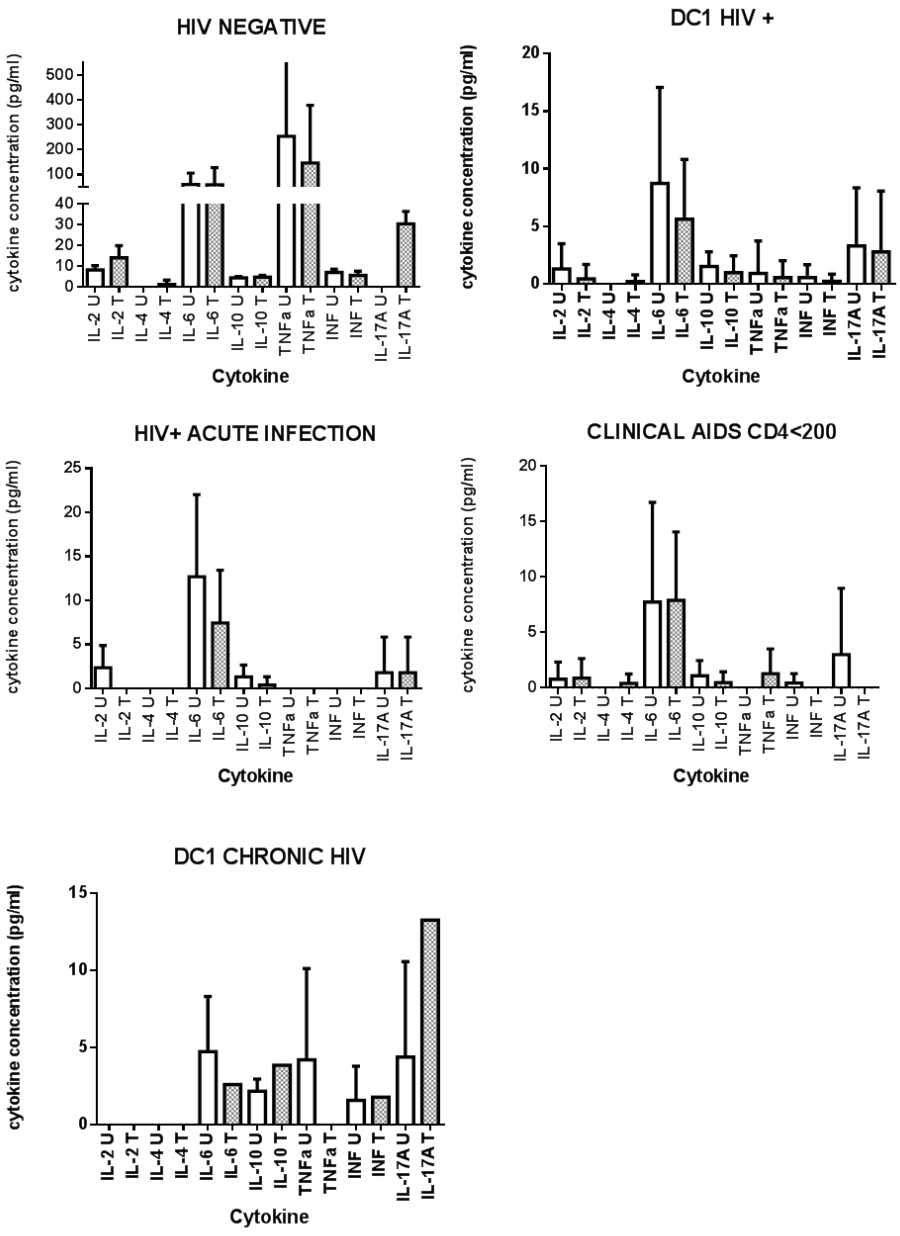


Figure 4.2.7: Cytokine profile from peripheral mononuclear cells of HIV infected individuals as well as HIV negative donors stimulated with 10 µg/ml DC1 antigens for a period of 7 days. Th1/Th2/Th17 cytokines were quantified using a multiplex cytokine bead array kit, which makes use of FACS Array. The obtained amounts of cytokines (pg/ml) were grouped according to the following; CD4 count, duration of infection and HIV status; and the means, medians and variation were compared.

In HIV negative patients there was a slight increase in IL-2 and IL-17 secretion after treatment, IL-6, IL-10 and INF were not influenced by treatment. TNFα was decreased by treatment with DC1. In HIV positive individuals, there is high variation in IL-6 secretion but treatment leads to a decrease in patients at the acute and chronic stages of HIV infection.

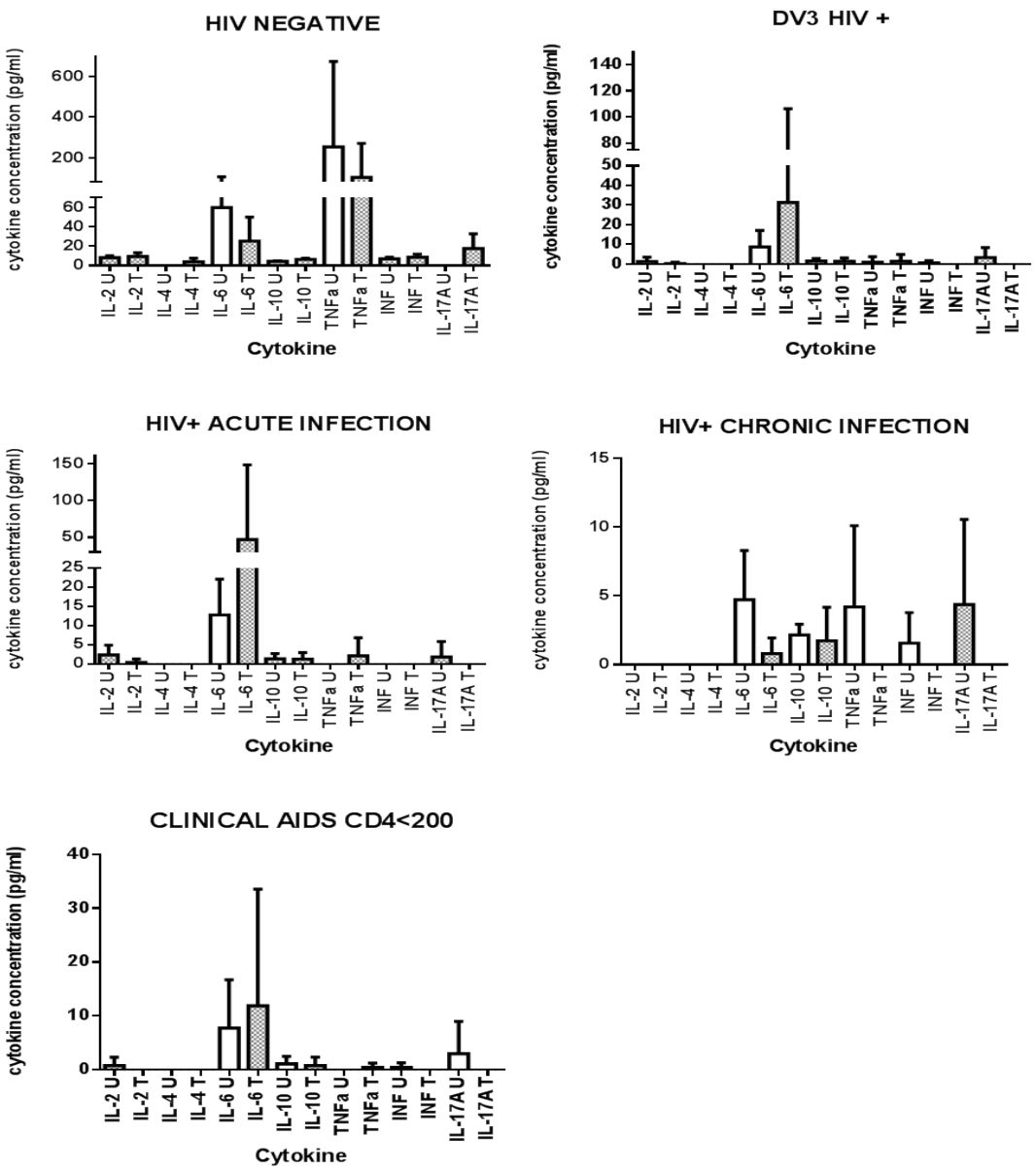


FIGURE 4.2.8: Cytokine profile from peripheral mononuclear cells of HIV infected individuals as well as HIV negative donors stimulated with 10 µg/ml DV3 antigens for a period of 7 days. Th1/Th2/Th17 cytokines were quantified using a multiplex cytokine bead array kit, which makes use of FACS Array. The obtained amounts of cytokines (pg/ml) were grouped according to the following; CD4 count, duration of infection and HIV status; and the means, medians and variation were compared.

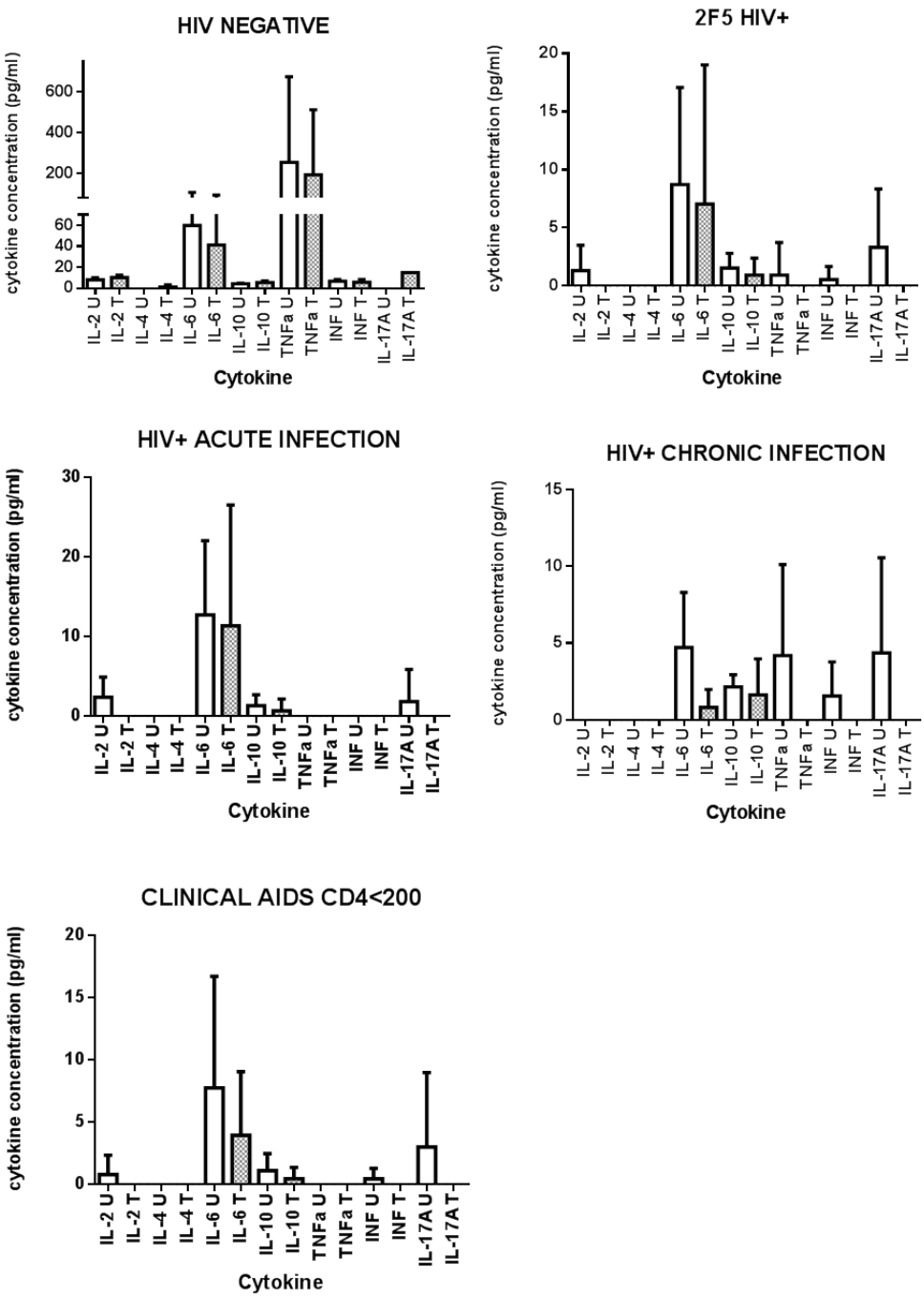


FIGURE 4.2.9: Cytokine profile from peripheral mononuclear cells of HIV infected individuals as well as HIV negative donors stimulated with 10 µg/ml 2F5 antigens for a period of 7 days. Th1/Th2/Th17 cytokines were quantified using a multiplex cytokine bead array kit, which makes use of FACS Array. The obtained amounts of cytokines (pg/ml) were grouped according to the following; CD4 count, duration of infection and HIV status; and the means, medians and variation were compared.

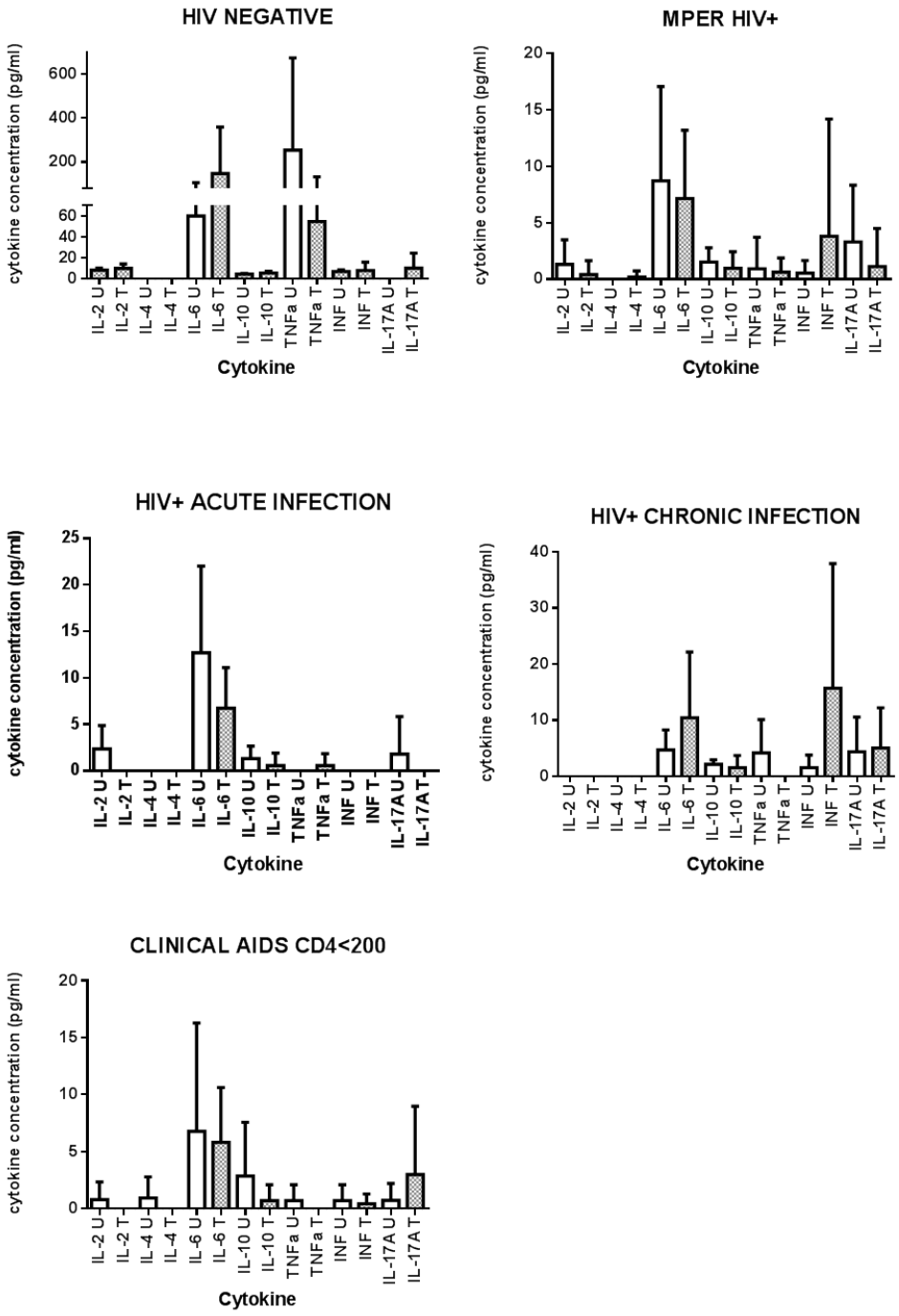


FIGURE 4.2.10: Cytokine profile from peripheral mononuclear cells of HIV infected individuals as well as HIV negative donors stimulated with 10 µg/ml MPER antigens for a period of 7 days. Th1/Th2/Th17 cytokines were quantified using a multiplex cytokine bead array kit, which makes use of FACS Array. The obtained amounts of cytokines (pg/ml) were grouped according to the following; CD4 count, duration of infection and HIV status; and the means, medians and variation were compared.

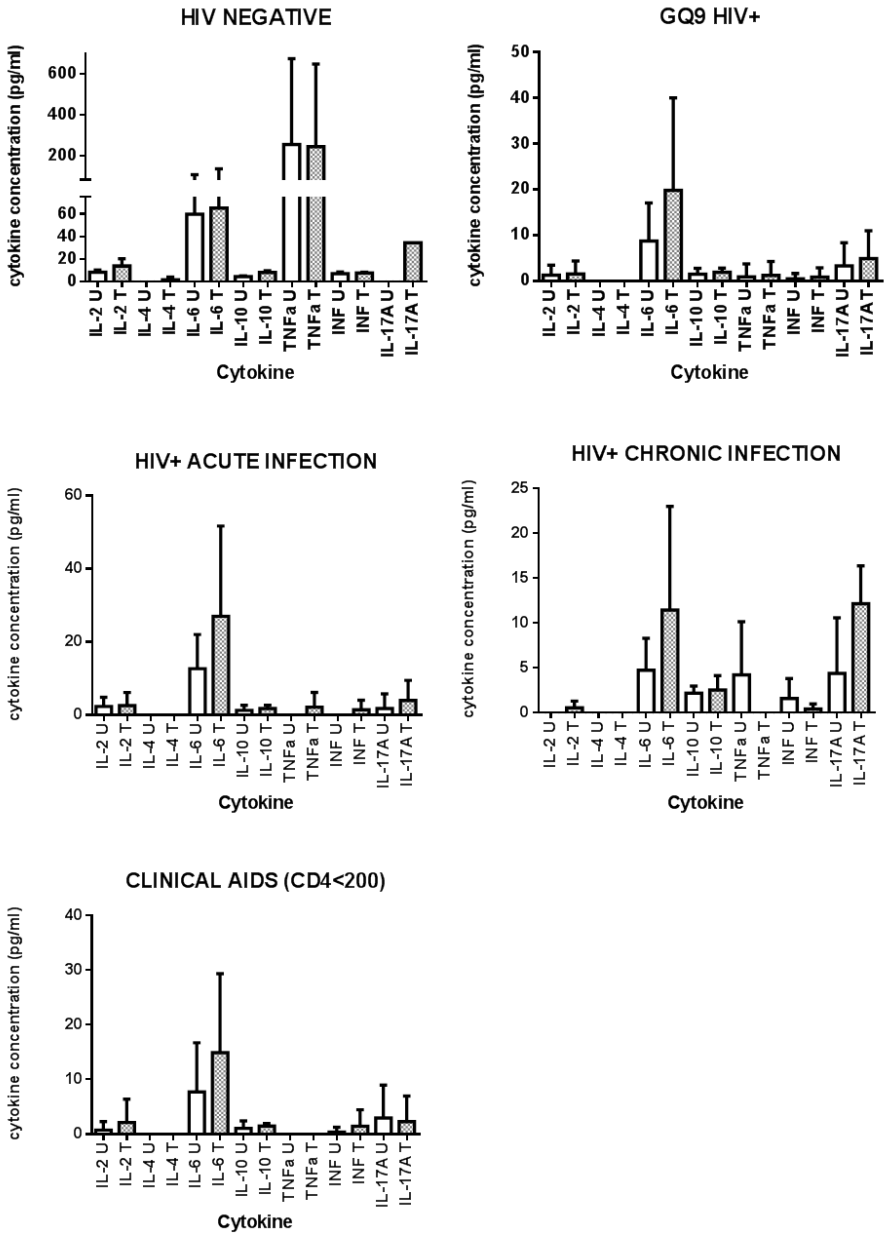


Figure 4.2.11: Cytokine profile from peripheral mononuclear cells of HIV infected individuals as well as HIV negative donors stimulated with 10 µg/ml GQ9 antigens for a period of 7 days. Th1/Th2/Th17 cytokines were quantified using a multiplex cytokine bead array kit, which makes use of FACS Array. The obtained amounts of cytokines (pg/ml) were grouped according to the following; CD4 count, duration of infection and HIV status; and the means, medians and variation were compared.

4.3 Viability- XTT

Viability (and proliferation) of PBMCs from HIV positive and HIV negative individuals was assessed using XTT. Viability dyes are not the most sensitive means for determining proliferation. The best assay uses bromodeoxyuridine (BrdU) or tritiated thymidine incorporation, these radioactive labels are incorporated into DNA during cell division and stimulation indices of 2 or more are considered positive. XTT measures viability of cells by assessing the metabolic state which relies on live cells, and is an indication of proliferation.

Table 2: Viability data as determined by XTT metabolism by donor PBMCs

PEPTIDE	HIV STATUS	N	STIMULATION INDEX ± SD
HOST PEPTIDES			
(C) R7V	HIV +	12	0.96 ± 0.10
	HIV -	7	0.84 ± 0.06
(C) S7K	HIV +	13	1.01 ± 0.13
	HIV -	7	0.78 ± 0.10
(C) F7E	HIV +	10	1.15 ± 0.04
	HIV -	5	1.10 ± 0.04
β2M	HIV +	21	0.99 ± 0.03
	HIV -	7	1.33 ± 0.24
HM	HIV +	14	1.24 ± 0.14
	HIV -	7	1.16 ± 0.11
MODIFIED HOST PEPTIDES			
R7V-3	HIV +	4	1.00 ± 0.18
	HIV -	5	0.89 ± 0.09
S7K-4	HIV +	-	-
	HIV -	7	1.25 ± 0.13

F7E-8	HIV +	-	-
	HIV -	5	0.93 ± 0.11
VIRAL PEPTIDES			
DV3	HIV +	25	1.09 ± 0.26
	HIV -	7	0.99 ± 0.19
MPER	HIV +	25	1.62 ± 0.46
	HIV -	7	1.21 ± 0.16
DC1	HIV +	25	0.91 ± 0.40
	HIV -	7	0.94 ± 0.18
2F5	HIV +	25	0.90 ± 0.35
	HIV -	7	0.96 ± 0.12
GQ9	HIV +	24	1.08 ± 0.35
	HIV -	7	1.04 ± 0.08
***PM**	HIV +	8	1.47 ± 0.18
	HIV -	7	1.03 ± 0.08
***PHA**	HIV +	27	1.95 ± 0.12
	HIV -	7	1.85 ± 0.06

*positive control

The stimulation index presented in table 2 represents the proliferation of treated PBMCs as a fraction of untreated cells. It is evident, that none of the peptides were toxic to PBMCs at 10 µg/ml, following stimulation over a 7 day period. The host peptides only stimulated proliferation when they were used as a cocktail (S7K, F7E and R7V), host peptide mix (designated as HM), but were unable to do so on their own or in their modified forms. B2M was able to induce proliferation in HIV negative individuals; however, this was not significant. Of the viral peptides under investigation MPER was the only one that was able to induce proliferation (SI of 1.62). The positive controls were PHA, a lectin, which was able to stimulate proliferation in both negative as well as HIV positive donors; and an env cocktail pepmix^{TM} (JPT, USA) designated

as PM which stimulated the proliferation of HIV positive PBMCs only (with a mean SI of 1.47). TZM-bl cells were also treated with peptides at different concentrations (10, 20, 80 and 100 µg/ml), see figure 4.3.1 No dose-response was evident and there were no significant differences observed except in the case of S7K. S7K showed toxicity to TZMbl cells following treatment at or above 80 µg/ml.

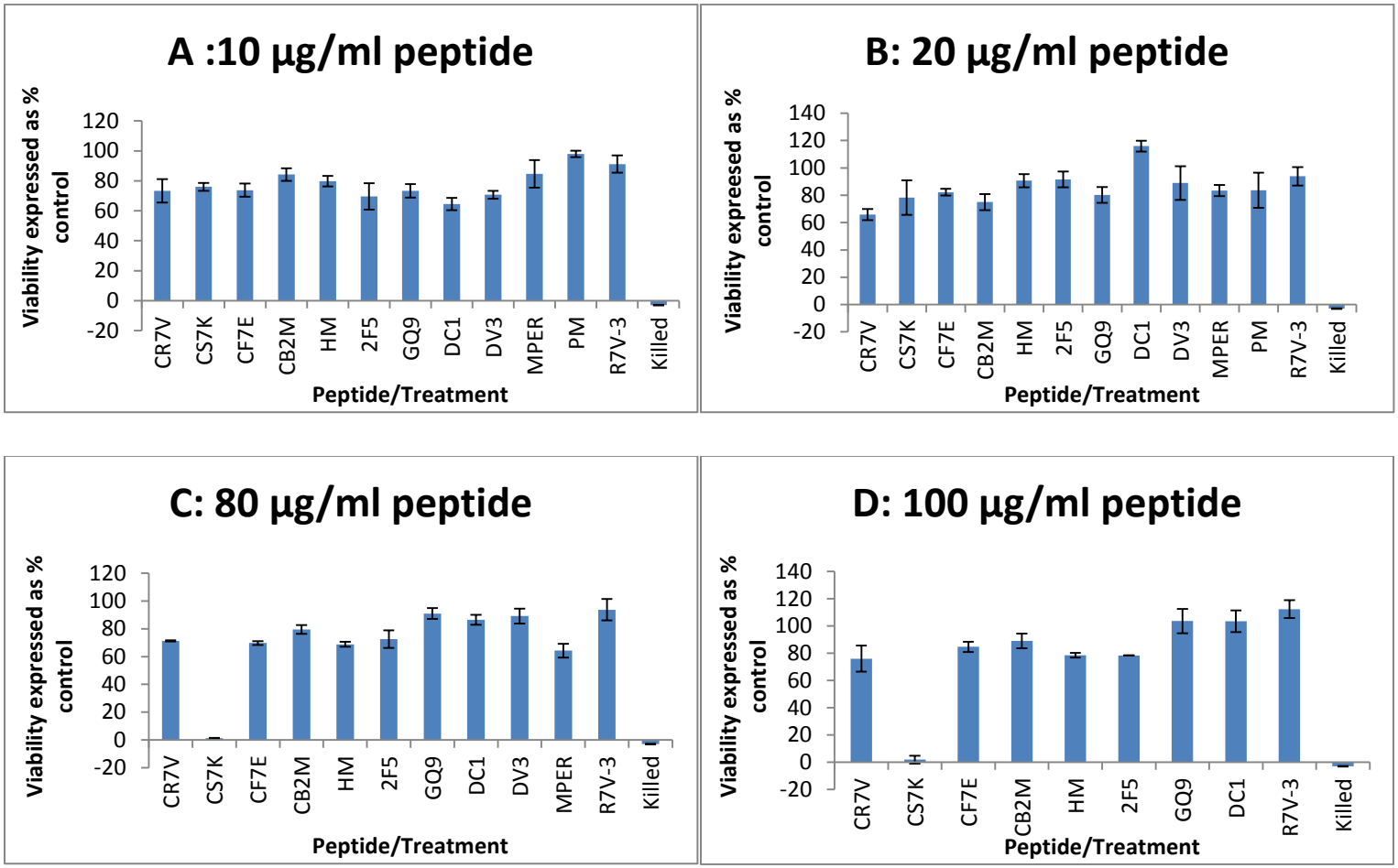


Figure 4.3.1: Graph showing TZMbl viability following stimulation with different concentrations of peptides. As the concentration is increased, the viability decreases slightly in some cases but not significantly ($p>0.05$). No dose-response relationship observed. One of the peptides (S7K) suddenly loses viability at concentrations beyond 80 ug/ml.

4.4 Proliferation as measured by flow cytometry

Flow cytometry is a more sensitive method than XTT and provides information about proliferation of cells of interest through the use of proliferation tracking dyes such as CFSE. The CSFE dye allows for discrimination of cell generations along with the enumeration of the total frequency of cells in division, this dye also reports the number of times cells divide and allows for the calculation of the frequency of the starting population that divided. Data generated using flow cytometry is displayed using multi-parameter acquisition and display software platforms. Histograms corresponding to each of the parameters of interest were analyzed using statistical tools to calculate the percentages of cells manifesting specific fluorescence, and fluorescence intensity. This information was also used to look at fluorescence expression within subpopulations of cells in a sample. Cells were initially labelled with CFSE, cultured *in vitro* for several days in the presence of a stimulant (peptide) and the dye was progressively diluted among proliferating cells. Gating is an important principle of flow cytometry (shown in Figure 4.4.1) because it facilitates the selection of the cells of interest and allows for the exclusion of dead cells and debris during data analysis.

In the top left hand corner of figure 4.4.1 is a forward scatter (FSC) / side scatter (SSC) plot for PBMCs obtained from human blood. Size is estimated by FSC and complexity by SSC. The physical properties of lymphocytes, granulocytes and monocytes can therefore be distinguished from each other. The lymphocytes have been gated and on the right the same cells are now plotted as SSC on the y-axis versus Comp-PerCP-cy5-A fluorescence on the x-axis. PerCP is a fluorophore that allows for the tracking of the propidium iodide dye which was used to stain dead cells. The fluorescence intensity was used to gate the dead cells which were then excluded from the population of interest. The proliferation node was then applied to the viable cells in relative terms; viable lymphocytes have a low PerCP fluorescence intensity. Frame 4 is a representative image of what proliferating cells look like, the parent population is on the far left with the highest fluorescence intensity, the daughter populations follow as the dye is progressively diluted for up to a maximum of 8 generations generated from the parent population.

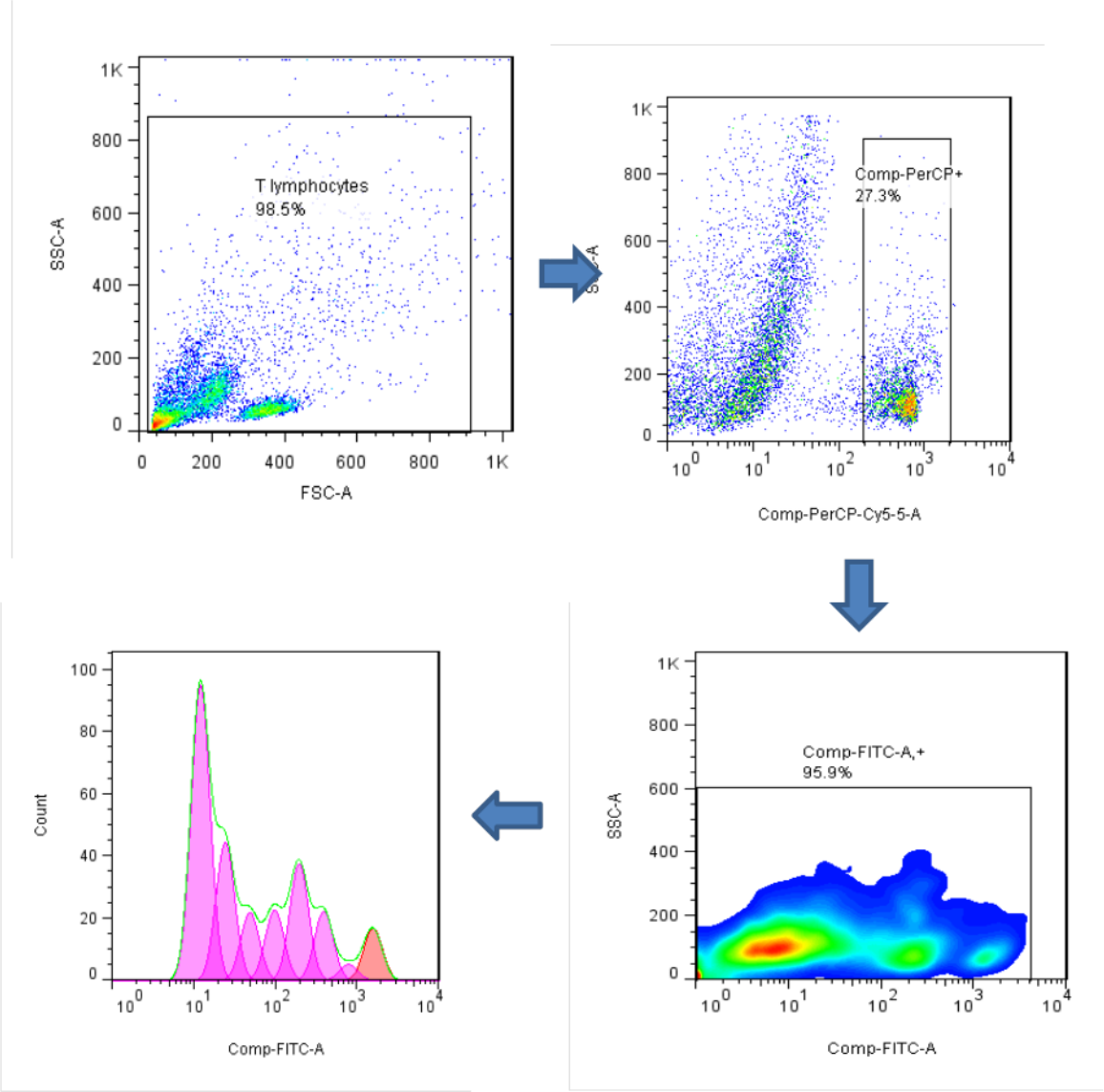


Figure 4.4.1: Gating strategy used for CFSE analysis using Flow Cytometry. The first frame is a density plot in which, each point represents an individual cell that has been picked up and analysed by the instrument, based on the size and granular complexicity, T lymphocyte population was selected. In frame 2 the dead cells are gated and the FLOWJo software allows these to be excluded from the analysis. The third frame is the the viable T lymphocytes in the population of interect, these are then analysed for proliferation generating frame 4. Frame 4 is a representative image of what proliferating cells look like, the parent population is on the far left with the highest fluorescence intensity, the daughter populations follow as the dye is progressively diluted for up to a maximum of 8 generations generated from the parent population.

Proliferation indices were calculated following flow cytrometric analysis of cell proliferation in the presence of peptides. These were much higher than the stimulation indices obtained using XTT demonstrating the increased sensitivity of flow cytometry.

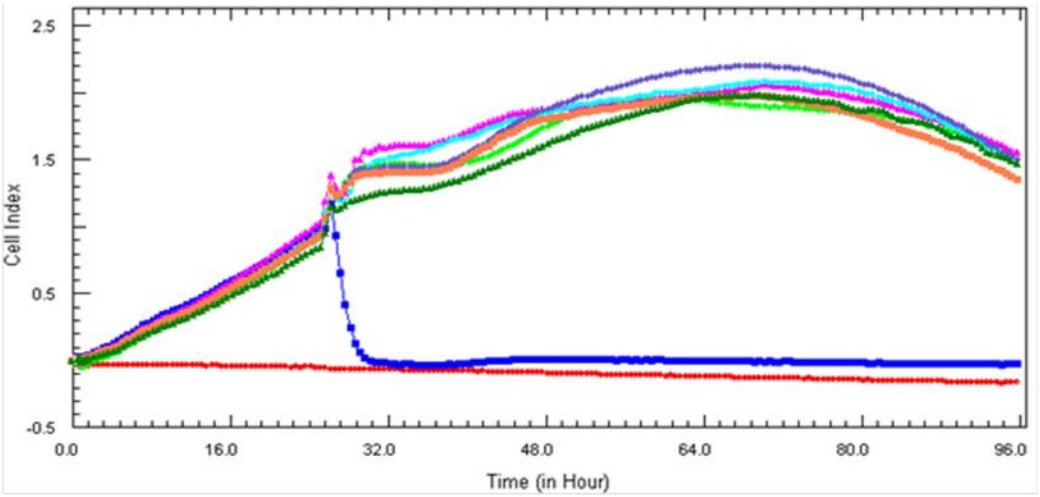
Table 3: Proliferation induced by peptides as detected by CFSE and flow cytometry

PEPTIDE 10 g/ml	% DIVIDED	PROLIFERATION INDE X
B2M	100	7
R7V	100	7
DC1	75	1.14
DV3	100	7
GQ9	85.3	4.90
Host mix	82.5	1.01
ENV MIX	86.5	5.35
MPER	63.7	4

Proliferation index as determined by Flowjo software analysis. Percent Divided indicated the number of cells that had divided at least once. Proliferation Index is the average number of divisions of just the responding cells (cells that underwent at least one division). The data was obtained following treatment of HIV positive PBMCs of two patients.

4.5 Real Time Cell Electronic Sensing RT-CES

The XCELLigence system, also called Real Time Electronic Sensing, is a real time cell analyzer. This label-free, cell-based assay was used to monitor TZM-bl cells following treatment with peptides. The cell index reflects, among other biological processes, the viability of these cells. As seen in figure 4.5.1 the peptides did not kill cells, proliferation in the presence of peptides is visible but not as clear as in the more sensitive flow Cytometric assay depicted in table 3



COLOUR	RED	DARK BLUE	LIGHT GREEN	DARK GREEN	BRIGHT RED	LIGHT BLUE	PINK
PEPTIDE	MEDIA ONLY	KILLED CELLS	UNTREATED CELLS	S7K	F7E	DV3	MPER

Figure 4.5.1 Real Time Cell Electronic Sensing RT-CES data of 500 cells/well, the colour key is indicated.

4.6 Immunogenicity tests

As explained in the methods, New Zealand white rabbits were immunized with the peptides to assess the ability of the peptides to induce an immunogenic response. All the peptides were conjugated to KLH prior to immunization but peptide specific antibodies were produced in every case. Very high antibody titers were observed with responses detectable at antibody dilutions as high as 1:512000

Table 4.6.1: Anti-R7V-3

	Dilution	Pre-immune serum (A450nm)		Purified antibody (A450nm)	
		Anti-R7V-3			
		Rabbit #5651	Rabbit #5652	Rabbit #5651	Rabbit #5652
1	1:1,000	0.077	0.160	2.710	2.622
2	1:2,000	0.074	0.112	2.648	2.560
3	1:4,000	0.066	0.088	2.642	2.555
4	1:8,000	0.064	0.093	2.611	2.544
5	1:16,000	0.064	0.087	2.577	2.510
6	1:32,000	0.062	0.082	2.274	2.173
7	1:64,000	0.065	0.056	1.822	1.940
8	1:128,000	0.064	0.084	1.500	1.500
9	1:256,000	0.058	0.082	0.882	1.077
10	1:512,000	0.061	0.094	0.764	0.689
11	Blank	0.073	0.073	0.073	0.073
12	Blank	0.073	0.073	0.073	0.073
	Titer	<1:1,000	1:1,000	>1:512,000	>1:512,000

Table 4.6.2: Anti-S7K 4 antibody titers

	Dilution	Pre-immune serum (A450nm)		Purified antibody (A450nm)	
		Anti-S7K-4			
		Rabbit #5629	Rabbit #5630	Rabbit #5629	Rabbit #5630
1	1:1,000	0.191	0.066	3.092	3.053
2	1:2,000	0.115	0.062	3.004	3.031
3	1:4,000	0.082	0.053	2.967	2.970
4	1:8,000	0.068	0.059	2.803	2.788
5	1:16,000	0.060	0.055	2.648	2.763
6	1:32,000	0.057	0.053	2.506	2.669
7	1:64,000	0.059	0.056	2.354	2.546
8	1:128,000	0.062	0.058	2.148	2.198
9	1:256,000	0.063	0.058	1.759	1.677
10	1:512,000	0.078	0.063	1.317	1.206
11	Blank	0.071	0.071	0.071	0.071
12	Blank	0.071	0.071	0.071	0.071
	Titer	1:1,000	<1:1,000	>1:512,000	>1:512,000

Table 4.6.3: Anti-F7E-8 antibody titers

	Dilution	Pre-immune serum (A450nm)		Purified antibody (A450nm)	
		Anti-F7E-8			
		Rabbit #5631	Rabbit #5632	Rabbit #5631	Rabbit #5632
1	1:1,000	0.128	0.275	3.004	2.776
2	1:2,000	0.097	0.136	3.004	2.721
3	1:4,000	0.096	0.074	2.931	2.721
4	1:8,000	0.082	0.086	2.815	2.681
5	1:16,000	0.074	0.079	2.766	2.604
6	1:32,000	0.072	0.072	2.437	2.481
7	1:64,000	0.071	0.081	2.239	2.121
8	1:128,000	0.072	0.090	1.651	1.733
9	1:256,000	0.081	0.074	0.969	1.135
10	1:512,000	0.071	0.072	0.666	0.698
11	Blank	0.084	0.084	0.084	0.084
12	Blank	0.084	0.084	0.084	0.084
	Titer	<1:1,000	1:1,000	>1:512,000	>1:512,000

Table 4.6.4: Anti-DC1 antibody titers

	Dilution	Pre-immune serum (A450nm)		Purified antibody (A450nm)	
		Anti-DC1			
		Rabbit #5633	Rabbit #5634	Rabbit #5633	Rabbit #5634
1	1:1,000	0.189	0.102	2.726	2.909
2	1:2,000	0.106	0.087	2.651	2.896
3	1:4,000	0.099	0.096	2.452	2.896
4	1:8,000	0.097	0.085	2.236	2.884
5	1:16,000	0.097	0.085	1.720	2.602
6	1:32,000	0.092	0.080	1.017	2.106
7	1:64,000	0.086	0.085	0.689	1.410
8	1:128,000	0.087	0.090	0.599	0.913
9	1:256,000	0.087	0.076	0.304	0.565
10	1:512,000	0.085	0.086	0.204	0.325
11	Blank	0.105	0.105	0.105	0.105
12	Blank	0.105	0.105	0.105	0.105
	Titer	<1:1,000	<1:1,000	1:256,000	1:512,000

Table 4.6.5: Anti-2F5 antibody titers

	Dilution	Pre-immune serum (A450nm)		Purified antibody (A450nm)	
		Anti-2F5c			
		Rabbit #5649	Rabbit #5650	Rabbit #5649	Rabbit #5650
1	1:1,000	0.088	0.057	2.918	2.750
2	1:2,000	0.080	0.057	2.815	2.740
3	1:4,000	0.069	0.061	2.815	2.686
4	1:8,000	0.064	0.056	2.752	2.678
5	1:16,000	0.059	0.054	2.732	2.678
6	1:32,000	0.059	0.066	2.563	2.669
7	1:64,000	0.059	0.053	2.262	2.368
8	1:128,000	0.057	0.052	1.773	2.251
9	1:256,000	0.055	0.062	1.204	1.767
10	1:512,000	0.058	0.073	0.795	1.192
11	Blank	0.069	0.069	0.069	0.069
12	Blank	0.069	0.069	0.069	0.069
	Titer	<1:1,000	<1:1,000	>1:512,000	>1:512,000

Table 4.6.6: Anti-DV3 antibody titer

	Dilution	Pre-immune serum (A450nm)		Purified antibody (A450nm)	
		Anti-DV3c			
		Rabbit #5623	Rabbit #5624	Rabbit #5623	Rabbit #5624
1	1:1,000	0.118	0.127	2.171	2.043
2	1:2,000	0.108	0.112	1.792	1.563
3	1:4,000	0.096	0.098	1.268	1.068
4	1:8,000	0.091	0.088	1.110	0.971
5	1:16,000	0.081	0.077	0.995	0.815
6	1:32,000	0.064	0.075	0.874	0.660
7	1:64,000	0.080	0.084	0.733	0.545
8	1:128,000	0.092	0.084	0.421	0.318
9	1:256,000	0.088	0.084	0.337	0.209
10	1:512,000	0.089	0.084	0.184	0.135
11	Blank	0.091	0.091	0.091	0.091
12	Blank	0.091	0.091	0.091	0.091
	Titer	<1:1,000	<1:1,000	1:256,000	1:256,000

Table 4.6.7: Anti-MPER antibody titer

	Dilution	Pre-immune serum (A450nm)		Purified antibody (A450nm)	
		Anti-MPERm			
		Rabbit #5625	Rabbit #5626	Rabbit #5625	Rabbit #5626
1	1:1,000	0.092	0.095	2.929	2.810
2	1:2,000	0.084	0.096	2.826	2.760
3	1:4,000	0.078	0.074	2.800	2.748
4	1:8,000	0.073	0.068	2.764	2.638
5	1:16,000	0.080	0.073	2.654	2.557
6	1:32,000	0.081	0.071	2.344	2.253
7	1:64,000	0.078	0.088	1.857	1.881
8	1:128,000	0.088	0.073	1.412	1.461
9	1:256,000	0.095	0.077	0.985	1.060
10	1:512,000	0.091	0.082	0.642	0.682
11	Blank	0.090	0.090	0.090	0.090
12	Blank	0.090	0.090	0.090	0.090
	Titer	<1:1,000	<1:1,000	>1:512,000	>1:512,000

Table 4.6.8: Anti GQ9 antibody titers

	Dilution	Pre-immune serum (A450nm)		Purified antibody (A450nm)	
		Anti-GQ9b			
		Rabbit #5627	Rabbit #5628	Rabbit #5627	Rabbit #5628
1	1:1,000	0.066	0.064	3.001	3.019
2	1:2,000	0.068	0.067	2.963	2.927
3	1:4,000	0.062	0.055	2.611	2.850
4	1:8,000	0.061	0.056	2.483	2.739
5	1:16,000	0.061	0.057	2.253	2.578
6	1:32,000	0.056	0.053	1.712	2.383
7	1:64,000	0.062	0.064	1.219	2.320
8	1:128,000	0.057	0.066	0.814	1.938
9	1:256,000	0.061	0.056	0.458	1.380
10	1:512,000	0.068	0.058	0.284	0.939
11	Blank	0.067	0.067	0.067	0.067
12	Blank	0.067	0.067	0.067	0.067
	Titer	<1:1,000	<1:1,000	1:512,000	>1:512,000

4.7 Neutralisation assay

The ability of the peptides to neutralize pseudovirus (ZM53) was assessed. The host-derived peptides exhibited no ZM53 inhibition or neutralization. The positive control (a known neutralizating compound- IBU21) was able to show dose-dependant pseudovirus neutralization. What appears to be neutralization by F7E at 62.84 ug/ml was not found to be reproducible.

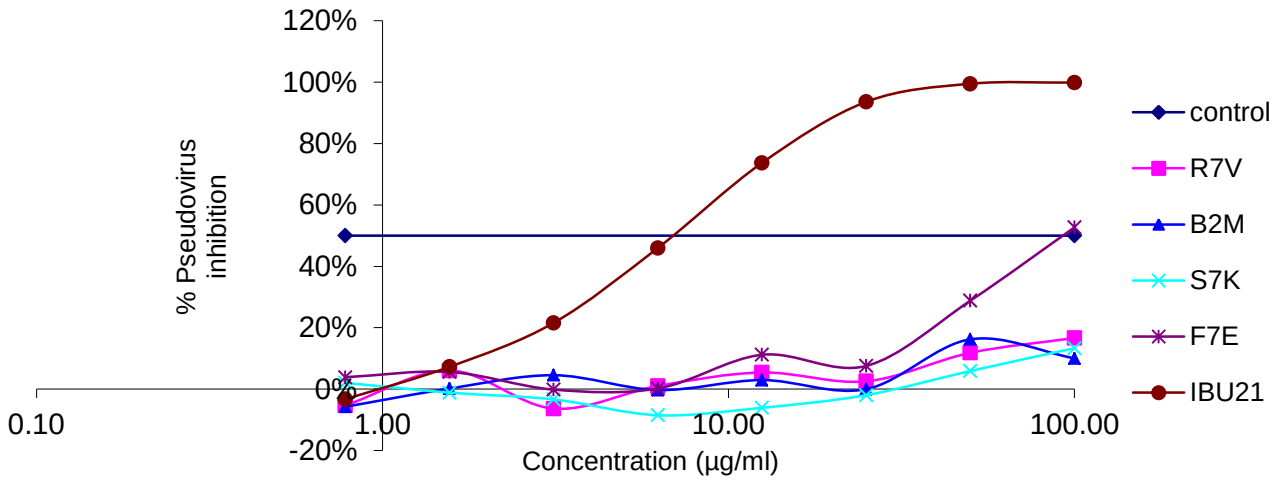
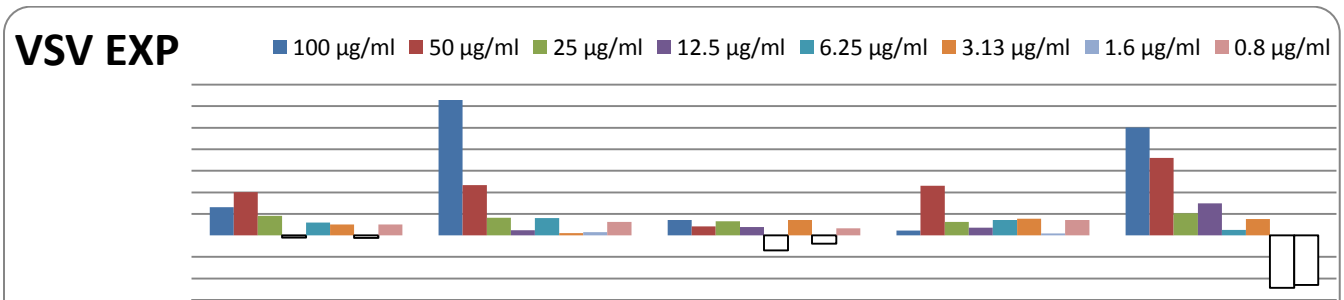


Figure 4.7.1: Dose response curvesobtained during testing to ascertain if host-derived peptides and a positive control were able to neutralize pseudovirus ZM53 *in vitro*. The line at 50% represents the IC50; titres below this line show no virus neutralization. The positive control (IBU21) was able to neutralise the virus at various concentrations, whereas no neutralization was exhibited by the host-derived peptides.



	R7V	F7E	B2M	SK7	IBU
100 µg/ml	13.11921477	62.84	7.16	2.31	50.04
50 µg/ml	20.06066784	23.42	4.14	22.99	35.94
25 µg/ml	9.06811568	8.24	6.54	6.26	10.30
12.5 µg/ml	-0.977426303	2.33	3.95	3.54	14.86
6.25 µg/ml	5.917991549	8.00	-6.90	7.12	2.59
3.13 µg/ml	5.063051806	1.03	7.21	7.76	7.60
1.6 µg/ml	-1.207602387	1.43	-3.82	0.95	-24.37
0.8 µg/ml	5.115663483	6.33	3.33	7.11	-23.00

Figure 4.7.2: The positive control IBU demonstrates neutralization. What appears to be neutralization by F7E at 62.84 ug/ml was not found to be reproducible.

The figures below show percent viability as a function of virus neutralization capability of each treatment with a known drug (Nevirapine) compared to the HIV viral peptides. This provides an indirect indication of virus neutralization of each treatment (figure 4.7.3 and 4.7.4). The ability of polyclonal antibodies elicited against the viral peptides to neutralize ZM53 pseudovirus was tested at concentrations of 5, 10 and 50 µg/ml. When virus was present cells were not viable, in the presence of a virus neutralizing molecule, cell viability was retained depending on the efficacy of the neutralizer.

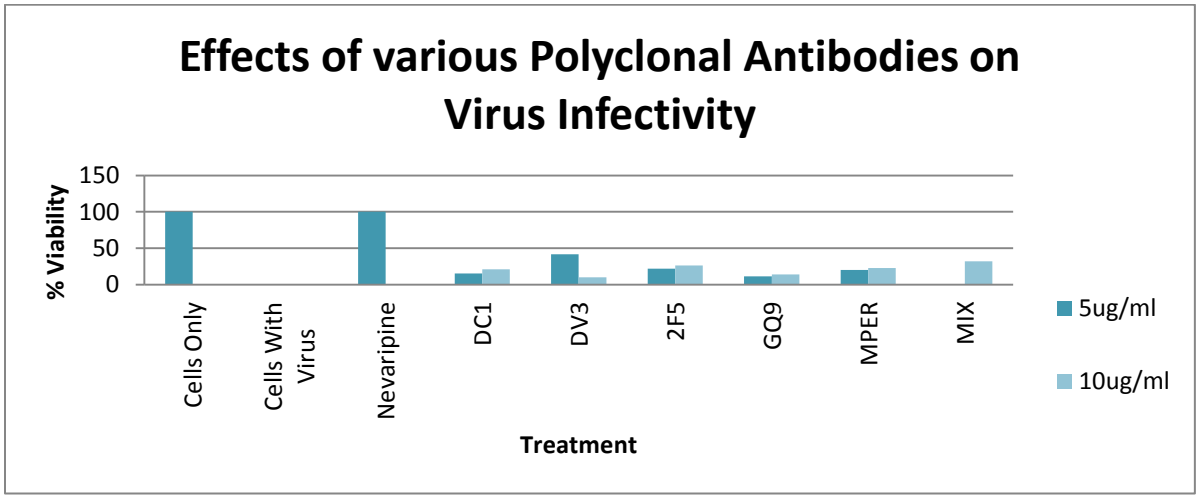


Figure 4.7.3: The viability is shown as an indirect indicator of virus neutralization, preliminary data n=2. The ability of polyclonal antibodies elicited against the viral peptide to neutralize ZM53 pseudovirus is shown here. Peptides tested against ZM53 pseudovirus at concentrations of 5, 10. When virus is present cells are not viable, in the presence of a virus neutralizing molecule, cell viability is retained depending on the efficacy of the neutralizer.

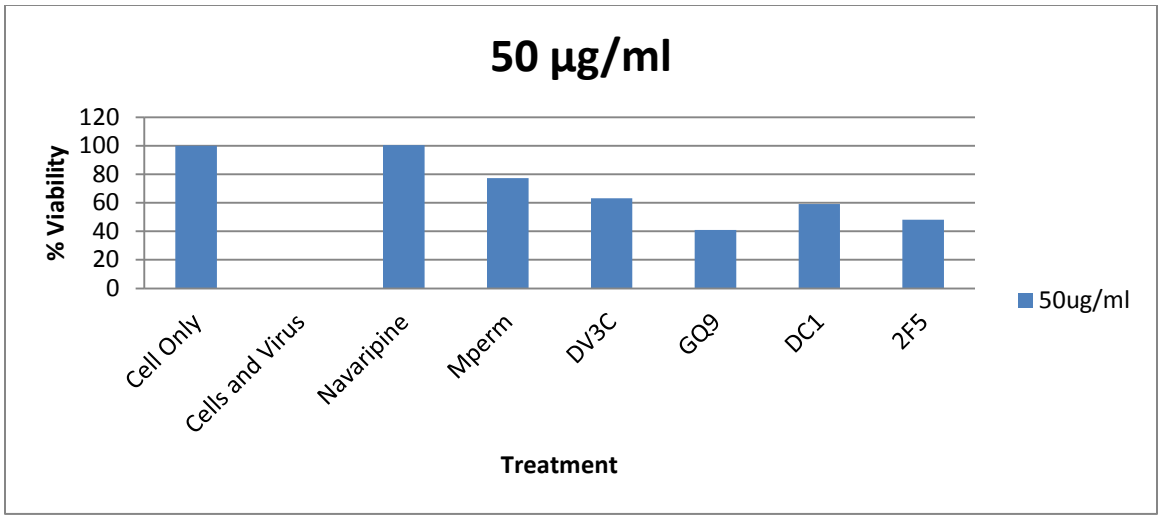


Figure 4.7.4: neutralisation assay n=2. The ability of polyclonal antibodies elicited against the viral peptide to neutralize ZM53 pseudovirus is shown here. Antibodies tested against ZM53 pseudovirus at a concentration of 50 µg/ml.

Antibodies at 50 µg/ml were able to neutralize virus induced antibodies and antibodies produced during natural infection, to neutralize pseudovirus (ZM53) was assessed.

CHAPTER 5 DISCUSSION

According to the constructed helical wheels, some peptides are visibly hydrophobic on one side (e.g DV3, DC1) while others are populated with hydrophilic residues. Hydrophobic regions in peptides are associated with buried residues which in the context of antigenicity are thought to be hidden from the immune system and therefore not expected to be antigenic. Peptides containing hydrophilic amino acids are expected to be exposed on the surface of the native protein sequence and are more likely to be antigenic (Hopp and Woods 1982, Kyte and Doolittle, 1982). The amino acid composition determines the secondary structure of a peptide as well as its chemical properties. Antigens and antibodies bind to each other as a consequence of the specific attraction between their epitopes and paratopic sites. Hydrophobic and electrostatic interactions play an important role in primary epitope-paratope interactions (Van Oss 1994). Epitopes located on prominent bends of an antigenic molecule are the most favoured to make contact with corresponding paratopes. Hydropathy plots and helical wheels data suggested the peptides to not be wholy antigenic but also not completely without potential as antigens.

Construction of synthetic peptides for use as vaccines to induce either humoral or cell-mediated immune responses requires an understanding of the nature of T-cell and B-cell epitopes. Ideally, vaccines for inducing humoral immunity should include peptides that form immunodominant B-cell epitopes. Such epitopes can be identified or designed by determining the dominant antibody in the sera of individuals who are recovering from disease and then testing various synthetic peptides for their ability to react with that antibody with high affinity. A successful vaccine must also generate a population of memory T helper cells; therefore the peptide should include immunodominant T-cell epitopes. All HIV diagnostic tests are based upon detection of one or more of the molecules that make up a virus particle or detection of the antibodies that human hosts make against HIV particles. Peptides (such as HIV Gag p24 peptides) are currently being used for HIV diagnosis; therefore the use and further development of peptides for prognostic/diagnostic markers, is conceivable.

5.1 Antibody detection

5.1.1 Host-derived peptides

Previous studies have made use of techniques such as baculovirus technology (Haslin *et al*., 2007) to produce recombinant anti-R7V antibodies. For this work, Dr Mervyn Beukes developed recombinant scFv fragments using phage display technology, these recombinant antibody fragments were able to bind to B2M epitopes and served as positive controls for the in-house ELISA assay. Work on host-derived peptides has focused on R7V as the immunodominant epitope. We investigated a total of three overlapping heptamers as well as a partial B2M peptide (designated as B2Mp) the antibody prevalence of which was originally thought to be associated with long term non-progressor status (Galea *et al.,* 1996).

Duration of HIV-infection has been reported (Haslin and Chermann 2007 b; Ravanini *et al.*, 2007; Sanchez *et al*., 2008), as influencing the R7V antibody presence. Galea *et al.* (1999) proposed that the presence of R7V antibodies were prognostic and indicative of slower progression of HIV-1 infection. A prospective study conducted by Margolick *et al.* (2010) revealed that individuals with R7V antibodies early in infection presented with high viral load and significantly lower CD4 T cells than those who were R7V antibody negative. Contrary to previous studies, Margolick *et al*. 2010 showed no association was found between the presence of antibodies against the R7V epitope and slower disease progression. Furthermore, the study conducted on R7V in our group (Bremnaes, MSc, 2010) showed no difference in the presence of R7V antibodies between individuals on HAART compared to treatment-naïve individuals or progressors, when compared to non-progressors. The current work makes a comparison between the influence of virus-derived and host-acquired HIV viral envelope epitopes when used as peptides to assess antibodies as indicators of infection.

This work revealed the presence of host-derived antibodies to be greatest in sera obtained from newly diagnosed individuals. This makes sense because in recent or new infections or when treatment is not yet taking effect, virus continues to be made and the body is continuously exposed to viral antigens leading to antibody production. As treatment takes hold, viral production is lowered and less or no antibodies are being made.

5.1.2 Virus-derived peptides

DC1 was the only viral peptide for which no antibodies were detected in the sera of subtype C infected individuals. This is probably due to the fact that this antigen was derived from regions of subtype A and B (Luchese *et al.*, 2011). Although only trace amounts of antibody were detected for DC1, the other virus derived envelope peptides were very antigenic (as expected from previous work) and consistently showed tendencies of distinguishing infected from uninfected controls as in a diagnostic response while the consistent lack of antibodies to the host-derived peptides confirmed the fact that most of our samples were not recently infected. With MPER being a close second, the region that generated the highest antibody response in rabbits was based on the peptide mimicking an epitope on the V3 loop of env of HIV subtype C (DV3). Responses against GQ9 peptide were observed in mainly recently infected individuals, suggesting (as described by Sengupta *et al.*, 2011) that HIV infection may have a role in promoting gene expression of human endogenous retroviral sequences. Sengupta *et al.* also demonstrated that during HIV infection not only are HERV-K transcripts present in the plasma of infected individuals, but that patients who were recently infected also exhibited CD8+ T cell responses that were absent in HIV negative donors.

5.2 Viability and Proliferation

Once an immune reaction is triggered in response to a pathogen, PBMCs undergo proliferation as part of the immune system response. The addition of the peptides to PBMCs is done to test for proliferation of cells *in vitro* due to a memory response being triggered. According to the viability dye data, the cells were viable indicating that the peptides were not toxic but the proliferation of HIV infected PBMCs was limited which was possibly due to the XTT assay being the lowest sensitivity assay for detecting proliferation. Proliferation is expected if memory cells in infected individuals recognize the epitopes from previous exposure. Table 2 showed XTT proliferation data of patient PBMCs following seven days of incubation with 10 µg/ml of each peptide. This data was validated and confirmed using the CFSE assay which is a more sensitive assay utilizing flow cytometry detection.

CFSE data showed that a memory response was triggered by some peptides but that the conserved DC1 peptides was not well remembered which is expected because structures on the viral envelope glycoproteins that are conserved among diverse viral strains are, in general, poorly exposed to the humoral immunes system (Wyatt and Sodroski, 1998).

5.3 Cytokine secretion

HIV is reported to alter cytokine production during immune disruption in order to increase virus production. Cytokine analysis was performed using multiplexing technology for the analysis of the human Th1/Th2/Th17 cytokinome through flow cytometric detection.

Secreted cytokine levels were measured following the isolation and stimulation of PBMCs obtained from infected individuals on HAART as well as HIV negative donors. The Human Th1/Th2/Th17 CBA kit (BD Biosciences, San Jose, California) allows for the simultaneous detection of IL-2, IL-4, IL-6, IL-10, TNF-a, IFN-ϒ and IL-17A. Secreted cytokine levels in HIV positive PBMCs were treated with 10 µg/ml peptide and incubated for 7 days after which the supernatant was used for cytokine quantification on the FACS Array. Patients were grouped according to CD4 count (CD4>350 cells/ml and CD4<200cells/ml) as well as disease progression or clinical stage of infection. Some cytokines showed no increase following peptide treatment (IL-4 and IL-2). It has been reported that chronic progressors lose IL-2 expression. Throughout, IL-6 was the cytokine that was secreted significantly following stimulation. IL-6 was already classified as a discriminatory cytokine in previous work from this group (Williams *et al.*, 2012). According to Williams *et al.*, IL-6, IL-10 and IL-17 were the interesting cytokines between infected and uninfected sera. The observations made were that IL-6 and IL-10 concentrations were significantly ($p = 0.001$and $p = 0.025$) different between the HIV positive and HIV negative groups. IL-17A levels that were reported to be higher than other cytokines were similar in levels between HIV negative and HIV positive individuals in this study.

The current study generally detected an increase in IL-6 production in cells compared to the absence of peptide, higher levels were secreted in cases where longer peptides (15 amino acids) were used as the stimulus. IL-6 is a pleiotropic cytokine that is produced against chronic intracellular infections as it is involved in immune restoration (Romagnani, 2000). This finding

supports the use of peptides as a vaccine component to counteract immune exhaustion by boosting IL-6 production in HIV positive individuals.

Th1 cells are reported to produce interferon (IFN), IL-2 and TNF, these cells function by inducing cell-mediated immunity and phagocyte-dependent inflammation. Th2 cells, which produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, evoke strong antibody responses (including those of the IgE class) and eosinophil accumulation (Romagnani, 2000). The response of PBMCs to treatment with the peptides show influences on Th2 cytokine production as opposed to Th1. Increases in Th2 cytokine production are associated with HIV infection, the cytokines in the Th2 response push humoral responses which are thought to provide protection against extracellular pathogens.

5.4 Neutralization of pseudovirus

Antibodies generated against host-derived peptides, including the positive control antibody fragment were unable to neutralize pseudovirus. The viral peptides were able to produce antibodies that showed potential to neutralise ZM53 pseudovirus at high concentrations. This was not surprising because these peptides (DV3, MPER) were made to mimic a portion of the envelope that is known to be reactive and is based on the principal neutralising domain. These antibodies show promise for consideration in a vaccine.

5.5 CONCLUDING REMARKS

This study looked at whether detection of the humoral immune response can be improved upon by using host and viral peptides with the ultimate aim of providing new data to improve upon the disadvantages and short comings of current diagnostic/prognostic tools. Synthetic peptides based on HIV envelope proteins (gp120/gp160 or incorporated B2M) stimulated the production of selected cytokines and detected specific humoral immune responses.

ANSWERS TO RESEARCH QUESTIONS

In this study it was hypothesized that synthetic peptides based on epitopes of HIV envelope proteins (host-derived & viral antigens) could be used to characterize the immune response during HIV infection. The research questions that were formulated to verify this hypothesis and the answers developed through data collection are presented below.

Were the peptides antigenic? Peptides were observed to be antigenic based on the properties obtained through computational analysis (Table 4.1). The peptides were *in vitro* stimulants of proliferation (flow cytometry data) and detected antibodies in the sera of HIV infected patients.

Were these peptides able to stimulate the proliferation of PBMCs and TZM-bl cells *in vitro*? An immunologic memory response was observed following stimulation of PBMCs with peptides; proliferation of lymphocytes in response to peptide stimulation was observed (flow cytometry).

Did the synthetic peptides have an influence on cytokine production of PBMCs?

Treatment with peptides favoured a Th2 cytokine profile in HIV infected individuals, and in HIV negative individuals IL-6 production was influenced.

Were the peptides able to elicit an immune response?

Following immunization of New Zealand white rabbits with peptides, strong antibody titers were detected. This work focused on the humoral immune response i.e. the presence of antibodies following immunization with the peptides.

In the event that peptides were immunogenic were the antibodies functional? The virus-derived antibodies showed potential in that they were all able to provide 50% neutralization of pseudovirus at 50 µg/ml. the HERV-k peptide as well as the host-derived peptides were not able to do so.

Could the peptides distinguish stages of HIV-1 infection? The detected antibodies were found primarily in a specific stage of disease, recently diagnosed, which is presumed to be earlier in infection therefore these antibodies (and by extension, the epitope) have prognostic potential.

5.6 FUTURE PERSPECTIVES

Among the limitations of this study was the small sample size which limits extrapolation of data and predicting responses in larger populations. The availability of longitudinal samples from the same patient to track progression of disease and follow effects on the immune system would be advantageous in work like this, although it must be highlighted that obtaining blood samples was not easy and the availability of up-to-date and/or any patient information for a given sample from the donor or clinics was not always forthcoming and would be very helpful in data interpretation. In some instances this information was not available, which influenced conclusions drawn. HIV negative patients were confirmed as being negative using rapid tests but other malignancies were not tested for.

Linear peptides were associated with low immunogenicity, a suggestion would be to increase the length of the peptides or testing modified peptides. T these could be modified by the addition of more amino acids or adapter molecules which would increase the antigenicity of the peptides. Missing from this study was a look at how CD8 T cells were affected; this could be done by doing ELISPOT assays for each peptide which would provide information about CD8 T cells through the detection of INFϒ. This assay would have allowed us to ascertain if peptide stimulation could contribute to T cell immunity which could contribute to peptide efficacy. This kind of project is labour intensive and requires a lot of hours in the laboratory, therefore adequate time management was essential, I would also suggest that writing be done parallel to the laboratory work.

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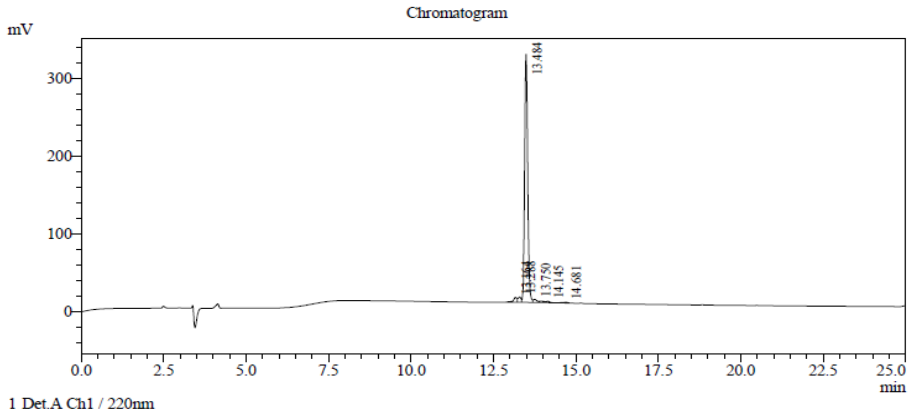
Appendix

This section provides all the supporting material to the findings of this dissertation in the form of graphs/figures/tables. Some of this material is referred to within the context of the dissertation, while the other material is extra work that was conducted to support this dissertation's findings.

1. Peptide production

The company (Genscript Corporation) provided High Performance Liquid Chromatography (HPLC) and Mass Spectroscopy (MS) data for the synthetic peptides, to confirm purity and molecular weighr. The purity was >80%



Peak Table

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	13.164	47588	6307	2.141
2	13.288	43823	6914	1.972
3	13.484	2058902	318581	92.636
4	13.750	33873	4026	1.524
5	14.145	28686	1790	1.291
6	14.681	9707	874	0.437
Total		2222578	338492	100.000

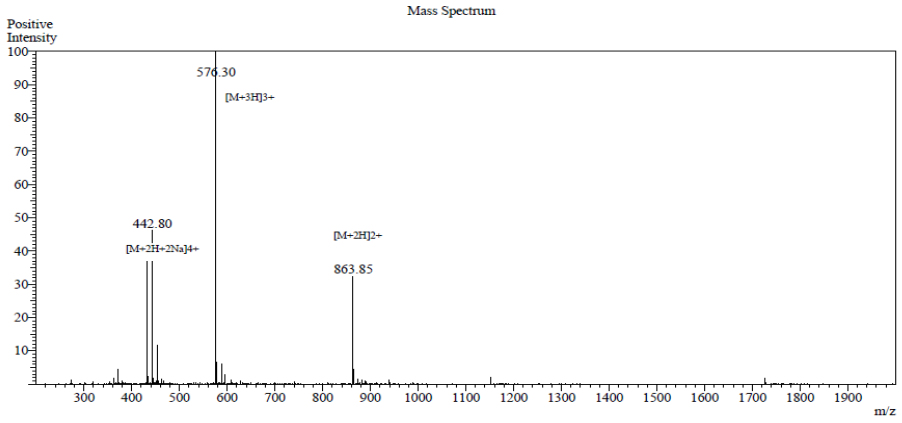
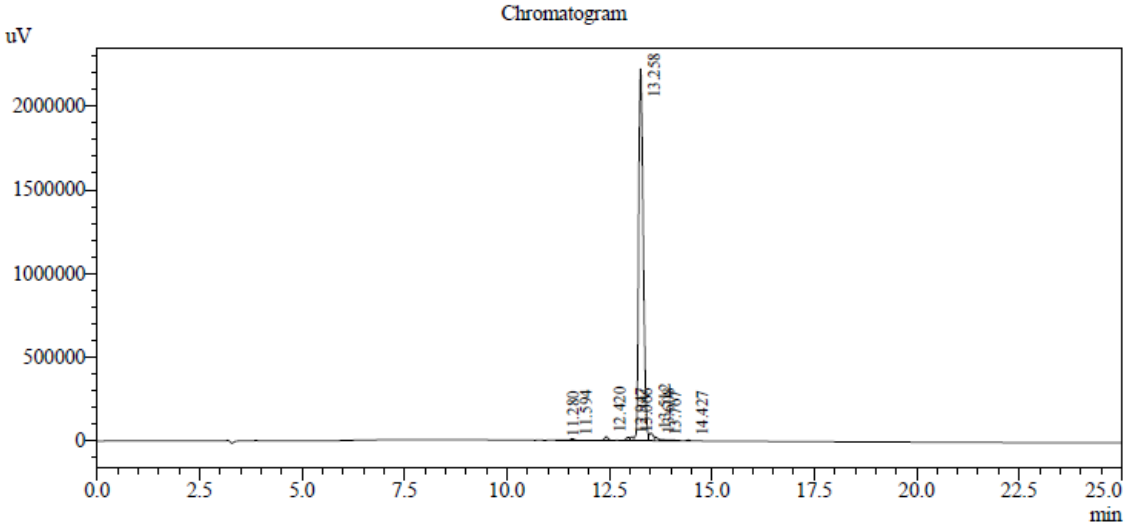


Figure A1: High Performance Liquid Chromatography (HPLC) and/or Mass Spectroscopy (MS) profiles of DV3.



PeakTable

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	11.280	7181	1385	0.039
2	11.594	61666	9475	0.337
3	12.420	129397	22786	0.707
4	12.947	108867	18577	0.595
5	13.066	120133	21620	0.656
6	13.258	17307423	2217807	94.577
7	13.512	301474	45445	1.647
8	13.608	124997	21472	0.683
9	13.767	108291	5985	0.592
10	14.427	30322	5108	0.166
Total		18299751	2369661	100.000

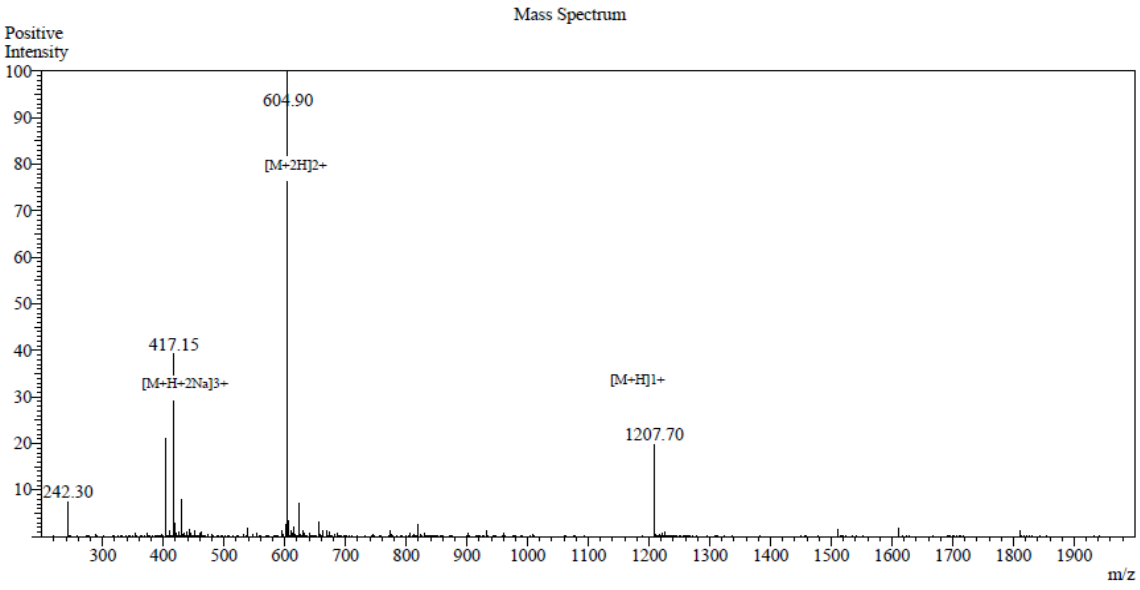
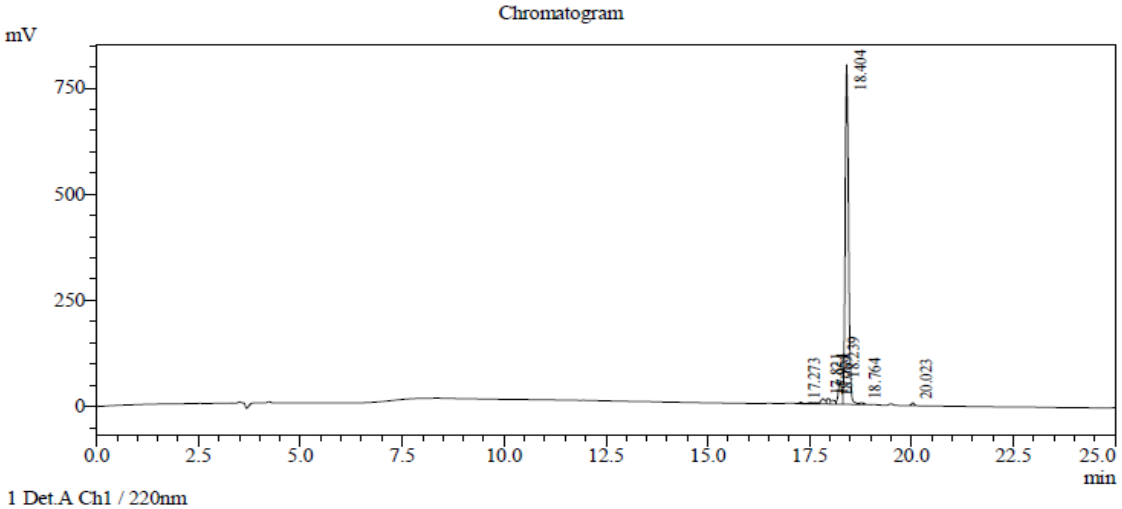


Figure A2: High Performance Liquid Chromatography (HPLC) and/or Mass Spectroscopy (MS) profiles of 2F5.



Peak Table

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	17.273	72469	3560	1.383
2	17.821	92872	12943	1.772
3	17.954	69333	13629	1.323
4	18.069	68182	9188	1.301
5	18.239	327631	53911	6.253
6	18.404	4538874	799477	86.626
7	18.764	36139	3991	0.690
8	20.023	34122	6512	0.651
Total		5239622	903211	100.000

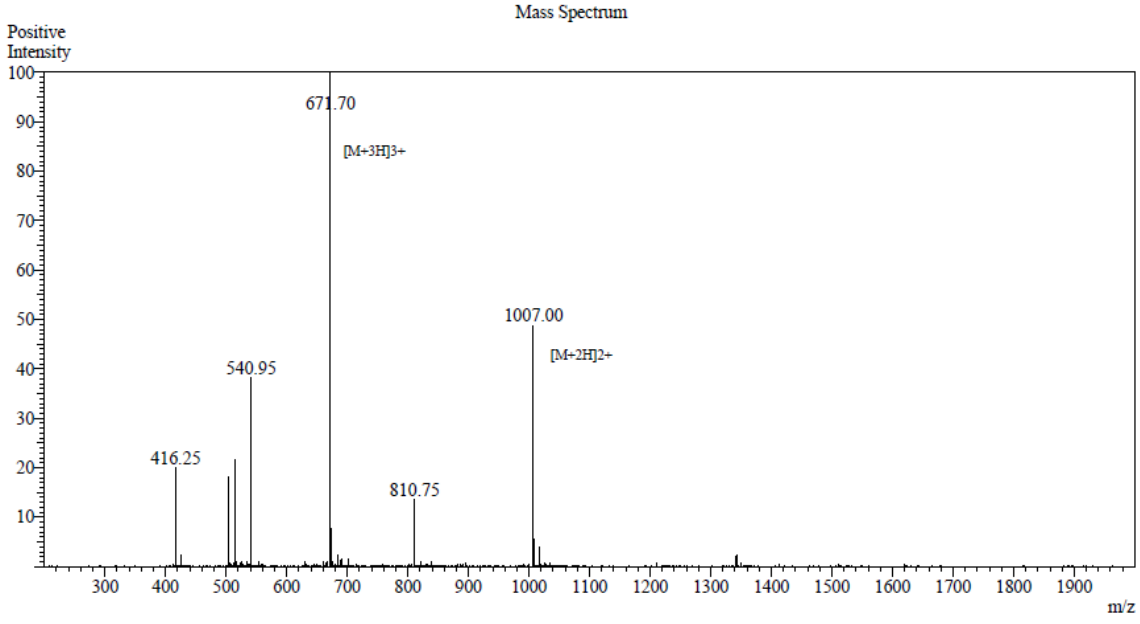
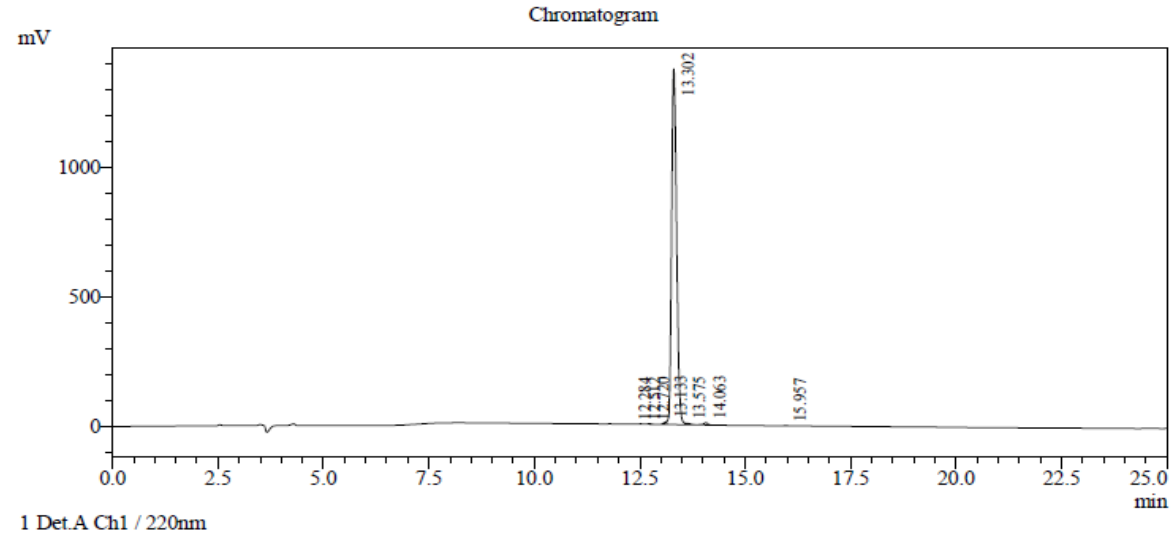


Figure A3: High Performance Liquid Chromatography (HPLC) and/or Mass Spectroscopy (MS) profiles of MPER.



Peak Table

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	12.284	4530	713	0.039
2	12.512	11557	1745	0.101
3	12.720	15379	2518	0.134
4	13.133	46764	11609	0.407
5	13.302	11282139	1373144	98.252
6	13.575	49614	6141	0.432
7	14.063	64899	9079	0.565
8	15.957	7924	1145	0.069
Total		11482806	1406095	100.000

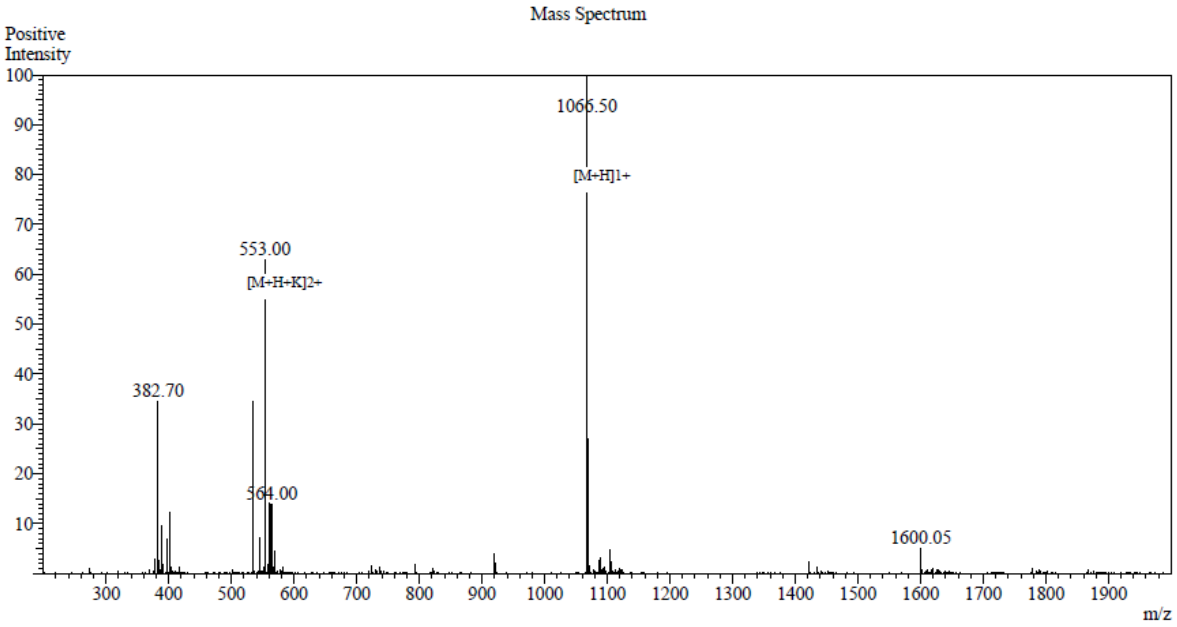
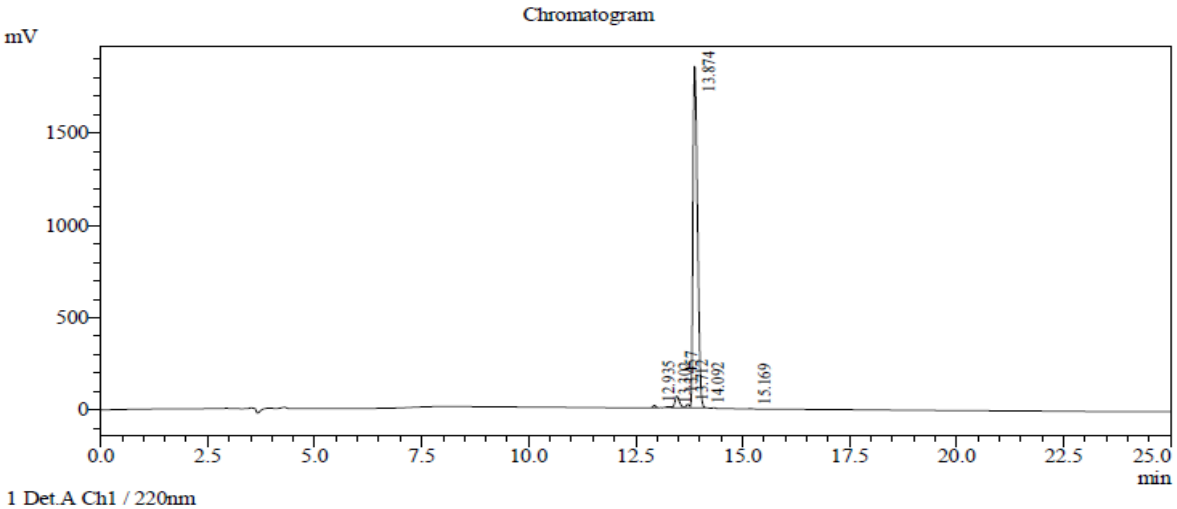


Figure A4: High Performance Liquid Chromatography (HPLC) and/or Mass Spectroscopy (MS) profiles of GQ9.



Peak Table

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	12.935	61323	13100	0.430
2	13.302	42247	5198	0.296
3	13.457	451469	63086	3.163
4	13.712	99002	18844	0.694
5	13.874	13591889	1849146	95.239
6	14.092	10307	6514	0.072
7	15.169	15090	3049	0.106
Total		14271326	1958936	100.000

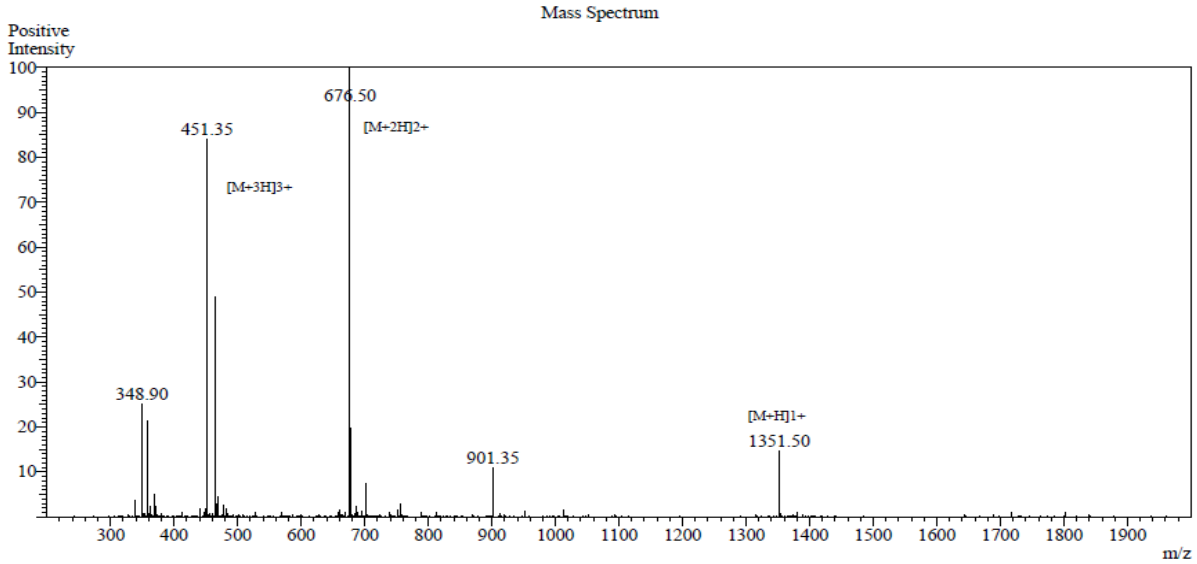
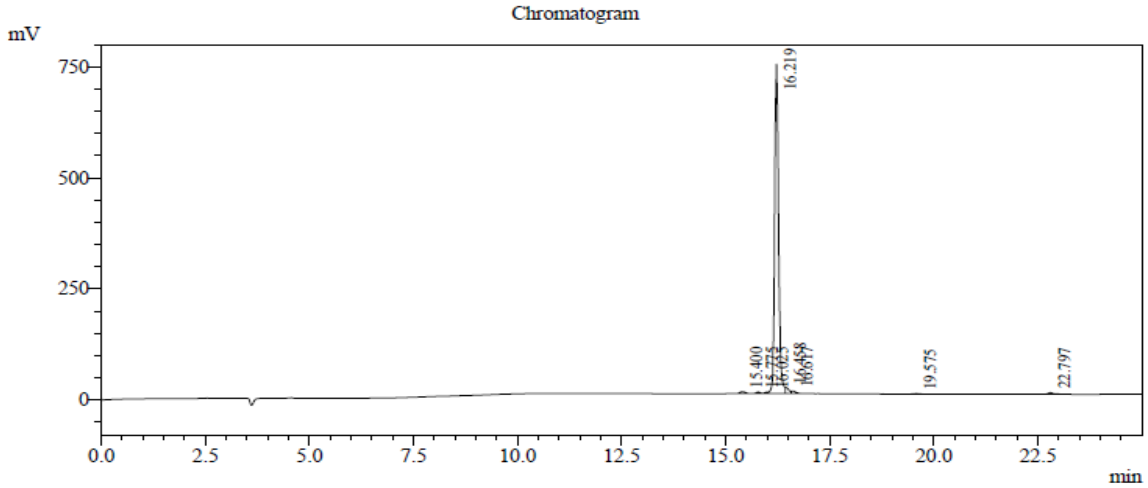


Figure A5: High Performance Liquid Chromatography (HPLC) and/or Mass Spectroscopy (MS) profiles of R7V-3.



Peak Table

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	15.400	37290	4037	0.731
2	15.775	21270	3228	0.417
3	16.025	19646	3392	0.385
4	16.219	4859154	741645	95.263
5	16.458	79705	13558	1.563
6	16.617	42203	5936	0.827
7	19.575	9814	1082	0.192
8	22.797	31708	3892	0.622
Total		5100790	776770	100.000

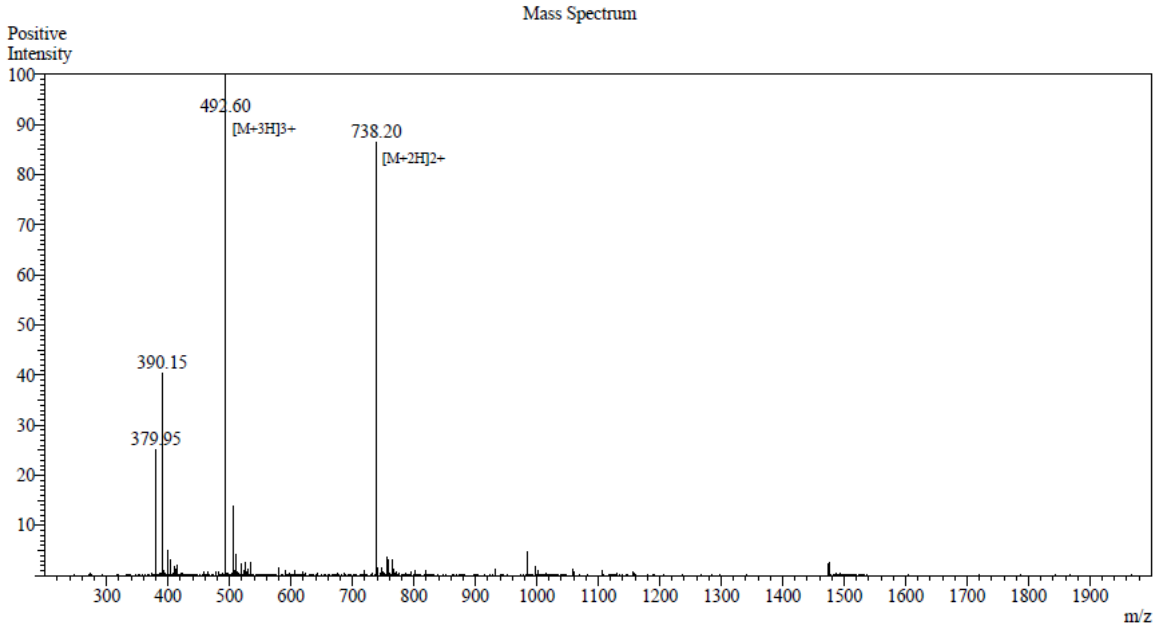
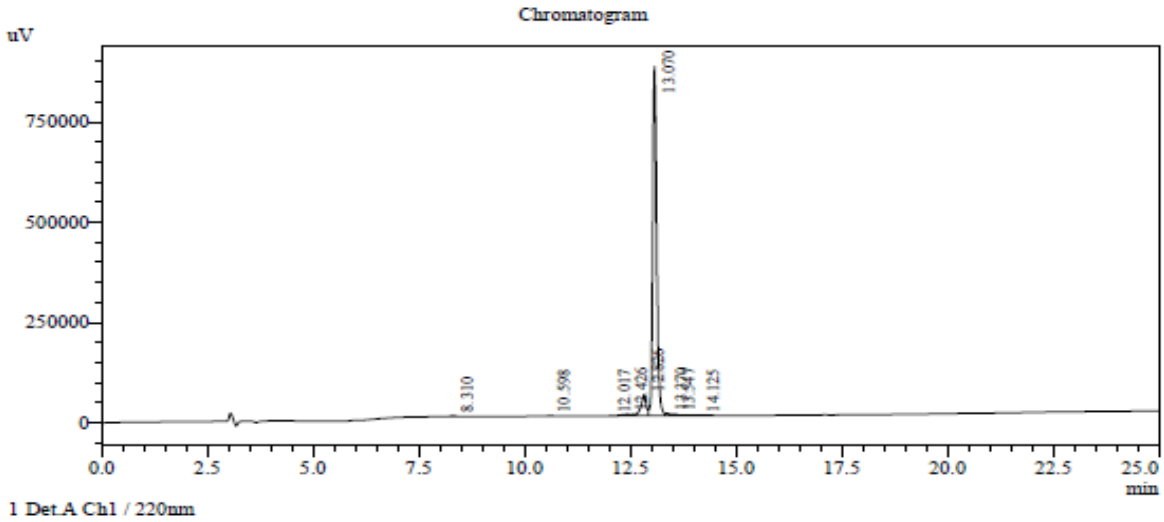


Figure A6: High Performance Liquid Chromatography (HPLC) and/or Mass Spectroscopy (MS) profiles of S7K-4.



Peak Table

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	8.310	22552	2896	0.357
2	10.598	16358	1854	0.259
3	12.017	1139	150	0.018
4	12.426	80170	4609	1.270
5	12.826	442374	52550	7.006
6	13.070	5645665	869369	89.407
7	13.370	45413	5715	0.719
8	13.547	52378	3691	0.829
9	14.125	8542	570	0.135
Total		6314590	941404	100.000

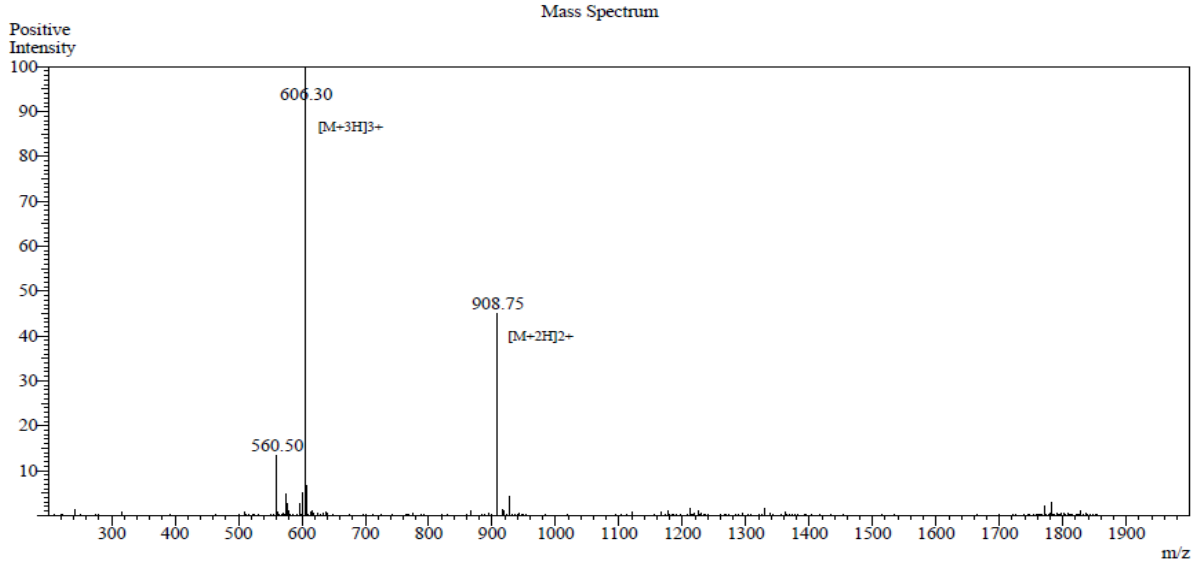
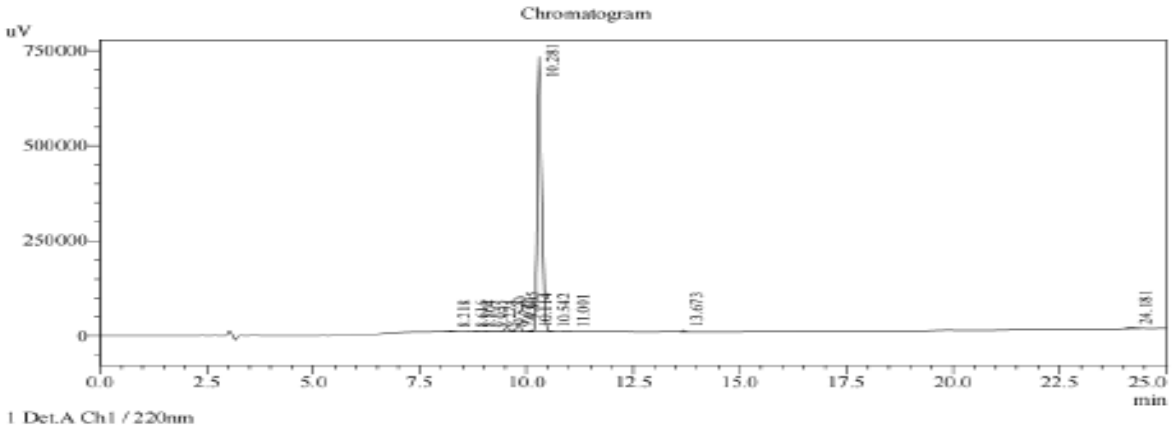


Figure A7: High Performance Liquid Chromatography (HPLC) and/or Mass Spectroscopy (MS) profiles of F7E-8.



Peak Table

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	8.218	11080	1478	0.183
2	8.616	2419	367	0.040
3	8.804	8409	900	0.139
4	9.055	9836	1378	0.162
5	9.274	5229	626	0.086
6	9.520	65124	9792	1.073
7	9.672	16078	3270	0.265
8	9.805	131944	23453	2.174
9	10.114	18415	2806	0.303
10	10.281	5735356	723855	94.512
11	10.542	15065	1626	0.248
12	11.001	3361	485	0.055
13	13.673	19850	3609	0.327
14	24.181	26223	1934	0.432
Total		6068387	775581	100.000

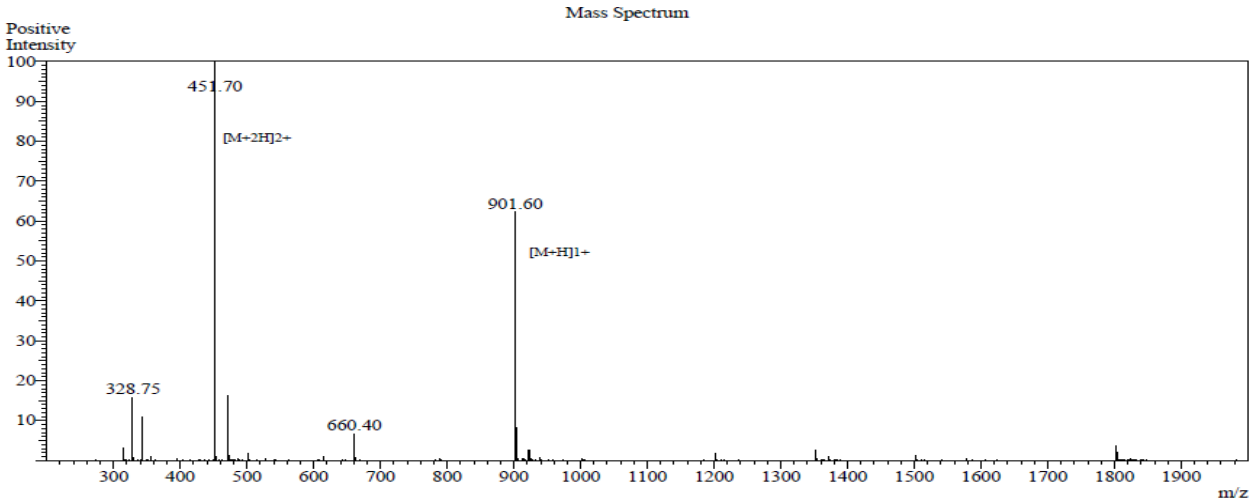


Figure A8: High Performance Liquid Chromatography (HPLC) and/or Mass Spectroscopy (MS) profiles of DC1.

Hydropathy plots and the net charge of all peptides are provided next.

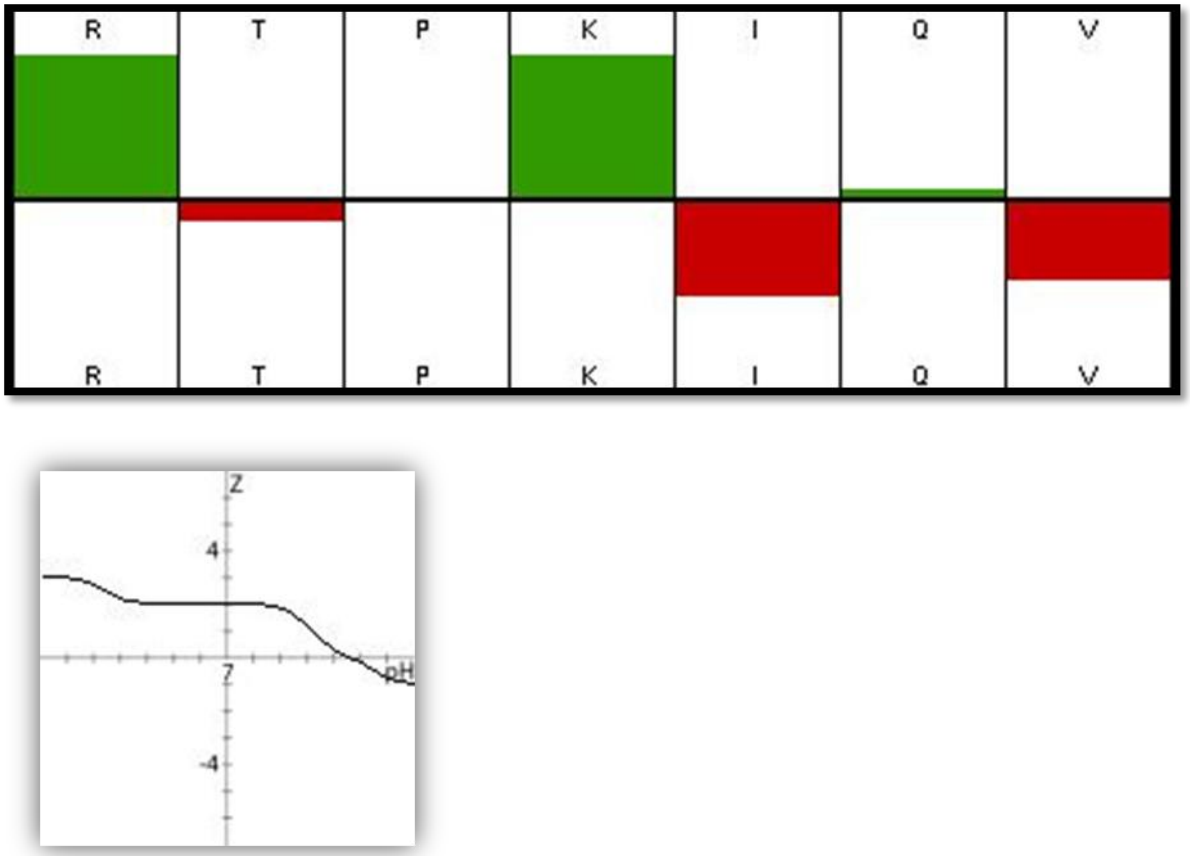
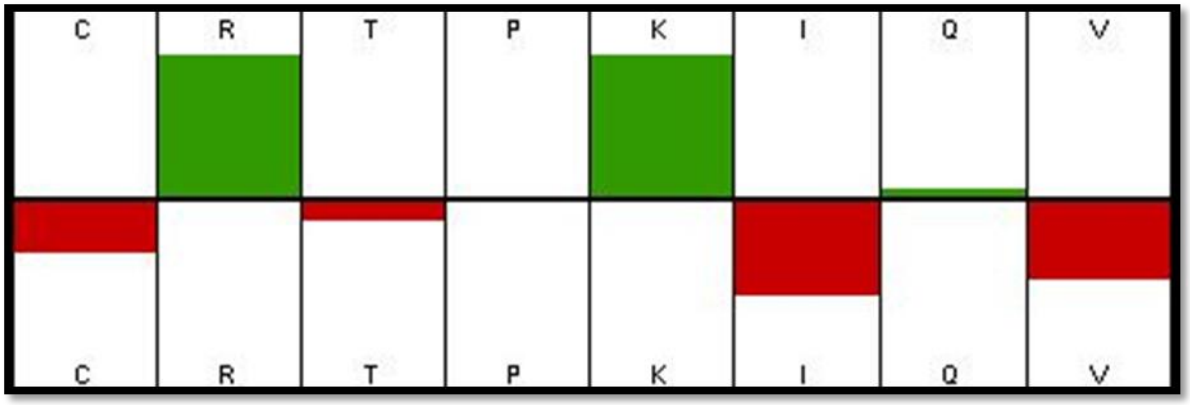


Figure A9: Hydrophobicity plots and Net Charge curve of R7V



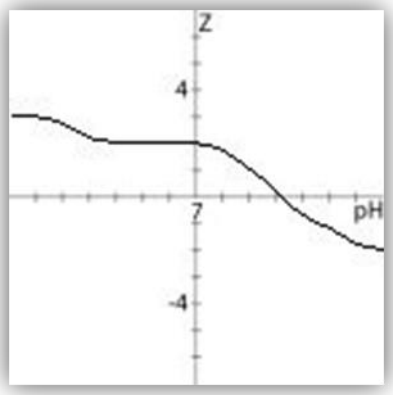
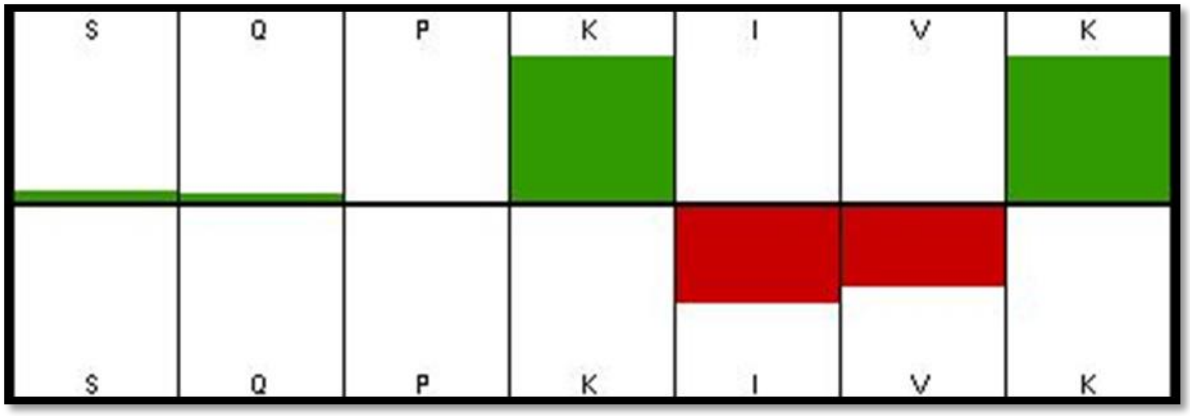


Figure A10: Hydrophobicity plots and Net Charge curve of CR7V



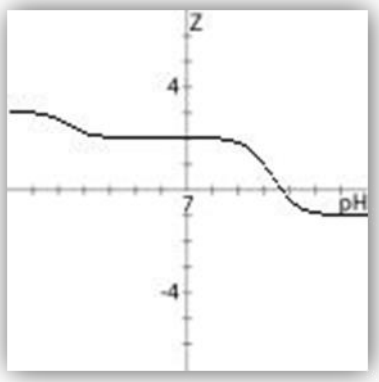
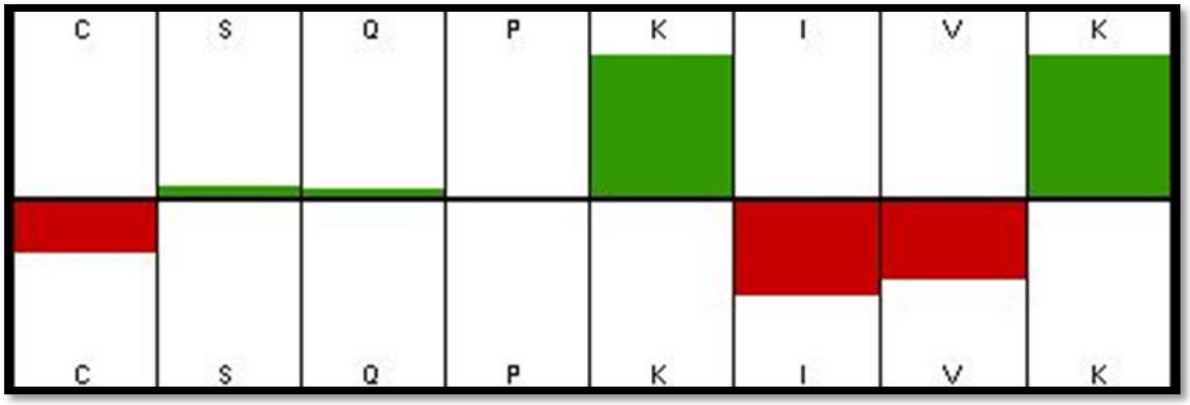


Figure A11: Hydrophobicity plots and Net Charge curve of S7K



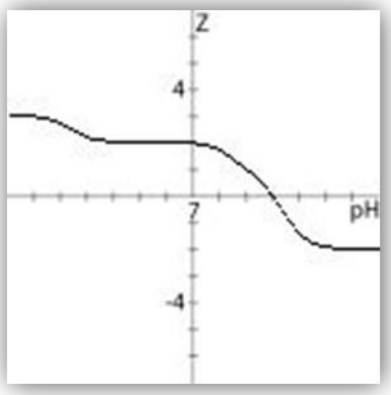
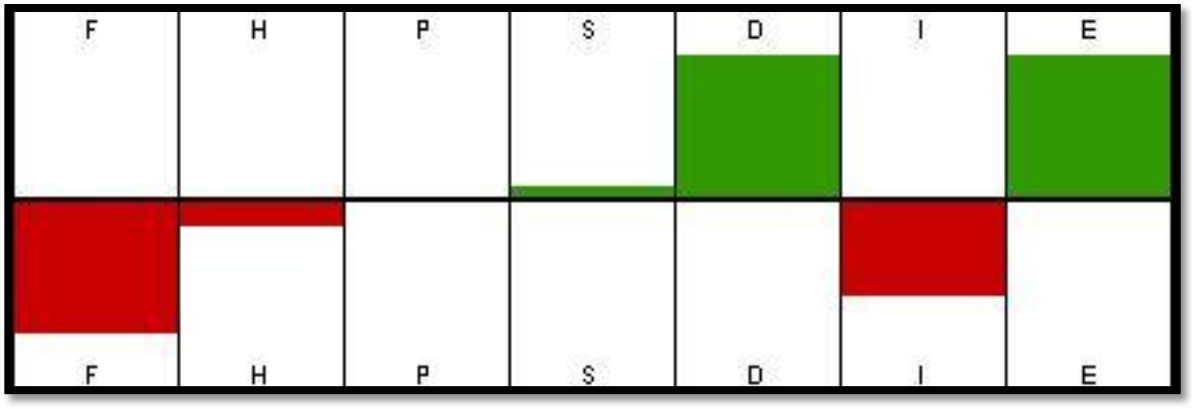


Figure A12: Hydrophobicity plots and Net Charge curve of Cys-S7K



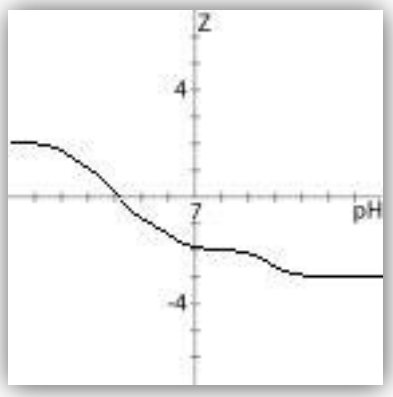
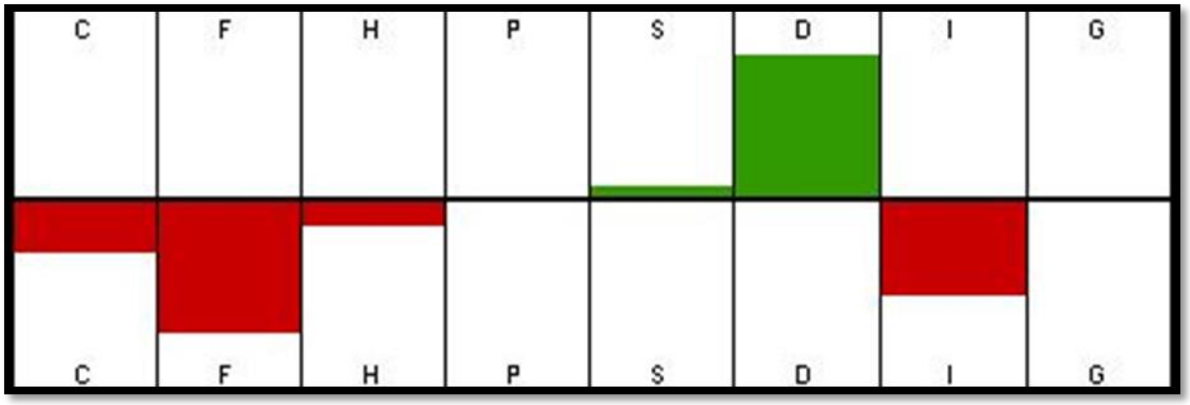


Figure A13: Hydrophobicity plots and Net Charge curve of F7E



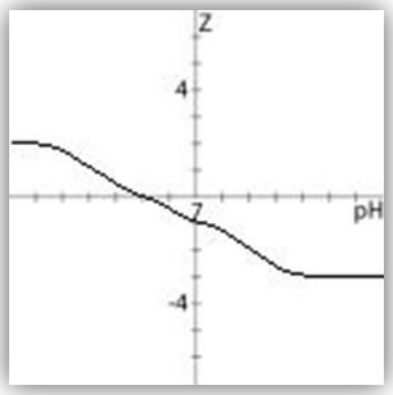
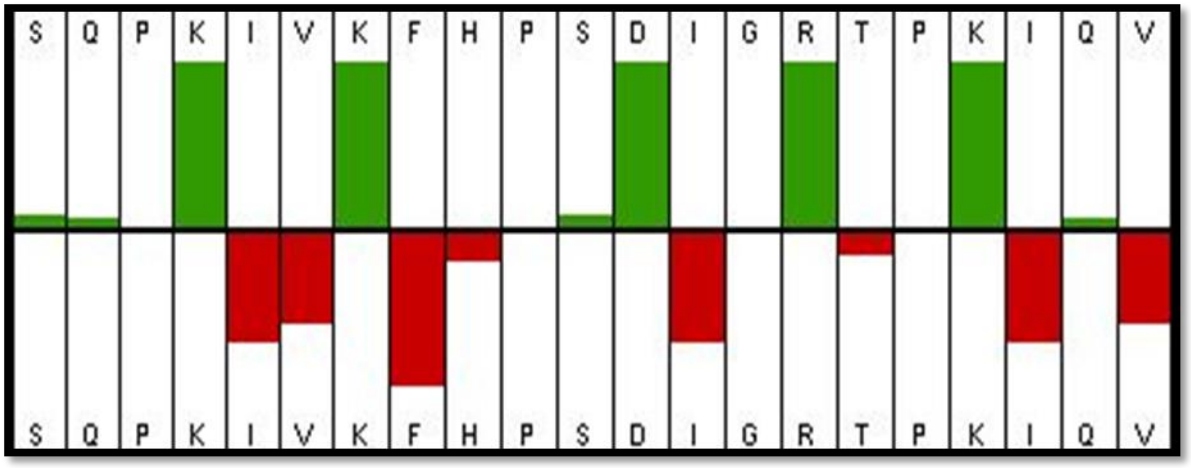


Figure A14: Hydrophobicity plots and Net Charge curve of Csy-F7E



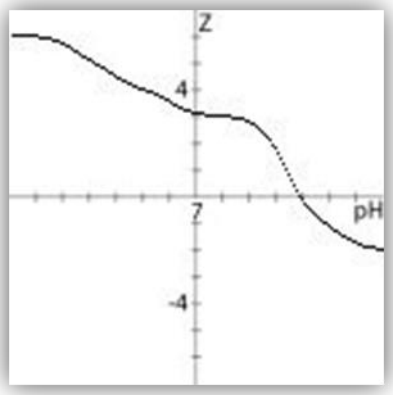
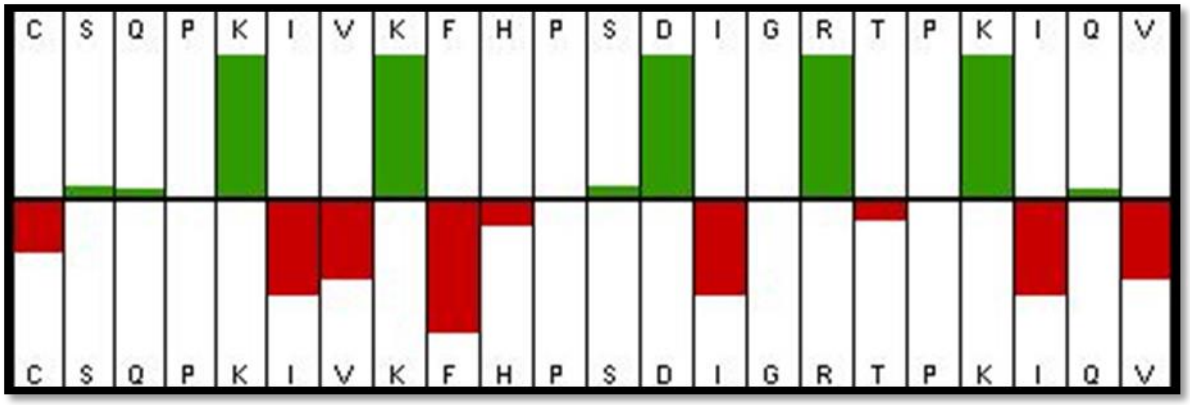


Figure A15: Hydrophobicity plots and Net Charge curve of β-2M



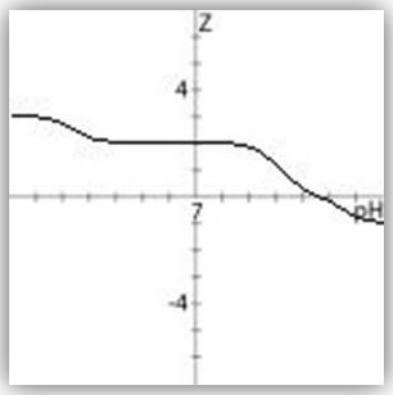
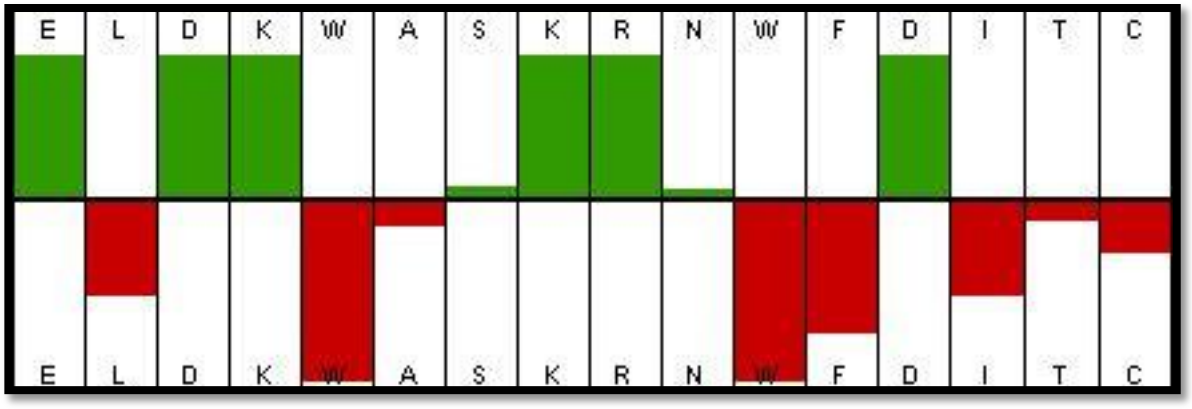


Figure A16: Hydrophobicity plots and Net Charge curve of Cys- β-2M



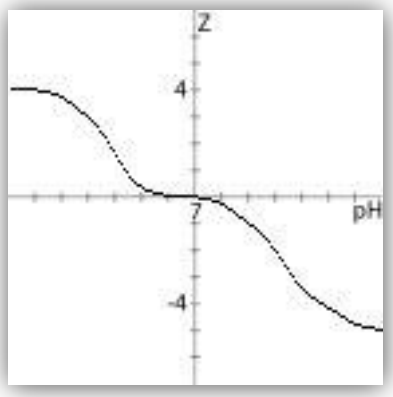
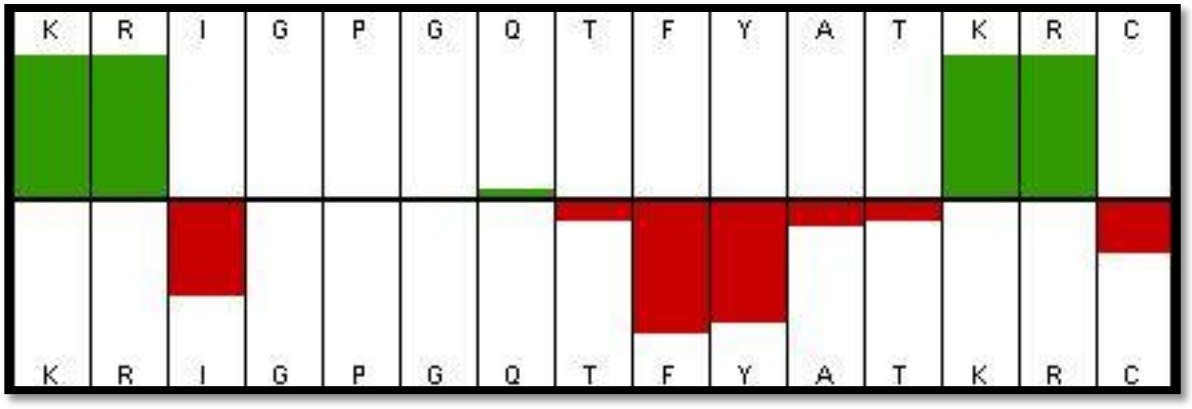


Figure A17: Hydrophobicity plots and Net Charge curve of MPER



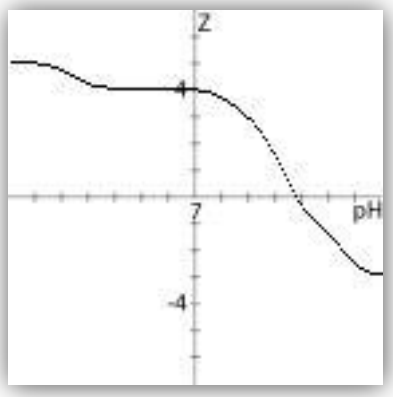
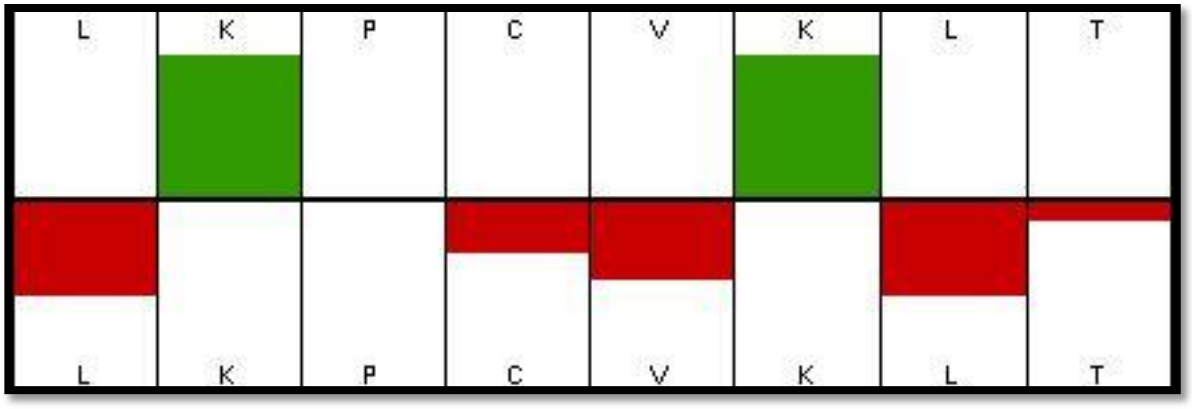


Figure A18: Hydrophobicity plots and Net Charge curve of DV3



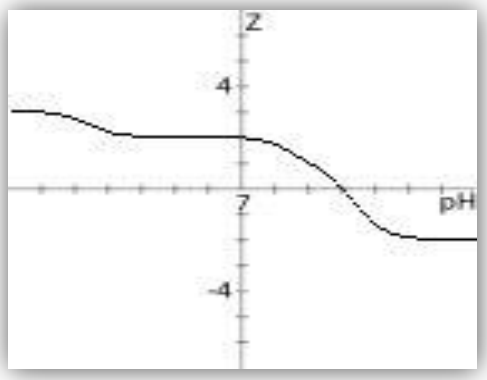
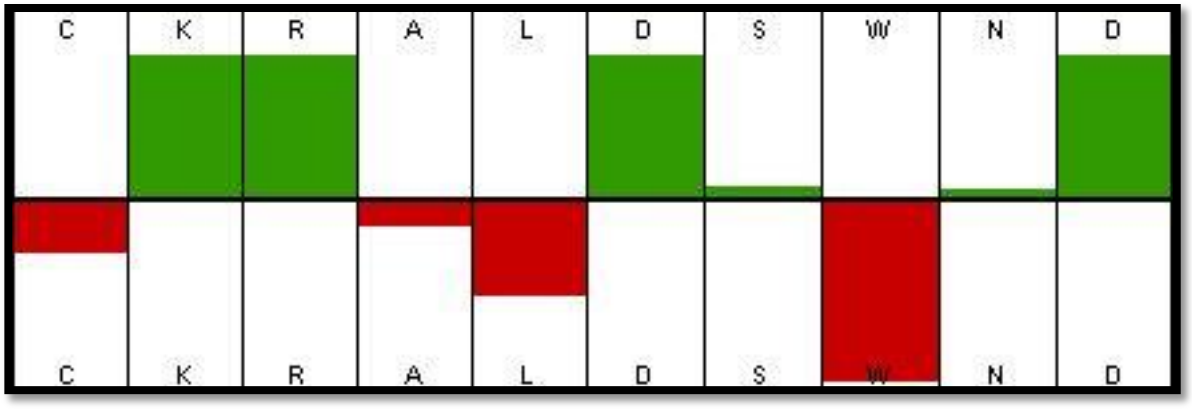


Figure A19: Hydrophobicity plots and Net Charge curve of DC1



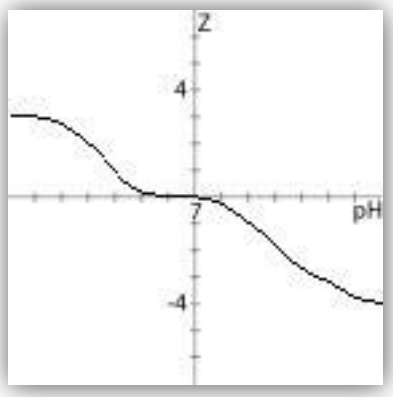
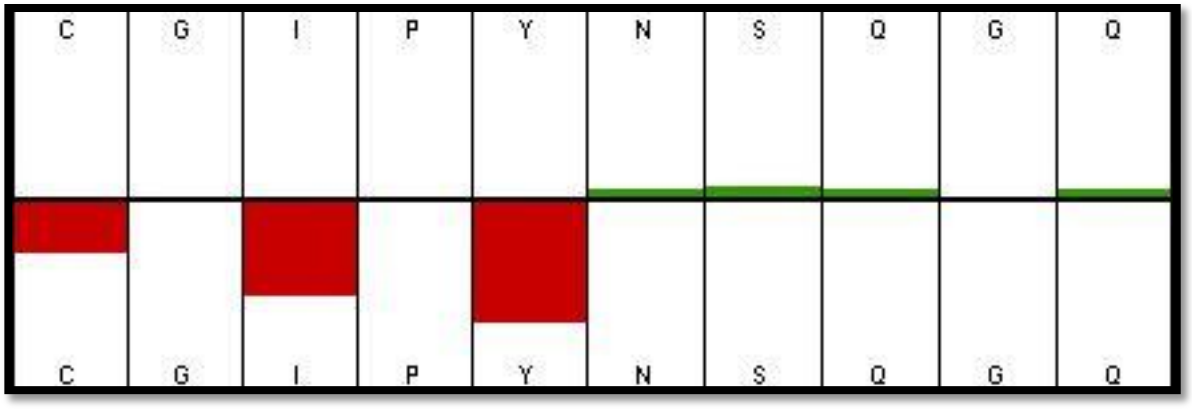


Figure A20: Hydrophobicity plots and Net Charge curve of 2F5



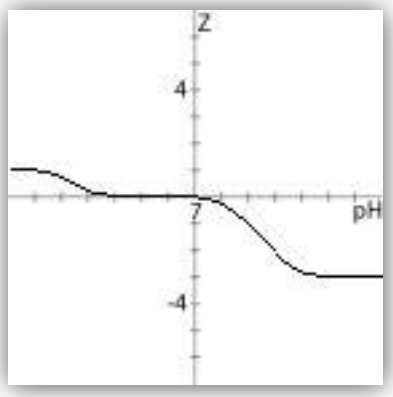
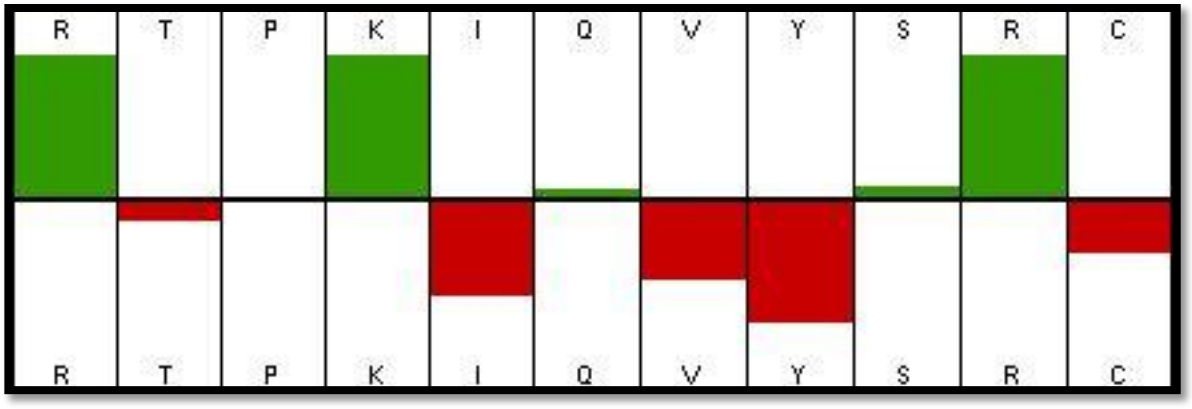


Figure A21: Hydrophobicity plots and Net Charge curve of GQ9



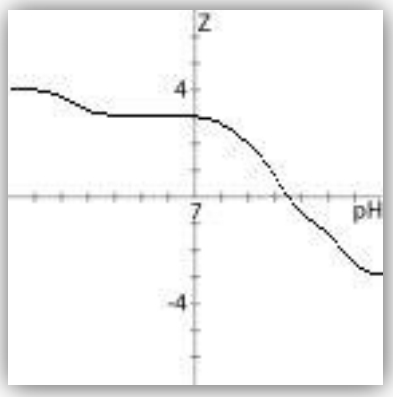
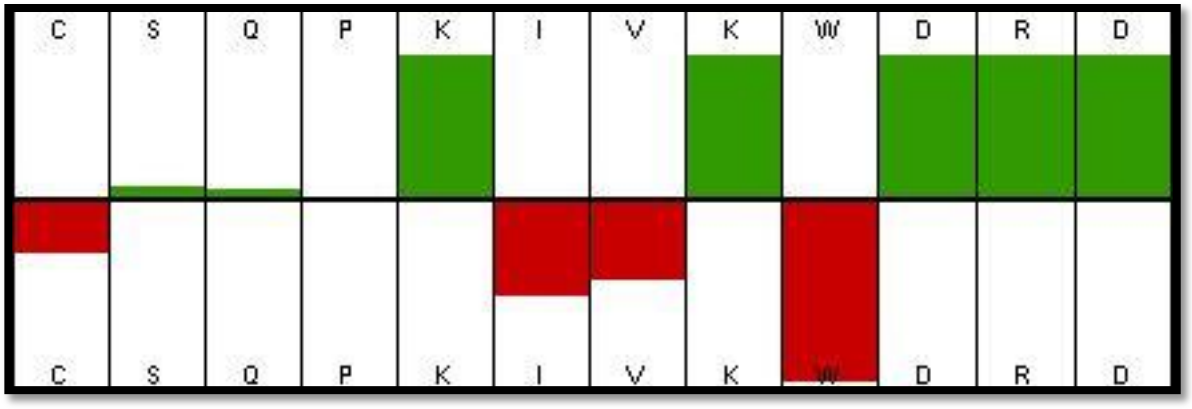


Figure A22: Hydrophobicity plots and Net Charge curve of R7V-3



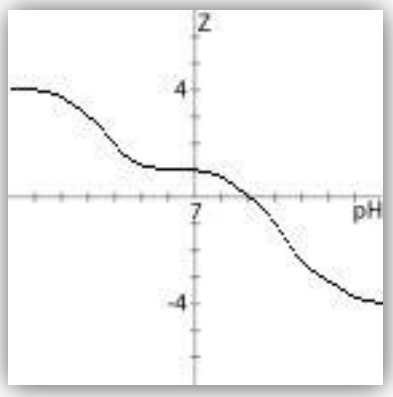
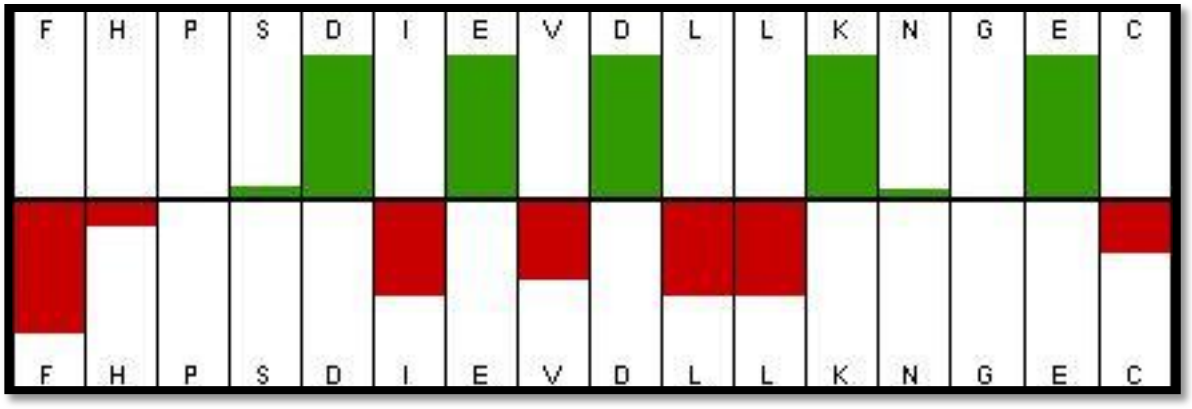


Figure A23: Hydrophobicity plots and Net Charge curve of S7K-4



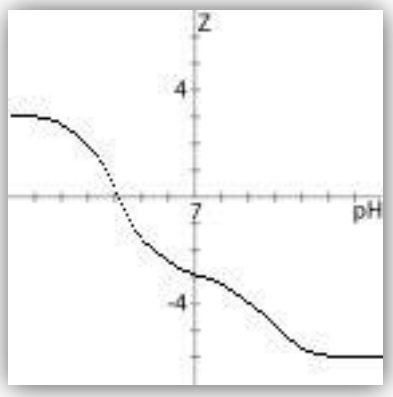


Figure A24: Hydrophobicity plots and Net Charge curve of F7E-8

The following is representative data showing the general trends observed after each assay performed.

1. **Antibody detection: indirect ELISA**

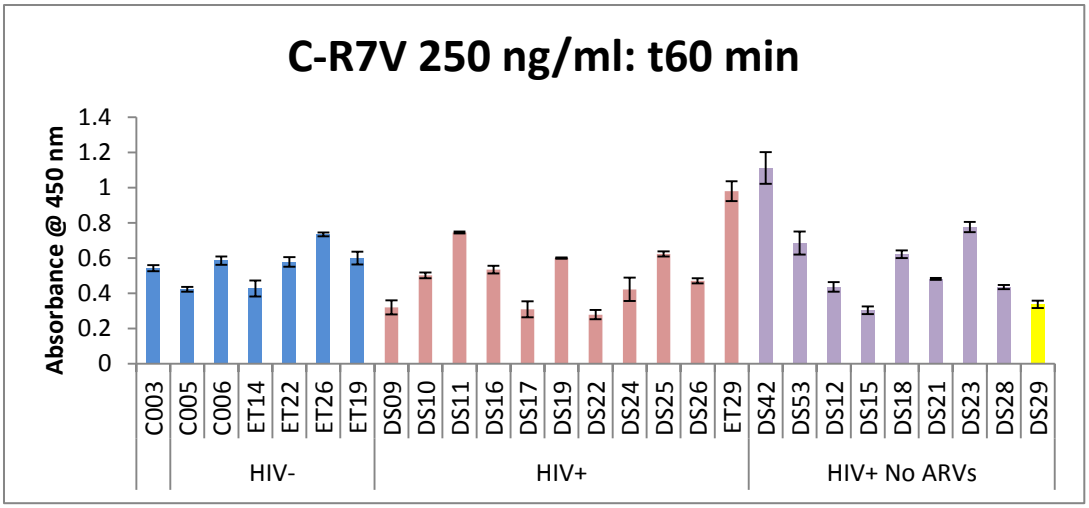


Figure A25: Seroprevalence of anti-C-R7V antibodies captured using 250 ng/ml of C-R7V as antigen. The OD was taken after 60 minutes following addition of the substrate at 450 nm. The blue bars represent antibodies detected in HIV negative serum, the pink represent HIV positive on HAART and the purple represent HIV positive not on treatment. Finally, the yellow bar represents an individual who was infected and not on treatment for ≥ 5 years since the sample bleed date.

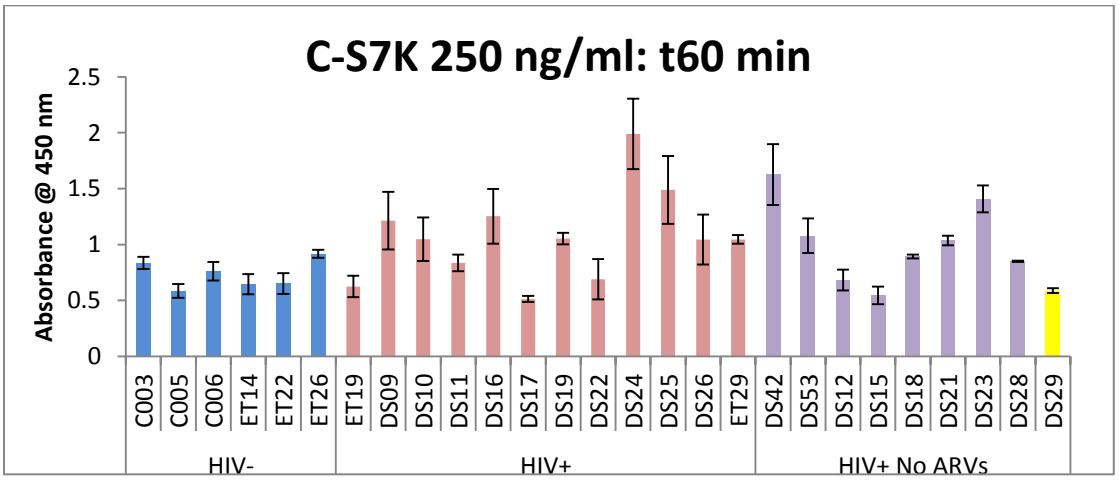


Figure A26: Seroprevalence of anti-C-S7K antibodies captured using 250 ng/ml of C-S7K The blue bars represent antibodies detected in HIV negative serum, the pink represent HIV positive on HAART and the purple represent HIV positive not on treatment. Finally, the yellow bar represents a long term non progressor (infected and not on treatment for ≥ 5 years).

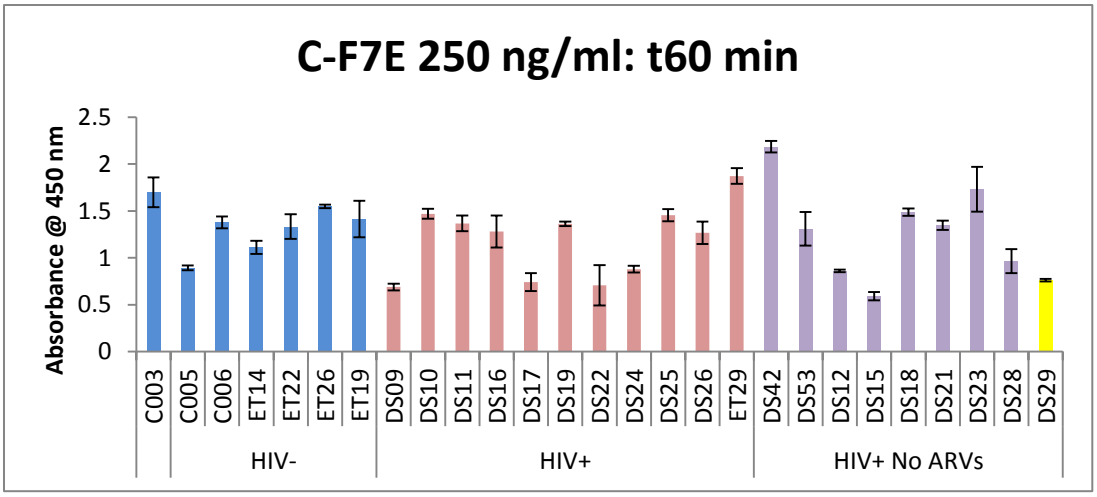


Figure A27: Seroprevalence of anti-C-F7E antibodies captured using 250 ng/ml of C-F7E. The blue bars represent antibodies detected in HIV negative serum, the pink represent HIV positive on HAART and the purple represent HIV positive not on treatment. Finally, the yellow bar represents a long term non progressor (infected and not on treatment for ≥ 5 years).

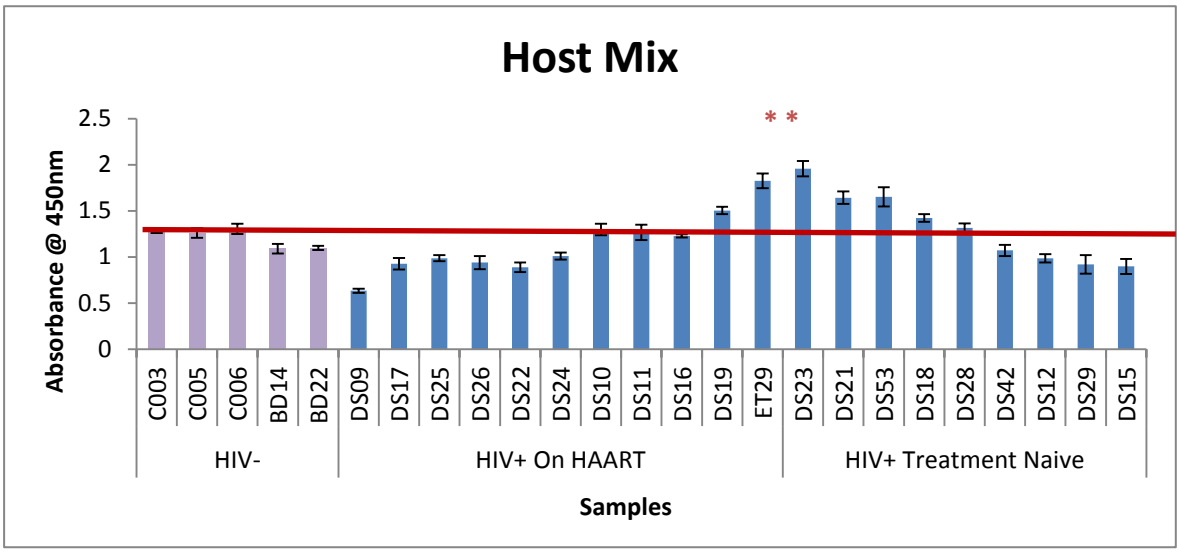


Figure A28: Seroprevalence of anti-Host Mix antibodies captured using 250 ng/ml of Host mix. The purple bars represent antibodies detected in HIV negative serum, the blue represent HIV positive on HAART and the asterix denotes recently infected individuals.

A summary of the findings for the three heptamers following several repeat experiments. Overall the antigenicity of te peptides were low if recently infected sera samples were not used.

Table A1: Anti-R7V indirect ELISA absorbance readings obtained after 60 minutes.

R7V(250 ng/ml): absorbance readings at 450nm after 60 min				
Patient number	**Sample bleed date (dd/mm/yy)**	**HIV status & treatment info**	**Response at 100x dilutions**	**Number of experiments**
Control 18	10/11/08	-	1.405	3
Control 034		-	1.487	1
Control 035		-	1.052	3
Control 036		-	0.728	3
Control 45	30/04/09	-	0.409	1
Control 98	08/10/09	-	0.935	3
51 FOH		+ On ARVs	1.054	4
56 FOH	04/02/09	+ On ARVs	0.957	3
57 FOH	04/02/09	+ On ARVs	1.357	5
58 FOH	04/02/09	+ On ARVs	0.872	3
59 FOH	04/02/09	+ On ARVs	1.448	3
60 FOH		+ On ARVs	1.519	1
64 FOH		+ On ARVs	1.067	1
DS 50	08/09/2009	+	0.280	1
DS 53	31/08/2010	+ LTNP	1.539	3
DS 59	14/09/10	+ LTNP	0.385	1

Table A2: Anti-S7K indirect ELISA absorbance readings obtained after 60 minutes.

S7K (250 ng/ml): absorbance readings at 450nm after 60 min				
Patient #	Sample bleed date (dd/mm/yy)	HIV status& treatment info	Response at 100x dilutions	# of experiments
Control 18	10/11/08	-	0.648	3
Control 45	30/04/09	-	0.542	3
Control 98	08/10/09	-	0.567	3
56 FOH	04/02/09	+ On ARVs	0.741	3
57 FOH	04/02/09	+ On ARVs	0.735	3
58 FOH	04/02/09	+ On ARVs	0.686	3
DS 50	08/09/2009	+	0.500	3
DS 53	31/08/	+ LTNP	0.713	3
DS 59	14/09/10	+ LTNP	0.555	3
DS 61		+ LTNP	1.564	2

Table A3: Anti-F7E indirect ELISA absorbance readings obtained after 60 minutes.

F7E (250 ng/ml): absorbance readings at 450nm after 60 min				
Patient #	Sample bleed date (dd/mm/yy)	HIV status& treatment info	Response at 100x dilutions	# of experiments
Control 18	10/11/08	-	0.501	3
Control 45	30/04/09	-	0.463	3
Control 98	08/10/09	-	0.368	3
56 FOH	04/02/09	+ On ARVs	0.490	3
57 FOH	04/02/09	+ On ARVs	0.645	3
58 FOH	04/02/09	+ On ARVs	0.502	3
DS 50	08/09/2009	+	0.445	3
DS 53	31/08/	+ LTNP	0.565	3
DS 59	14/09/10	+ LTNP	0.470	3

Cytokine data showed two cytokines, IL-6 and Il-10 to be influenced in PBMCs stimulated with peptides.

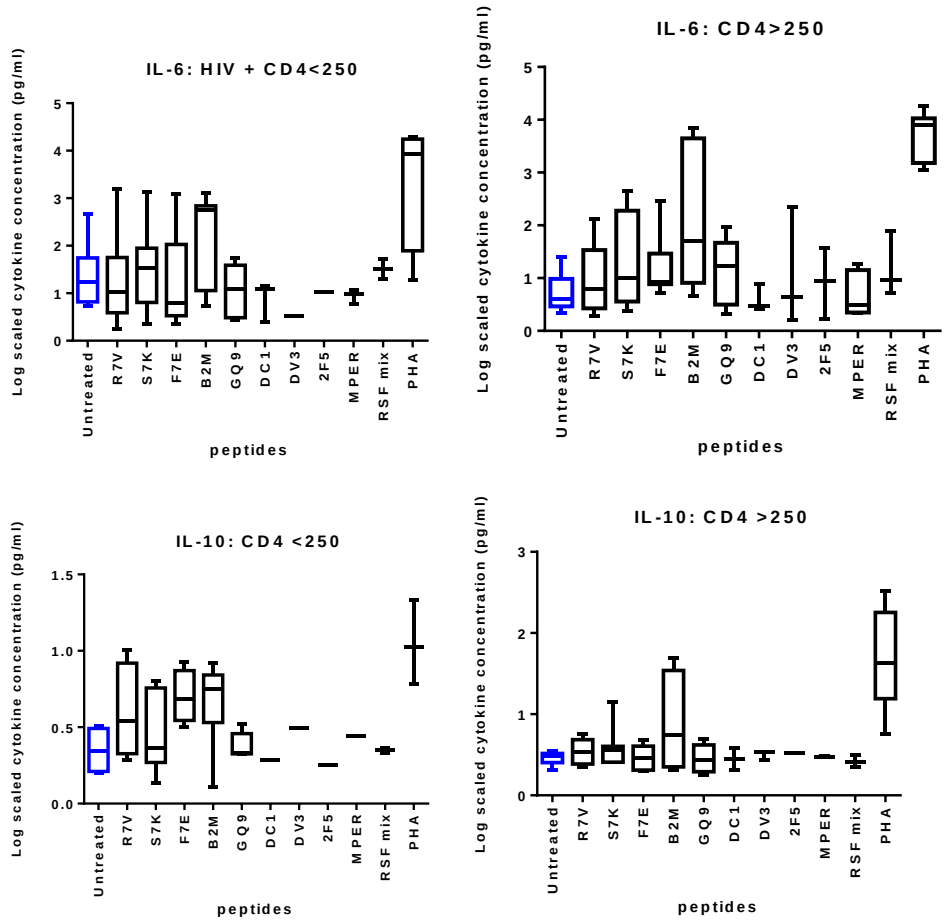


Figure A29: Data per patient demonstrating the effect of HIV disease status and treatment of PBMCs with peptides on the secretion of IL-6, IL-10. Different patients were averaged and box and whiskers plots were constructed to show variation.

Antioxidant activity as determined by the DPPH assay.

The peptides were not expected to be antioxidant response-inducers, which was confirmed by the DPPH assays. Only peptides produced from edible proteins are usually anti-oxidative. Ascorbic acid was the positive control for this experiment and had an IC50 of 3.67 µg/ml as shown in figure A39.

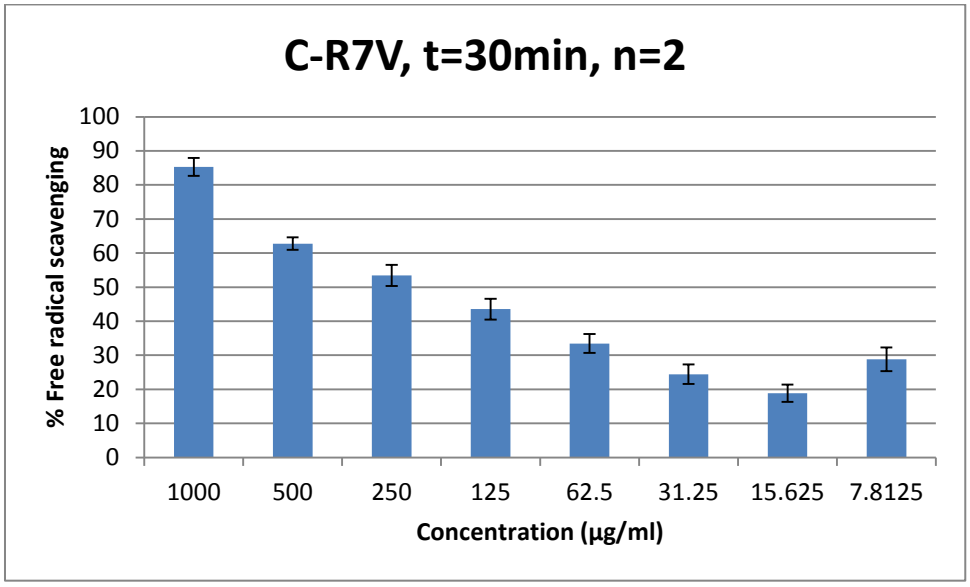


Figure A31:C- R7V, t=30min, n=2, IC50=116.6

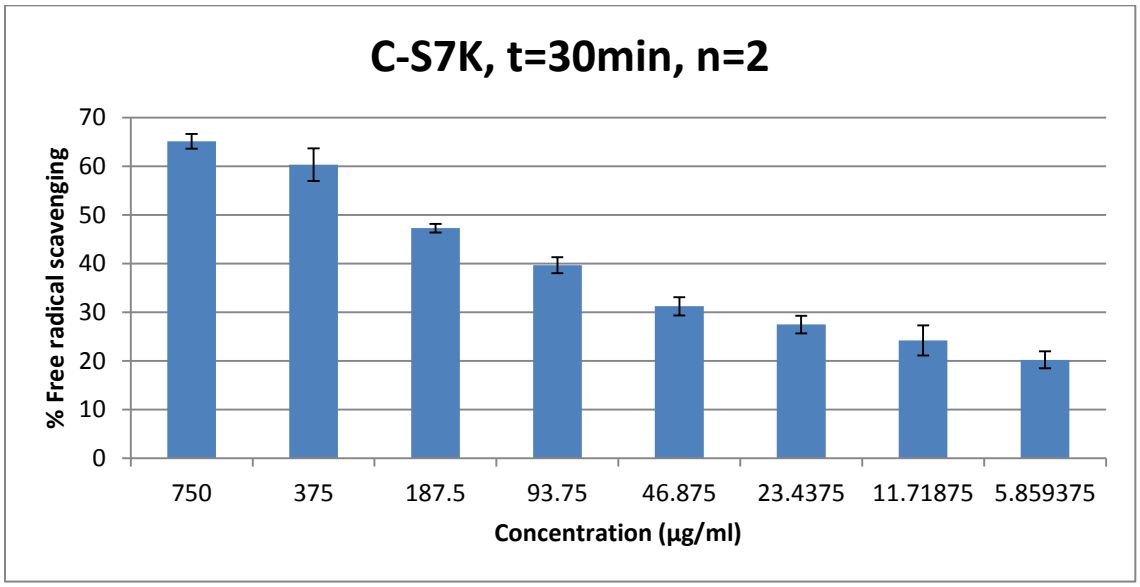


Figure A32:C- S7K, t=30min, n=2, IC50=33.34

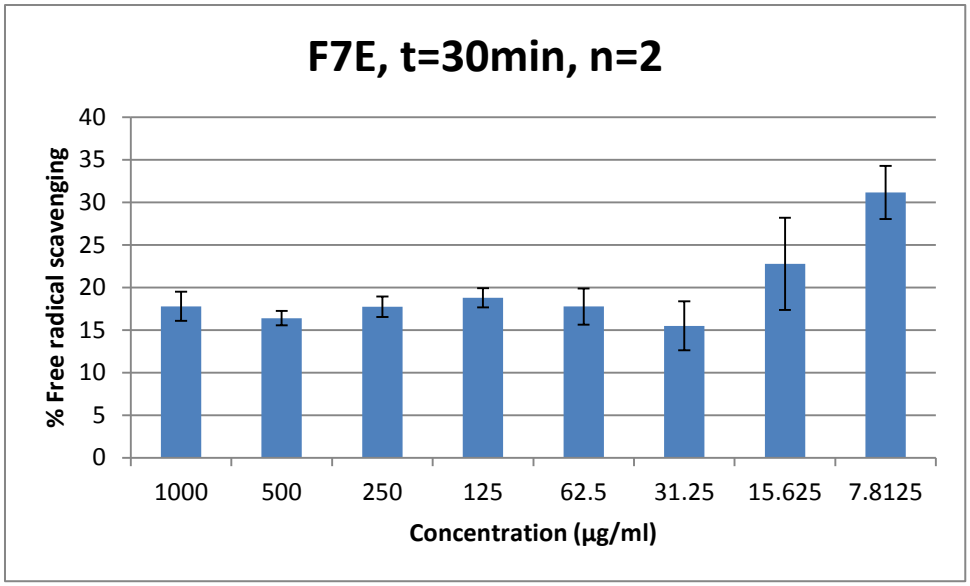


Figure A33: F7E, t=30min, n=2

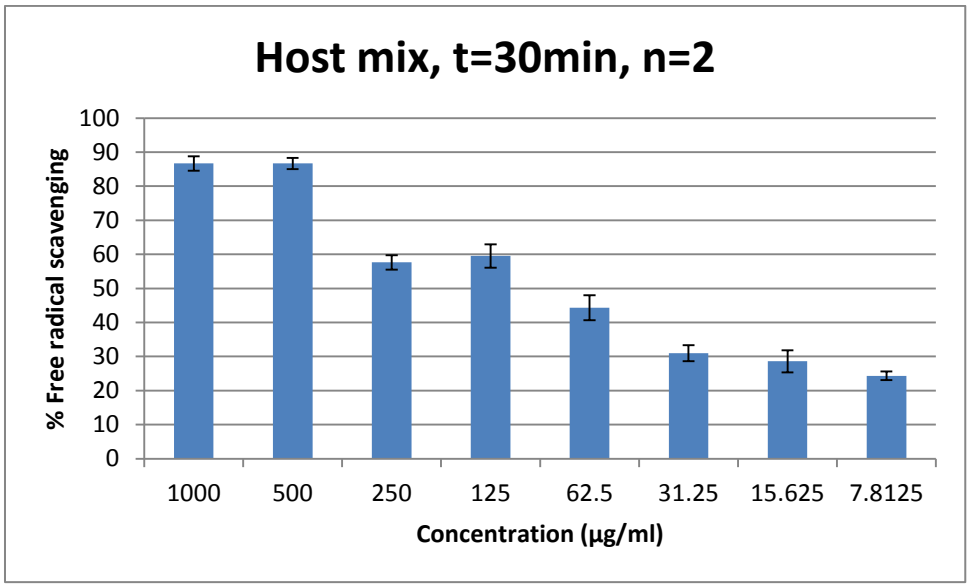


Figure A33: Host derived mix, t=30min, n=2, IC50=51.22

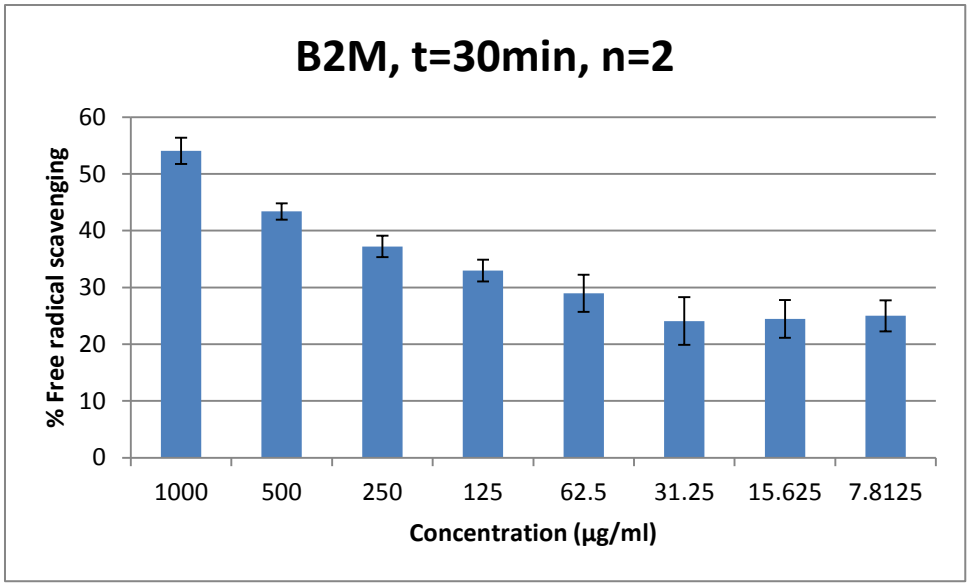


Figure A34: B2M, t=30min, n=2

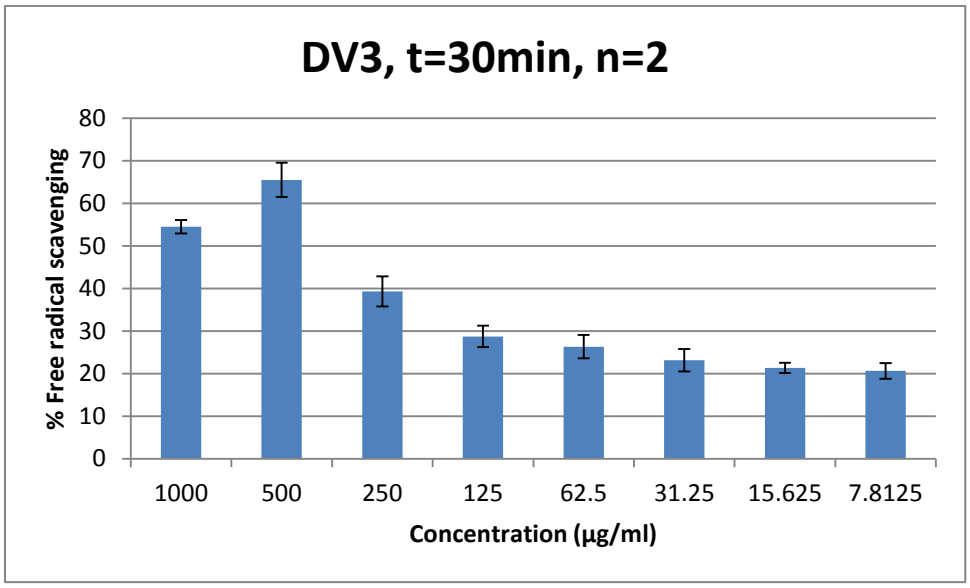


Figure A35:DV3, t=30min, n=2, IC50=87.94

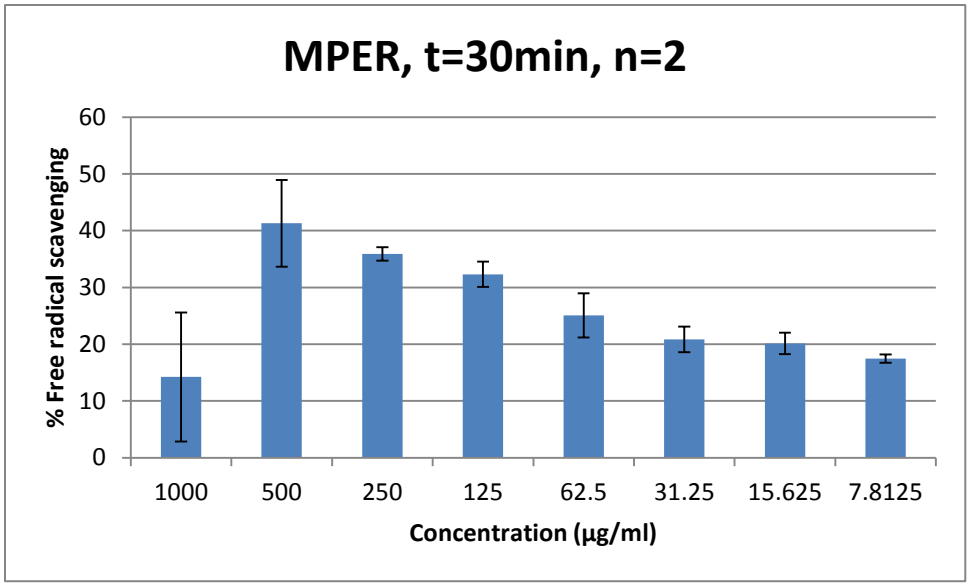


Figure A36: MPER, t=30min, n=2

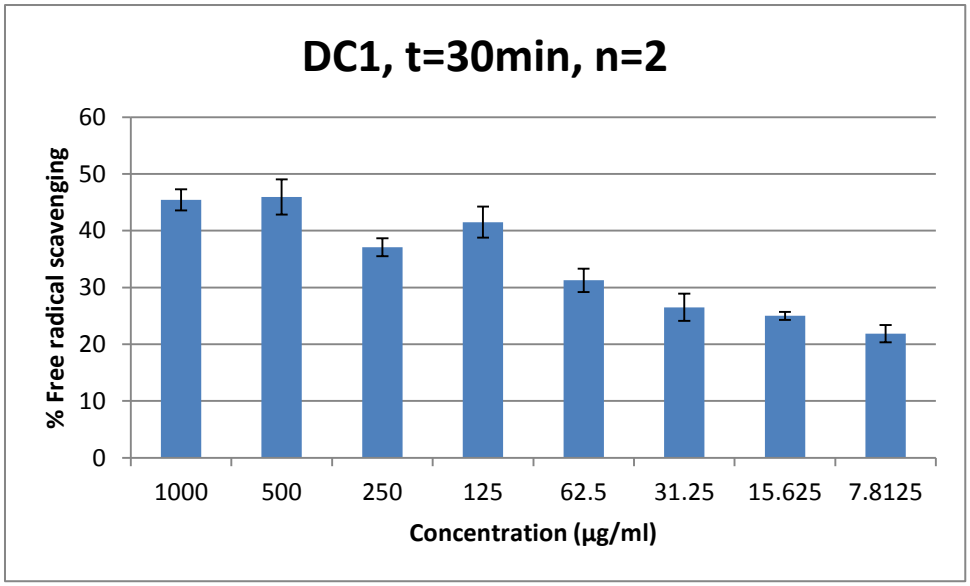


Figure A37:DC1, t=30min, n=2

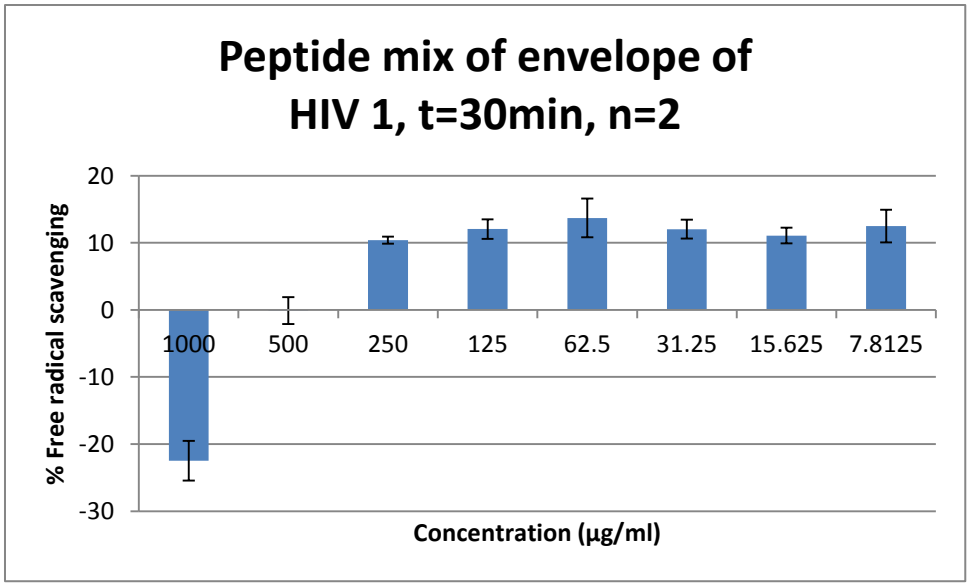


Figure A38: Peptide mix of envelope of HIV1, t=30min, n=2

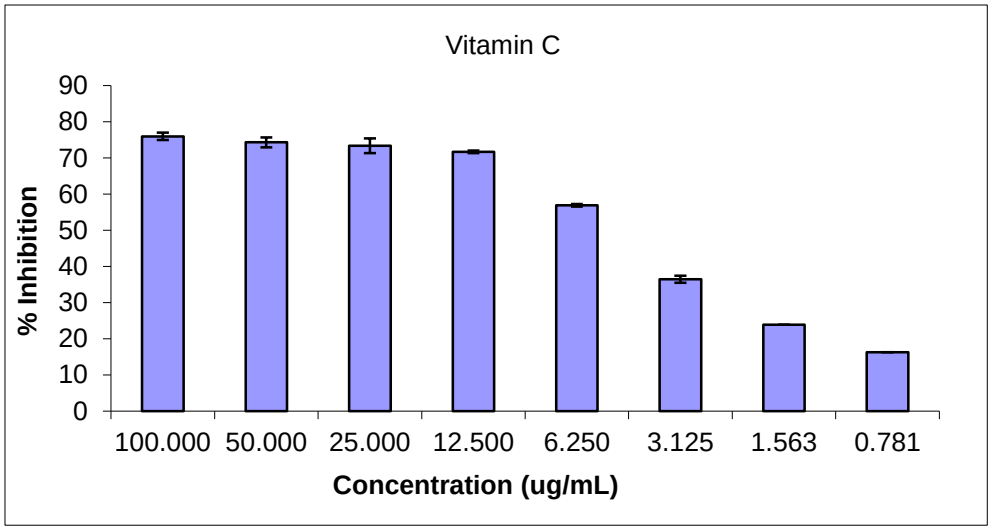


Figure A39: Ascorbic acid (Vitamin C), a known potent antioxidant that was the positive control for the DPPH assay. n=10 , IC50= 3.67

Summary of DPPH data in tabulated form

ORIGIN	PEPTIDE	RESPONSE	Active Concentration (µg/ml)
Positive Control	Ascorbic acid	Responsive	> 3.62
BASED ON HOST-DERIVED B2M	CB2M	Moderate	> 500
	CF7E	Responder	> 125
	CR7V	Responder	> 125
	CS7K	Responder	> 125
	CRS (HOST MIX)	Responder	> 62.5
HERV-K	GQ9	Responder	> 125

BASED ON REGIONS OF HIV-1 ENV PROTEINS	2F5	Responder	> 125
	DC1	Moderate	> 250
	DV3	Moderate	> 250
	MPER	Non responder	-
	JPT	Non responder	-

RESPONDERS (greater than 60% scavenging), Moderate between 50-60% scavenging, Non (below 50% scavenging after 30 minutes).

Manuscript

Title:

Synthetic peptide R7V characterises antibody responses in HIV-1 subtype C infection

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Abstract:

During budding, human immunodeficiency virus (HIV)-1 incorporates numerous host proteins into its viral envelope. An epitope designated R7V and derived from one of these host proteins, beta-2 microglobulin (β2m), has been shown to elicit an antibody response in HIV-infected individuals and predict a tendency towards long term non progression. Literature to date suggests that the presence of R7V antibodies in HIV-1 subtype A and B infected patients during the early stages of infection, signals slower progression to acquired immunodeficiency syndrome (AIDS) (Galea et al, 1996, le Contel 1996, Margolick et al 2010). In the current study the humoral immune response of HIV-1 infected patients living in South Africa was characterized using an R7V peptide in order to investigate whether HIV-1 subtype C incorporated the R7V epitope and elicited immune responses to the same extent as that of other subtypes. This was done using a synthetic R7V peptide as antigen in an "in-house" enzyme-linked immunosorbent assay (ELISA). The ELISA demonstrated an R7V antibody response in infected patients (n=92) compared to that of uninfected individuals (n=17). Statistically significant differences were observed between infected and uninfected individuals (p value ≤ 0.000152) but not between samples from patients on antiretroviral (ARV) treatment and those naïve of treatment. No differences were observed between progressors and non-progressors, the latter being defined here as being infected for more than

5 years and having never started treatment. There was an increase in R7V antibodies in individuals in the acute stage of infection and patients who had just started treatment. Polyclonal rabbit. Polyclonal Rabbit R7V antibodies and recombinant R7V antibody fragments (ScFv) were not able to neutralize HIV-1 subtype C isolate (Du151.2). The latter samples also neutralized a subtype B isolate (SF162) suggesting that the R7V epitope was more prominent in HIV-1 subtype B. R7V stimulated the production of IL-6 and IL-17 in HIV infected PBMCs, *in vitro*.

Keywords:
Prognostic marker, R7V, epitope, antibodies, β2m, HIV

Introduction

HIV incorporates a large number of host proteins in the virion particle during assembly and budding. These virus incorporated host proteins and their epitopes are believed to be present either inside or on the surface of HIV and may contribute to viral pathogenesis. (Ott 1997 and Ott 2008). HIV incorporates host proteins which are not subject to the variability associated with viral proteins and when they are presented as virus-associated components, these proteins provide potential targets for incorporation in novel diagnostic/prognostic tests or for investigation as vaccine components (Bremnaes C and Meyer D 2009). Diagnosis of HIV infection is achieved through antibody detection of core (p24) and surface (env) viral proteins provided the blood sample is collected following seroconversion. CD4 cell count is still the most successful indicator of AIDS progression even though these cells are affected by opportunistic infections and other conditions.

Beta-2 microglobulin (B2M) is one of the proteins that are acquired from the host during budding and is incorporated into HIV's envelope (Arthur *et al*. 1992; Ott 1997; Haslin *et al*. 2002). This protein is 12kDa in size and plays a role in major histocompatibility complex (MHC) class I presentation of antigen to T-cells in the host (Rosano *et al*. 2005). The levels of β2m found in urine and serum are implicated as a biomarker for diseases associated with renal dysfunction and viewed as indicative of HIV/AIDS prognosis, respectively (Fahey et al. 1990). R7V is an antigenic determinant of β2m and was identified to be presented on the exterior surface of HIV following budding (Le Contel *et al*. 1996). This epitope demonstrated the ability to elicit the production of HIV neutralizing antibodies (Le Contel *et al*. 1996; Galéa *et al*. 1999 a and b; Chermann 2001; Haslin and Chermann 2007b) and has consequently been

suggested as a possible vaccine candidate for HIV. The above mentioned observations have led to the suggestion that the antibodies detected by this peptide may have prognostic value (Galéa *et al*. 1996; Chermann 2001; Ravanini *et al*. 2007; Kouassi *et al*. 2007; Sanchez *et al*. 2008) or serve as a therapeutic tool (Haslin and Chermann 2002, 2004 and 2007 b; Haslin *et al*. 2007 a). However, the literature on R7V is still largely incomplete (Reviewed by Bremnæs and Meyer 2009). Most published work on this epitope makes no reference to HIV subtypes and if mentioned, referred to subtypes A and B. The presence of R7V antibodies in individuals infected with HIV-1 subtype C, which is dominant in South Africa (McCutchan, 2006), is yet to be clarified. More studies need to be done to ascertain the potential roles for this epitope in HIV. This study investigated the prognostic role of the R7V antibodies for individuals living in South Africa. This was done by characterizing the humoral immune response in HIV-infection using a synthetic peptide, designated R7V, representing the R7V epitope of the virus.

2. Materials and methods

2.1. Human blood samples

Blood samples collected from HIV-1 infected (treated or naïve of ARV treatment) and uninfected patients living in South Africa were obtained from the Steve Biko Academic Hospital (Pretoria, South Africa) and from the student clinic at the University of Pretoria (Pretoria, South Africa). In addition, HIV-1 positive blood was collected from patients attending the Fountain of Hope Clinic (Pretoria, South Africa), the King`s Hope Development Foundation (Olievenhoutbosch, South Africa) as well as Eesterust clinic (Pretoria, South Africa). Negative control blood was also obtained from volunteers at the Department of Biochemistry at the University of Pretoria (Pretoria, South Africa).

Owing to the fact that all the HIV positive donors in this study are from South Africa, It is assumed that blood samples collected from these individuals are HIV-1 subtype C (Van Harmelen, Van Der Ryst et al., 1999). The National Institute for Communicable Diseases (NICD, Johannesburg) also provided archived plasma and serum samples from HIV-1 infected South Africans. Blood samples acquired from HIV-1 infected individuals seropositive $\geq$ 5 years with a CD4 cell count $\geq$ 200 cells/µl in the absence of ARV treatment were considered as LTNPs.

2.2. Ethics

Ethical approval for the collection of whole blood from patients was obtained from the Faculties of Natural and Agricultural Sciences and Health Sciences at the University of Pretoria with the approval records E080-506-019 and 163/2008 respectively. Informed, consenting individuals were enrolled in this study with confidentiality and patient anonymity maintained throughout. Blood samples were collected from both genders and were representative of several races; the donors encompassed a wide age group (aged 21 and older).

2.3. Serum separation techniques

The blood was collected in red -capped vacutainers without anticoagulant and was allowed to clot (left at room temperature for 1 hour). Once clotted, the blood was centrifuged (1610 x g for 10 minutes) to recover the sera supernatant from the rest of the blood debris. Following which, sera were heat inactivated (56◦C) for 30min to remove interfering complement proteins and then stored in frozen aliquots at -70 °C until used.

2.4. Determination of CD4 T-cell count and plasma viral load

Monitoring $CD4^+$ T cell count and keeping records of viral load are important aspects of HIV disease management. Although several alternative methods have been described; such as The ELISA TRAx $CD4^+$ test kit (T Cell Diagnostics, Cambridge, MA, USA) and enumeration by way of dried blood specimens (Mwaba, Cassol et al 2003), flow cytometry remains the gold standard for $CD4^+$ T lymphocyte count measurement. This method makes use of fresh whole blood from which $CD4^+$ T cells are enumerated. The viral load represents the amount of HIV RNA in the blood and is measured from plasma using either real-time polymerase chain reaction (RT-PCR) test, isothermal nucleic acid based amplification (NASBA) assay or by the branched DNA signal amplification assay. Both viral load and $CD4^+$ cell count were obtained from practitioners where possible.

2.5. R7V peptides

Two peptides were made for this study and contained the seven amino acid region (Arg-Thr-Pro-Lys-Ile-Gln-Val) named R7V. Fmoc and Tboc chemistry was used for making one peptide by GenScript Corporation (Piscataway, USA) and the other by LifeTein LCC (Edison, USA).

2.6. R7V antibodies

Recombinant R7V antibody fragments (University of Pretoria) and polyclonal R7V rabbit antibodies (LifeTein LCC, Edison, USA) were made for this study.

2.6.1. Recombinant R7V antibody fragment - production

Recombinant single chain R7V antibody fragments consisting of only the binding domains of the heavy and light chains of the Fab region were made, based on a synthetic R7V sequence (GenScript Corporation), in the laboratory of Dr Mervyn Beukes, Department of Biochemistry at the University of Pretoria. Recombinant DNA technology and M13 phage display previously described by van Wyngaardt *et al*. (2004) was used with some modifications. These recombinant R7V antibody fragments were produced to serve as positive controls for the in-house ELISA.

2.6.2 Polyclonal R7V rabbit antibody production

The polyclonal antibodies were commercially manufactured by Lifetein LCC using AdjuboosterTM platform. New Zealand rabbits were injected with R7V peptide and AdjuboosterTM adjuvants as previously described by Bremnaes, C. (2009).

2.7. Detection of R7V antibodies using an "in-house" R7V ELISA

An indirect solid phase "in-house" R7V ELISA was developed to determine the presence of R7V antibodies in serum or plasma from naturally HIV-1 subtype C infected individuals. The standard protocol routinely used in the laboratory was performed as previously described (Hewer and Meyer 2002 and 2003; Sanchez *et al*. 2008). Briefly, 96-well Nunc-Immuno^{TM} Maxisorp^{TM} ELISA plates (Nunc, Roskilde, Denmark) were coated at 37°C for 1 hour with 100 µl/well of a 250 ng/ml or 1 µg/ml R7V (Arg-Thr-Pro-Lys-Ile-Gln-Val) peptide (GenScript Corporation, Piscataway, USA dissolved in 10 mM Phosphate Buffered Saline, PBS pH 7.4). Thereafter, the plates were incubated overnight at 4°C with 300 µl blocking solution. The blocking solution was 1% BSA Fraction V (Roche Diagnostics, Manheim, Germany), 5% Fetal Bovine Serum (FBS, Highveld Biological, Lyndhurst, South Africa), 0.1% Tween 20 (Highveld Biological, Lyndhurst, South Africa) in PBS. After washing 5 times with 300 µl/well wash buffer (0.5% Tween-20 in PBS) using a Wellwash 4 Labsystems (Thermo Labsystems, Helsinki, Finland), the plates were incubated for 1 hour at 37°C with 100 µl/well of 100x diluted human sera or plasma samples (diluted in blocking solution). Following a subsequent wash step as described above, 100 µl/well of a 5000x diluted (diluted in blocking buffer) horse radish peroxidise (HRP)-labelled secondary antibodies (goat

antihuman IgG H+L, Jackson ImmunoResearch laboratories, West Grove, USA) were added and the plates incubated for 1 hour at 37°C. A final washing step was followed by the addition of 100 µl/well of a O-Phenylenediamine (OPD) substrate (Invitrogen Corporation, Carlsbad, USA) diluted in substrate buffer which was 0.05 M Citric Acid monohydrate (Saarchem, Wadeville, South Africa), 0.1 M di-Sodium hydrogen phosphate anhydrous (Merck, Darmstadt, Germany pH 5.0) containing 0.03% hydrogen peroxide tablets (BDH Chemicals, Poole, England). One tablet substrate was dissolved in 12 ml substrate buffer. Enzymatic cleavage was determined on a Multiscan Ascent (Thermo Labsystems, Helsinki, Finland) plate reader with Multiscan Ascent software version 1.3.1, at a wavelength of 450 nm after 60 minutes. All samples were analysed in triplicate and experiments were repeated thrice.

2.7. Positive control

To detect the R7V antibody fragments in the ELISA, the same procedure as described was performed with the following modifications:

The amount antibody fragments and secondary antibodies per well was 50 µl (diluted in blocking solution). Two types of secondary antibodies (1 hour incubation at 37°C for each of the two) were used. The first incubation was performed with a rabbit-anti-c myc antibody (AbD Serotec, Oxford, UK) and the second incubation was done with a mouse-anti rabbit-HRP-conjugated antibody (AbD Serotec, Oxford, UK) following a wash step.

2.8. Evaluation of the virus neutralizing ability of R7V antibodies

The neutralization assay was carried out at the AIDS Unit of NICD using a protocol described by Montefiori (2004). Briefly, eight three-fold serial dilutions of inactivated HIV-1 subtype C human sera (with or without ARV treatment), recombinant R7V antibody fragments or polyclonal rabbit R7V antibodies were made in 100 µl growth medium (Dulbecco`s modified Eagle Medium with L-glutamine, sodium pyruvate, glucose and pyridoxine, Gibco BRL Life Technologies, Grand Island, USA) supplemented with 10% FBS, 50 µg/ml gentamycin and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) on 96-well Flat-bottom culture plates (Costar, AEC Amersham). To each well, 50 µl virus was added. The viral samples' dilutions used were 1:10 or 1:20 Du151.2 which contains two HIV-1 subtypes C isolates, 1:1500x diluted vesicular stomatitis virus (VSV-G) and 1:300x diluted subtype B virus entitled SF162. The plates were then incubated at 37°C and 5% CO_2 for 1 hour. VSV-G is a virus that contains the envelope of the vesicular stomatitis virus G and was used as a control and indicator of non-HIV-specific neutralization activity. SF162 is a very sensitive

HIV-1 subtype B virus strain which is easily neutralized. Following incubation, 100 µl of adherent TZM-bl cells resuspended at $1x10^5$ cells/ml in growth media containing diethylaminoethyl (DEAE) dextran (8 µg/ml) was added to each well. Controls incorporated in this assay were; cell control (cells in growth media), virus control (containing virus, cells and growth media) and positive control serum (with known neutralizing antibodies referred to as Bbpool). Following incubation for 48 hours at 37°C and 5% CO_2, 150 µl of cell-culture supernatant was removed from each well. Bright Glo™ Reagent (100 µl, Promega, USA) was added to each well followed by incubation for two minutes at room temperature. From each well, 150 µl supernatant was transferred to the corresponding wells of a 96-well Flat-bottom black plate (Nunc, AEC Amersham, Johannesburg, South Africa) which was read immediately on a luminometer (PerkinElmer 1420 Multilabel Counter, Victor^3™ with software Wallac 1420 Manager version 3.00). Percent neutralization was determined by the ratio in average relative luminometry units (RLU) according to the following equation:

$$\% \text{ neutralization} = 1 - [(\text{test wells} - cc) / (vc - cc)] \times 100$$

Neutralizing antibody titers were expressed as the reciprocal of the serum dilution required to neutralize 50% of the virus.

2.9. Influence of R7V on cytokine secretion

Cytokine analysis can provide valuable information about *in vivo* immune status; because cytokines are expressed in low concentrations, high-sensitivity assays are therefore required to permit detection and quantification. Cytokine quantification was performed using multiplexing technology for the analysis of human Th1/Th2/Th17 cytokines through flow Cytometric detection. We measured secreted cytokine levels following the isolation and stimulation of peripheral blood mononuclear cells (PBMCs) obtained from infected individuals on HAART as well as HIV negative donors.

The PBMCs from the abovementioned experimental groups were isolated fresh and treated with 10 µg/ml peptide and incubated for 7 days. Following incubation, secreted cytokines were quantified from the supernatant using the cytokine bead array (CBA) kit. This Human Th1/Th2/Th17 CBA kit (BD Biosciences, San Jose, California) allows for the simultaneous detection of cytokines using capture beads coated with antibody specific to IL-2, IL-4, IL-6, IL-10, TNF-a, IFN-ϒ and IL-17A. Briefly, 50 µl of pooled capture bead mixture was added to 50µl of test samples and standards respectively. A PE-conjugated detection antibody (50µl) was then added to these and the mixture was incubated in the dark for 3 hours to allow for

sandwich complexes to form. Samples were subsequently washed with 1 ml wash buffer and the pellet was resuspended in 300 µl wash buffer following centrifugation (258 x g). A volume of 200 µl/well of each sample was added to a PRO-BIND™ 96 well assay plate and analysed on a specialized flow cytometer (BD Biosciences, San Jose, California) with plate sampler used for the detection of cell-associated, secreted or lysate protein known as a FACS Array Bioanalyzer. Following data acquisition, debris was filtered out from the data and the bead populations and mean fluorescent intensities (MFI) were automated using FCAP FCS Filter and FCAP Array Software (BD Biosciences, San Jose, CA, USA) respectively. The Concentration of the respective cytokines in the test samples was obtained by fitting the MFI of the test samples into a 4-parameter logistic curve-fitting equation. Five independent experiments were performed.

2.10. Statistics

Calculations of the mean, standard deviation (SD) and percent relative standard deviation (% RSD) were made using Microsoft® Office Excel® 2007 (Microsoft Corporation, Redmond, USA). Data is presented with bars showing the actual data point value for each sample including error bars indicating the plus and minus SD. One-way Analysis of Variance (ANOVA) was performed using Statistical Package for the Social Sciences (SPSS) version 17 software. The statistically significant effect in the ANOVA was followed up by a Post hoc ergo propter hoc test (Benferroni multiple comparisons method) in order to assess which groups were different from which other groups. A result was declared as significant if the probability value, *p*, was less than 0.05.

3. Results

3.1. Seroprevalence of R7V antibodies in HIV-infected patients

A total of 109 individuals were tested using ELISA. Figure 1 shows heat-inactivated sera from 78 individuals tested using 250 ng/ml of antigen, 16 of these samples were HIV-negative and 62 were HIV-1 positive (32 on ARV treatment and 30 naïve of treatment with 11 considered LTNPs). Six recombinant R7V antibody fragment samples were used as positive controls and were very responsive with some clones exhibiting OD values above 2 even at dilutions of 1:10. Amongst the experimental samples, the HIV-negative sera consistently responded at OD values of 0.3 or lower. HIV-positive sera were considered positive for R7V

antibodies if OD values were above 0.3. Although the HIV-positive samples appear to have R7V antibodies present, few samples demonstrate OD values double that of the negative controls. No general trend was observed for the HIV-positive samples with or without ARV treatment. LTNP had the same R7V antibody levels of AIDS patients (progressors). No correlation between (1) viral load and $CD4^+$ cell count (R^2 0.1928 and 0.2425), (2) $CD4^+$ cell count vs. Abs at 450 nm (R^2 0.0744 and 0.2382) and (3) viral load vs. Abs at 450 nm (R^2 0.0048 and 0.3964) for either infected samples on treatment or naïve of treatment respectively was observed. Significant statistical differences were observed between HIV-negative and HIV-positive samples (P value $\leq$ 0.000152). No significant difference was observed between the infected samples on and without treatment or LTNPs (Table 1). In the case where 1 µg/ml of antigen was used (figure 2); it was the recently infected individuals or infected individuals recently on treatment that exhibited high antibody responses against R7V. Samples were considered positive for R7V antibodies if the OD values obtained were above 0.55, a threshold set by the representative HIV negative sample.

As mentioned in the introduction, R7V antibodies are reported to have HIV-1 neutralizing abilities. For that reason, archived sera (Philippeos, 2007) and plasma (NICD) with proven neutralizing antibodies were analysed for R7V content (Figure 3). All samples indicated the same level of R7V antibodies as those reported in Figure 3.

3.2. Virus neutralizing ability of R7V antibodies

All human sera tested positive for the ability to neutralize the HIV-1 subtype C isolate (Du151.2) at various titers as the titers were above IC50 (Figure 4). The recombinant R7V antibody response occurred in a scattered manner, and in the absence of a dose response (figure 5).The neutralizing ability against virus Du151.2 was detected at certain dilutions of the recombinant R7V antibody fragments, responses were either below IC50 or not significantly above it at a concentration of 4.7. R7V antibody fragment clone 2 was able to neutralize the sensitive HIV-1 subtype B virus (IC above 50) (Figure 5) and did not respond to the vesicular stomatitis virus (VSV-G), which would have been an indicator of nonspecific neutralization). The polyclonal rabbit R7V antibodies were unable to neutralize Du151.2 at any of the tested concentrations (Figure 6).

3.3 Influence of R7V on Th1/Th2/Th17 cytokine profile

One way ANOVA was carried out where multiple comparisons of all the secreted cytokines were analysed. A Kruskal-Wallis test was then employed which determines probability by comparing population medians; this revealed that IL-6 was the significantly altered cytokine in HIV positive patients. This observation is depicted in figure 7; IL-6 was the cytokine most secreted by HIV positive individuals, with recently infected patients and patients with $CD4^+$ counts below 250 cells/mm^3 exhibiting the highest amounts of secreted IL-6. Significantly higher amounts of cytokines were detected in HIV positive people than negative donors ($p<0.05$). HIV negative donors demonstrated almost double the amount of IL-2 compared to HIV positive patients but the IL-6 in these individuals was significantly lower with minor variances between individuals observed. The amount of IL-4, IL-10, TNFα, INFϒ and IL-17A detected in these individuals was negligible but increases (although not significant) were observed following stimulation of HIV negative PBMC with R7V.

Patients in the chronic phase of HIV progression showed an absence of IL-2 as well as IL-4.

The cytokines that were affected when PBMCs were stimulated with R7V peptides were IL-6 and IL-17A and IL-10 to a lesser extent.

4. Discussion

Since the early stages of the HIV epidemic, attempts have been made to determine factors which predict progression to AIDS and overall survival in those infected with the virus. The rate of progression and survival varies greatly, with some patients rapidly progressing to AIDS and death, while others remain asymptomatic for years and are designated LTNPs. The comparison of patient populations that fall into categories such as LTNPs and normal progressors allows for the identification of key factors that may contribute to HIV disease progression. The levels of antibodies directed to the R7V epitope of HIV-1 have previously been suggested to be a prognostic indicator for HIV-1 subtype A and B infected patients. In the current study it was hypothesized that a peptide representing the R7V epitope of HIV should be able to detect humoral immune responses in HIV-1 subtype C infected individuals (residing in South Africa).

To be able to detect the R7V antibodies in serum or plasma from HIV-infected individuals, an "in-house" R7V ELISA was used. This ELISA repeatedly showed negligible levels of R7V antibodies in negative control sera and marginal to high levels in HIV-1 infected individuals (Figure 1, 2). According to the literature (Galéa *et al*. 1996; Tagny *et al*., 2007; Ravanini *et al*.

2007; Kouassi *et al*. 2007; Sanchez *et al*. 2008), R7V antibodies can be prognostic markers based on the difference in R7V antibody levels observed in normal progressors compared to LTNPs.

An ANOVA test showed a significant statistical difference between R7V antibodies in HIV-negative and HIV-positive samples used in this study but not between the positive samples on treatment vs. not on treatment and LTNPs (Table 1). The significant statistical difference observed between uninfected and infected samples support the potential use of the prevalence of R7V antibodies as an indicator of immune status. It is important to mention that none of the published studies were done with specimens from HIV-1 infected individuals living in South Africa (primarily infected with HIV-1 subtype C, McCutchan 2006) , instead samples were collected from individuals living in the USA, Europe, Cameroon and the Ivory Coast, (individuals most likely infected with HIV-1 subtype A or B, McCutchan 2006). HIV-1 subtype C does not have the same characteristics as subtype B (Ping *et al.* 1999) and might not have the same β2m epitope incorporated or the epitope may be less prominent. Two other hydrophilic peptides (Le Contel *et al*. 1996), both 7 amino acid (S7K; Ser-Gln-Pro-Lys-Ile-Val-Lys and F7E; Phe-His-Pro-Ser-Asp-Ile-Glu) derived from β2m have also been found in the envelope of HIV-1 and were capable of reversing the neutralizing action of the monoclonal antibodies directed to β2m (Le Contel *et al*. 1996). These two epitopes were considered in another study investigating whether HIV-1 subtype C infected individuals had antibodies directed at these two epitopes and whether these antibodies were present to a larger extent than R7V antibodies. The data (not shown) showed R7V antibodies to be more prevalent.

The recombinant R7V antibody fragments were not able to neutralize a subtype C isolate but were capable of neutralizing a subtype B isolate (Figure 5). This supports the premise that the R7V epitope is more exposed in HIV-1 subtype B compared to subtype C. This can also be linked to the observed lack of differences in R7V antibody responses between normal progressors and LTNPs in sera from South Africans in this study compared to what is reported in the literature. An important observation (figure 2) was that the antibodies responded to the beta 2 microglobulin derived R7V peptide under very particular conditions; if the blood was obtained from recently infected individuals or infected individuals recently on treatment (so that treatment was not yet successful at lowering viral load or increasing $CD4^+$ count) then antibody responses were high. If blood was collected from long term non progressors or individuals for whom treatment had proven effective, then antibody responses

were low (Figures 1 and 2) and comparable to HIV negative samples. Duration of HIV-infection has been reported (Haslin and Chermann 2007 b; Ravanini *et al*. 2007; Sanchez *et al*. 2008), as influencing the R7V antibody presence. Our observations were in agreement with data reported by Margolick et al 2010 which showed that blood collected early on in infection of individuals who went on to become long term non progressors, had high levels of antibodies to R7V while later on in the patient's non progressor status, these antibodies were lower and eventually non detectable. In recent or new infections or when treatment is not yet taking effect, virus continues to be produced and the body is continuously exposed to viral antigens leading to antibody production. As treatment takes hold, viral production is lowered and less or no antibodies are being made.

It is noteworthy that the data in this study used a different ELISA than the previous studies which used their own "in-house" ELISA (Galéa *et al*. 1996) or the anti-R7V ELISA from Ivagen, Bernis France (Xu *et al*. 2002; Tagny *et al*., 2007; Ravanini *et al*. 2007; Kouassi *et al*. 2007; Haslin and Chermann 2007 b; Sanchez *et al*. 2008; Ergünay *et al*. 2008, Webber 2009). Ivagen re-called the ELISA from the market in 2008 concluding that the test could not be used as a prognostic tool. This could also suggest that the R7V antibodies play a less important role in slowing AIDS development as was previously believed.

The strong recombinant R7V antibody fragment response in this report`s "in-house" ELISA indicates that enough antigen was present in the wells to quickly detect concentrated R7V antibodies. The percent RSD between triplicates (8.3%, 6.5% and 5.8% for uninfected, infected on treatment and infected naïve of treatment respectively) and between tests (19.6%. 25.7% and 20.5 % for uninfected, infected on treatment and infected naïve of treatment respectively) in the "in-house" R7V ELISA is considered acceptable. However, the data provided in this paper are only based on ELISAs and further isolation and purification of these antibodies must be done in order to verify that the antibodies were true R7V antibodies.

It has previously been shown that purified R7V antibodies from rabbits immunized with the peptide R7V as well as in sera of HIV-1 infected non-progressors, neutralized various subtypes of HIV-1 including subtype C (Galéa *et al*. 1999 a and b; Haslin and Chermann 2007 b). In addition, recombinant R7V antibodies produced in insects were able to neutralize various subtypes of HIV-1 (Haslin and Chermann 2004; Haslin *et al*. 2007 a).

Possible reasons for why data collected here, differs from that in the literature is explained below. The polyclonal antibodies produced by Galéa *et al*. (1999 a) used a different carrier

protein and a different adjuvant and may have used different immunization and blood collection strategies. In addition, different virus stains were used in the neutralization assays. The recombinant antibody fragments used here were prepared by different means than those employed by Haslin and Chermann (2004) and Haslin *et al*. (2007 a). Antibodies produced in insects differ from antibodies produced in mammalian systems. Also the recombinant antibody fragments produced in the current study consist only of the variable fragments of the binding domains of the heavy and light chains of the Fab domain. The lack of a constant region could be the reason for it not binding to the virus. However, single-chain variable fragment (scFv) HIV-1 envelope gp120 human monoclonal antibodies have been produced with exceptional neutralizing activity and breadth of neutralization against different subtypes including subtype C (Zhang and Dimitrov 2007).

Archived sera and plasma containing strong neutralizing antibodies (responsive to other HIV-1 envelope epitopes) were found to have very little R7V antibodies (Figure 3) while selected human sera containing R7V antibodies (positive response in the ELISA) neutralized the HIV-1 subtype C isolate (Figure 4). This could suggest that the neutralization was not due to R7V antibodies or that the R7V epitope was not visible in the particular viral isolate.

The role of immune activation in understanding HIV pathogenesis is increasingly recognized for its importance and can be used for relating immune status or systematic inflammation to disease outcome. Cytokines function to maintain homeostasis of the immune system and are altered by HIV. Th1 cells produce IL-2, INFϒ and TNFα which initiate a cellular immune response to protect against intracellular pathogens. Here the Th1 cells secreted lower cytokine amounts in HIV positive patients. The highest level of TNFα was detected in newly infected patients, this was expected because during acute HIV, the virus makes copies of itself at a high rate and TNFα is an inflammatory cytokine which is used to control viral infections. IL-2 induces th1 cells to produce TNFα and INFϒ The absence of IL-2 at the chronic stage of the disease accounts for the low TNFα and INFϒ detected. HIV infection has been reported to result in the reduction of all Th1 cytokines, especially IL-2, due to loss of T cell function (Macon-Lemaitre et al, 2005). IL-4 drives $CD4^+$ T cells toward a humoral response governed by Th2 cells. These cells produce IL-4, IL-6 and IL-10, amongst others. HIV infection is known to increase Th2 cytokine production resulting in B cell activation and the production of antibodies. Stimulation of PBMCs with R7V was able to increase the production of IL-6. IL-6 was previously described by Williams' et al (2012) as being discriminatory for infected and uninfected individuals. The production of IL-17 by activated CD4 cells was also observed

to be promoted by treatment with R7V, observed mainly on individuals who presented with a CD4 count below 200 cells/mm3 (clinical AIDS).

Conclusion

Data presented here does not support the use of R7V antibodies as indicators of progression or non-progression to disease but rather suggest its use in distinguishing infected compared to uninfected individuals. R7V stimulated Th2 cytokine responses in vitro, especially the dual action IL-6. The data presented here using recombinant R7V antibody fragments suggests the epitope to be more prominent in subtype B.

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Abbreviations used

AIDS, acquired immunodeficiency syndrome; ARV, antiretroviral; β2m, beta-2 microglobulin; ELISA, enzyme-linked immunosorbent assay; HIV, human immunodeficiency virus; LTNP, long term non- progressor; MHC, major histocompatibility complex; rec., recombinant.

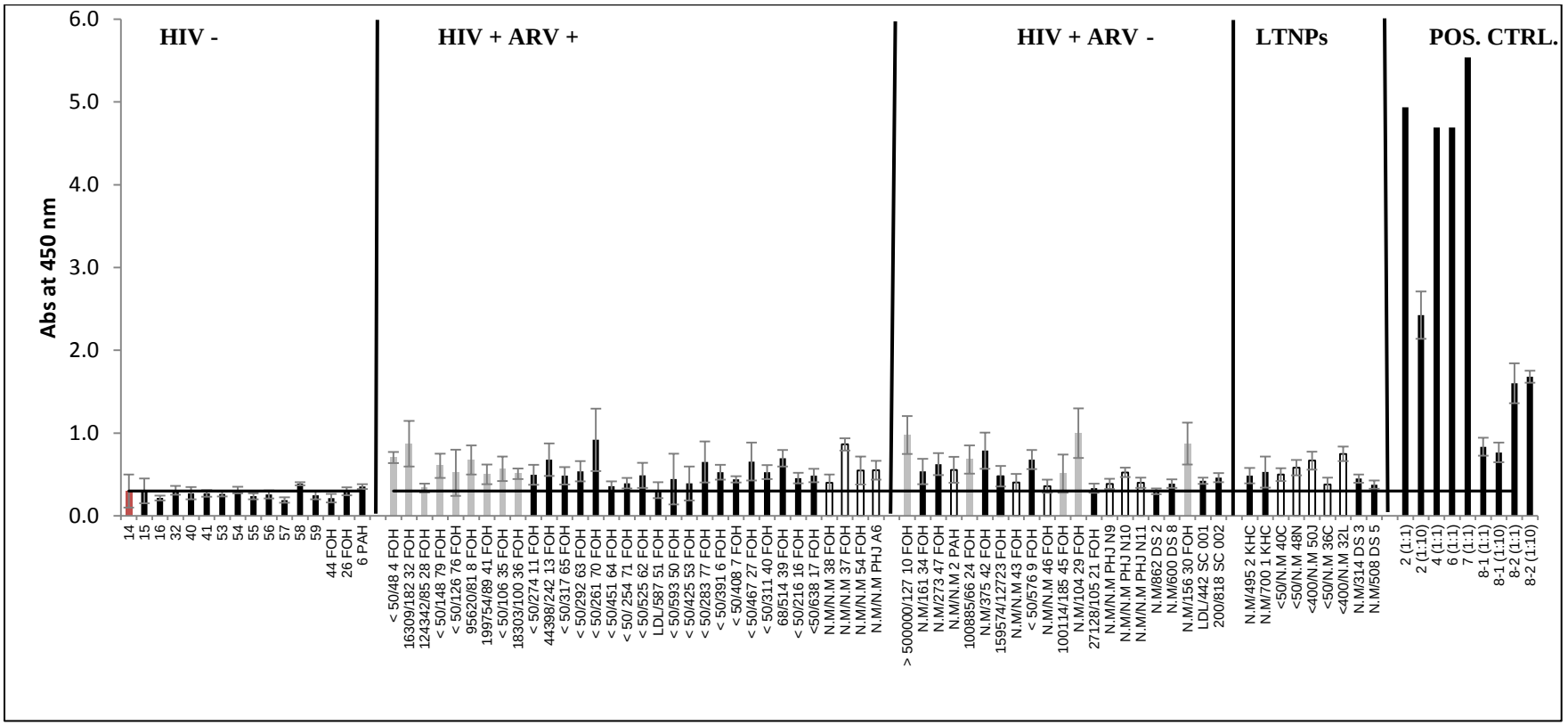

Figure1: Seroprevalence of R7V antibodies in heat-inactivated sera from naturally HIV-1 infected (HIV +) with (+) or without (-) ARV treatment, LTNPs and uninfected (HIV -) individuals. The negative controls are numbered 14-59 (including 44 and 26 FOH and 6 PAH). For HIV positive samples, viral load and CD4 cell counts are indicated in front of the sample number. Not mentioned (N.M) is indicated where information is not available. Colour codes for infected samples indicate the following: gray, individuals with AIDS; black, AIDS not yet developed (or recovered from AIDS after ARV); white, AIDS status unknown because of unknown CD4 cell counts. The line indicates negative control behaviour. Samples with OD values above 0.3 are considered positive for R7V antibodies. Samples with OD values below or equal to 0.3 are considered negative for R7V antibodies. An elevated but marginal reaction was observed for the HIV-positive samples. No general trend was observed for the HIV-positive samples with or without ARV treatment. LTNPs demonstrated the same R7V antibody level of AIDS patients (progressors).

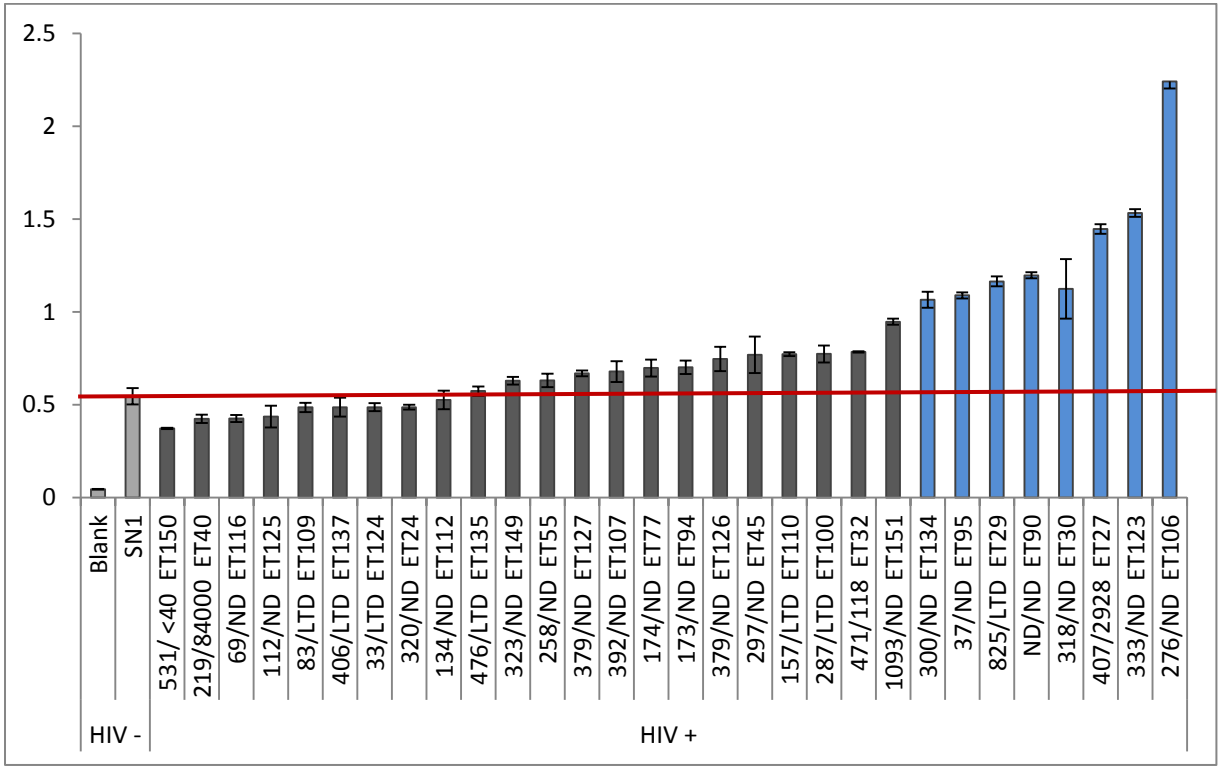

Figure 2: Seroprevalence of R7V antibodies as indicated by an Indirect anti-R7V (1 µg/ml) ELISA. The HIV- samples labelled grey were used as experimental negative controls. The blue represent HIV+ patients diagnosed within a year or less of their bleed date and are recently infected. The black bars represent rest of the HIV positive. The red bar represents the highest negative control and threshold for this assay; individuals with OD readings beyond the red line are considered to have R7V antibodies. The signal was obtained at an absorbance of 450 nm after 60 min incubation period. n=3

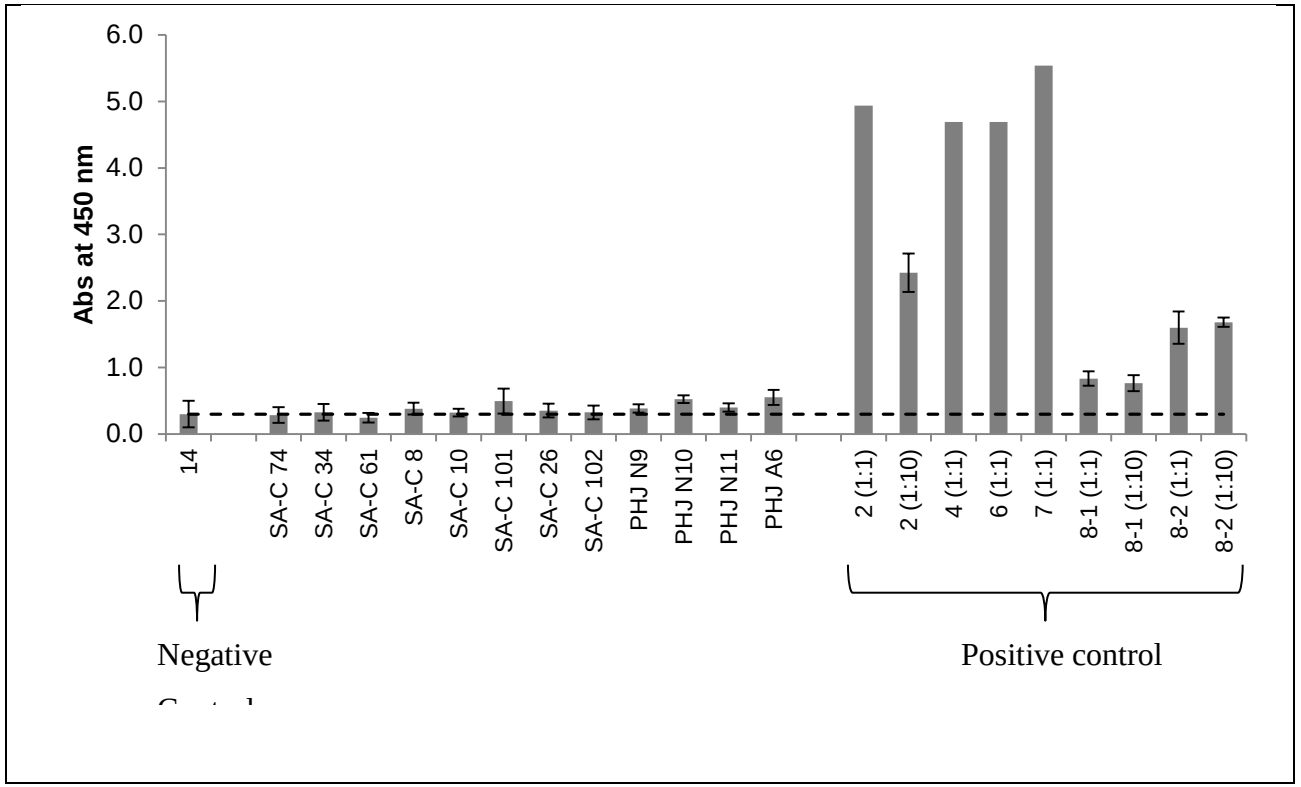

Figure 3. Seroprevalence of R7V antibodies in heat-inactivated serum or plasma from naturally HIV-1 infected individuals tested positive for strong neutralizing antibodies at NICD. The PHJ samples are samples that strongly respond to peptides from the gp41 membrane-proximal external region (MPER). The PHJ samples were sera from patients naïve of ARV treatment except for PHJ A6. The remaining samples (SA-C) were plasma samples from individuals' naïve of treatment. The negative control is uninfected serum and the positive controls are recombinant R7V antibody fragments with different dilutions indicated. Should I only show one? The line indicates expected negative control behaviour. Samples with OD value above 0.3 are considered positive for R7V antibodies. Samples with OD value below or equal to 0.3 are considered negative for R7V antibodies. All samples containing strong neutralizing antibodies did not respond with a strong R7V antibody response.

Table 1. One-way ANOVA test analyzing the statistical significant differences between the sample groups: uninfected (HIV Neg), infected on treatment (HIV + ARV), infected naïve of treatment (HIV + No ARV) and LTNPs.

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.=P-value	95% Confidence Interval	
					Lower Bound	Upper Bound
HIVNeg	HIV+ARV	-.273281*	,046873	,000001	-,40036	-,14621
	HIV+No ARV	-.274535*	,052599	,000010	-,41713	-,13194
	LTNP	-.262729*	,058461	,000152	-,42122	-,10424
HIV+ARV	HIVNeg	.273281*	,046873	,000001	,14621	,40036
	HIV+No ARV	-,001253	,045104	1,000000	-,12353	,12102
	LTNP	,010552	,051820	1,000000	-,12993	,15104
HIV+No ARV	HIVNeg	.274535*	,052599	,000010	,13194	,41713
	HIV+ARV	,001253	,045104	1,000000	-,12102	,12353
	LTNP	,011806	,057052	1,000000	-,14286	,16648
LTNP	HIVNeg	.262729*	,058461	,000152	,10424	,42122
	HIV+ARV	-,010552	,051820	1,000000	-,15104	,12993
	HIV+No ARV	-,011806	,057052	1,000000	-,16648	,14286

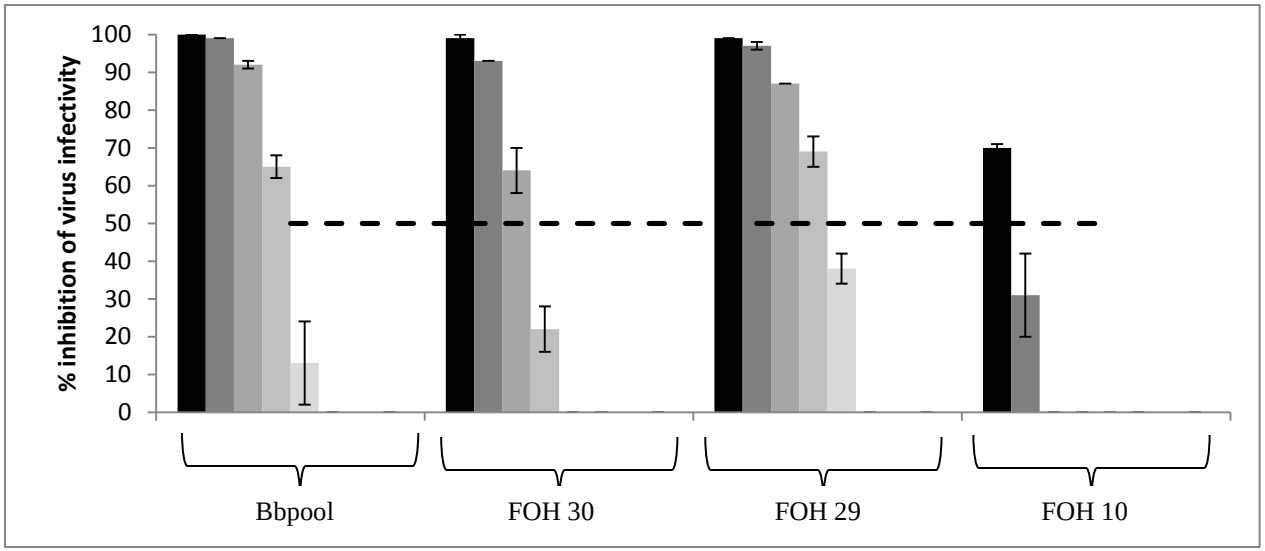

Figure 4. Neutralization ability of serum from naturally HIV-1 infected humans (FOH 10, FOH, 29 and FOH 30) and naïve of ARV treatment. Positive control sample was the Bbpool with known neutralizing ability. The start dilutions of the samples were 1:20 with further 8 three-fold dilutions where the black bar indicates the strongest dilution. The samples were tested for neutralizing ability against the viral isolate Du151.2 (HIV-1 subtype C). The line represents the IC50. No neutralization ability is obtained if the % inhibition is below IC50. The serum samples neutralized the virus at various titers.

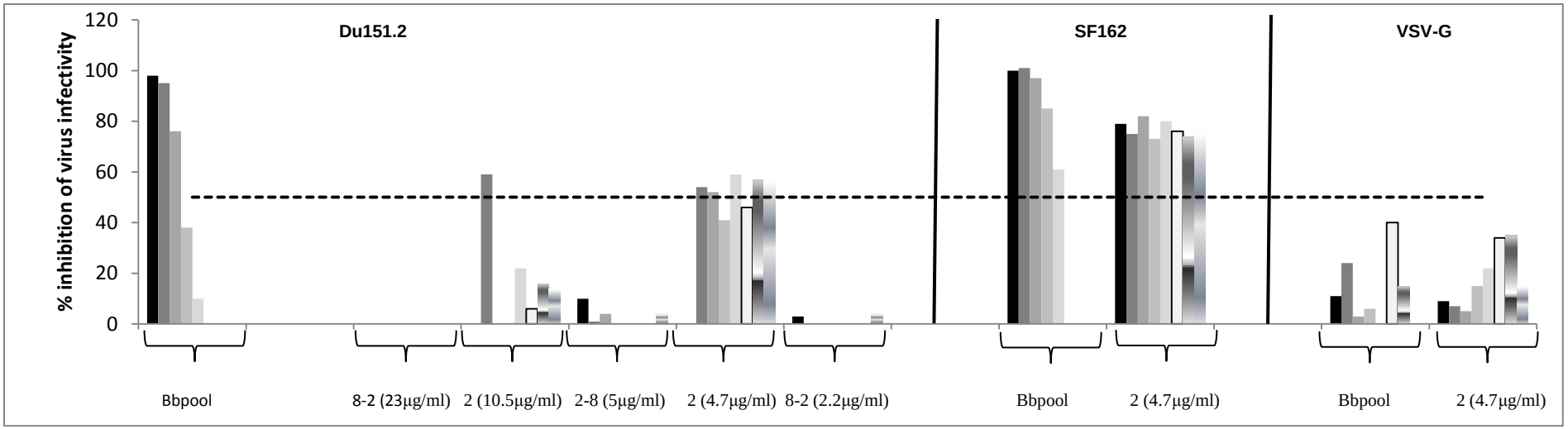

Figure 5. Neutralization ability of recombinant R7V antibody fragments (Clone 2 and 8-2). The start dilution of each sample is indicated in the graph which was followed by 8 three-fold dilutions where the black bar indicates the strongest dilution. Internal positive control sample was Bbpool with known neutralizing ability. The samples were tested for neutralizing ability against the viral isolates; Du151.2, SF162 (sensitive HIV-1 subtype B) and VSV-G (vesicular stomatitis virus). The line of 50% represents the IC50. Titers below IC50 indicate no neutralization ability. No neutralizing ability of Du151.2 was detected for either dilution of the different recombinant R7V antibody fragments. Clone 2 did not respond to VSV-G and was able to neutralize SF162.

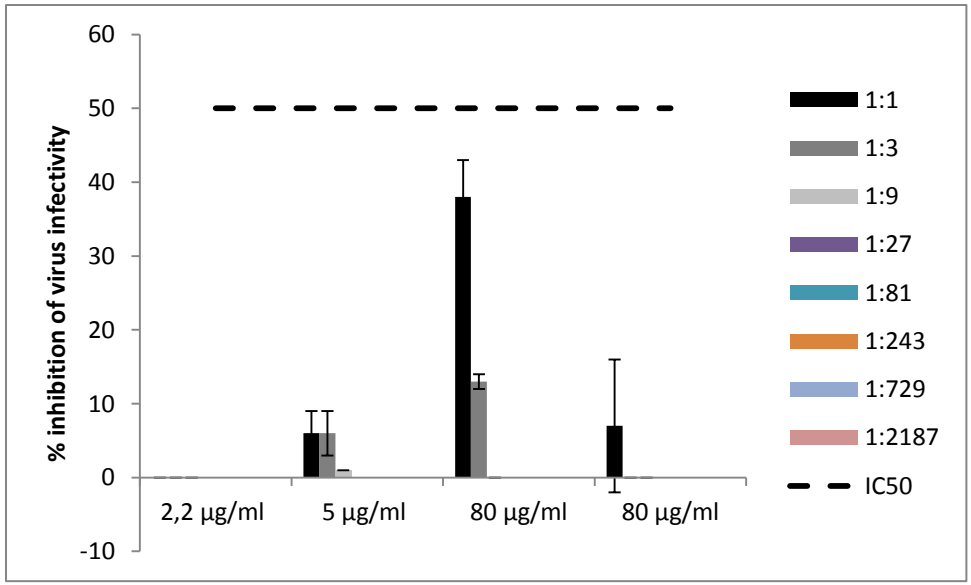

Figure 6. Neutralization ability of polyclonal R7V antibodies from immunized rabbits. Start dilution of each sample is indicated in the graph with further 8 three-fold dilutions. The antibodies were tested for neutralizing ability against the viral isolate Du151.2. Titers below IC50 indicate no neutralization ability. The rabbit R7V antibodies did not neutralize Du151.2 at the different concentrations.

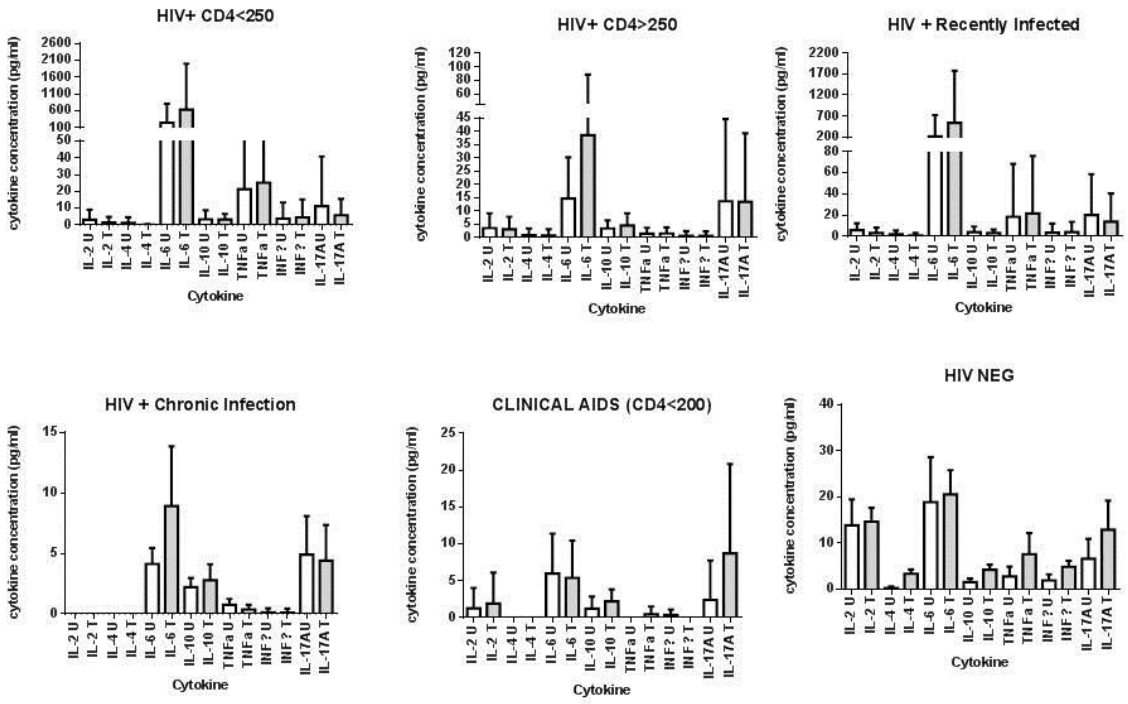

Figure 7: Cytokine profile from peripheral mononuclear cells of HIV infected individuals (n=17) as well as HIV negative donors (n=6) stimulated with 10 µg/ml R7V antigen for a period of 7 days. Th1/Th2/Th17 cytokines were quantified using a multiplex cytokine bead array kit, which makes use of FACS Array. The obtained amounts of cytokines (pg/ml) were grouped according to the following; CD4 count, duration of infection and HIV status; and the means, medians and variation was compared.