Immunology and Virology of HIV-1 Infection in Cameroon

By

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"My help comes from the LORD, the maker of heaven and earth" (Ps 121: 02).

I dedicate this thesis to my mother, brother and sister who have always supported and encouraged me.

To my late father and brother: I will never forget you. Rest in Peace.

To God Be the Glory!!!

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LIST OF ACRONYMS AND ABBREVIATIONS

aa	- amino acid
ADCC	- Antibody Dependent Cell-mediated Cytotoxicity
ADCVI	- Antibody Dependent Cell-mediated Virus Inhibition
agm	- African green monkey
AIDS	- Acquired immunodeficiency syndromes
ANC	- Ancestral/Ancestor
ART	- Antiretroviral Treatment
ARV	- Antiretroviral
APOBEC3G	-ApoliPOprotein B mRNA-editing, Enzyme Catalytic polypeptide-like 3G
Bp	- Base pair
bNAb	- Broadly Neutralizing Antibody
CA	- Capsid
CCR5	- C-C chemokine receptor type 5
cDNA	- Complementary deoxyribonucleic acid
CD4	- Cluster of differentiation 4
CD8	- Cluster of differentiation 8
CEF	- Cytomegalovirus, Epstein-Bar virus and Influenza virus
Chimp	- Chimpanzee
CTL	- Cytotoxic T-lymphocyte
Cons	- Consensus
COT	- Center-of-tree
CRF	- Circulating recombinant form
CXCR4	- C-X-C chemokine receptor type 4

DC-SIGN	- Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DMSO	- Dimethylsulphoxide
DNA	- Deoxyribonucleic acid
DNases	- Deoxyribonucleases
dNTP	- Deoxynucleotide triphosphate
EDTA	- Ethylenediaminetetraacetic acid
ELISpot	- Enzyme Linked ImmunoSpot
Env	- Envelope
FCS	- Foetal Calf Serum
Gag	- Group specific antigen
Gor	- gorilla
Gp	- Glycoprotein
HAART	- Highly Active Antiretroviral Treatment
HIV	- Human Immunodeficiency Virus
HLA	- Human leucocyte antigen
HRP	- Horseradish Peroxidase
IAVI	- International AIDS Vaccine Initiative
IFN-γ	- Interferon gamma
IgG	- Immunoglobulin G
IL-	- Interleukin-
IQR	- Interquantile Range
INT	- Integrase
kD	- KiloDalton
LANL	- Los Alamos National Library
LTNP	- Long-Term Non-Progressors
LTR	- Long terminal repeat

mAb - Monoclonal antibody

MA - Matrice

MEGA - Molecular Evolutionary Genetics Analysis

MIP-1α - Macrophage Inflammatory Protein one-Alpha

MIP-1β - Macrophage Inflammatory Protein one-Beta

mM - Milimolar

ml - Millilitre

MRCA - Most Recent Common Ancestor

MPER - Membrane-Proximal External Region

NC - NucleoCapside

Nef - Negative factor

NK - Natural Killer

NNRTI - Non-Nucleoside Reverse Transcriptase Inhibitor

PBMC - Peripheral Blood Mononuclear Cells

PBS - Phosphate Buffer Saline

PCR - Polymerase Chain Reaction

PrEP - Pre-exposure prophylaxis

PHA - Phytohaemagglutinin

Pol - Polymerase

PTE - Potential T cell Epitope

Ptt - Pan troglodytes troglodytes

p-value - Probability value

Rev - Regulator of expression virion

RNA - Ribonucleic acid

RNases - Ribonucleases

RPMI - Roswell Park Memorial Institute media

RT	- Room temperature (15^0C-25^0C) or Reverse Transcriptase
SIV	- Simian Immunodeficiency Virus
SFU	- Spot Forming Units
SHIV	- Simian/Human Immunodeficiency Virus
sm	- sootey mangabey
STLV	- Simian T-Cell Leukemia Virus
Tat	- Transcriptional transactivator
TCR	- T cell receptor
TNF-α	- Tumor Necrosis Factor-alpha
UNAIDS	- United Nations Common Programme on HIV/AIDS
UV	- Ultraviolet
URF	- Unique Recombinant Form
Vif	- Viral infectivity factor
Vpr	- Viral protein r
Vpu	- Viral protein u
µg	- Microgram
µl	- Microlitre
^{0}C	- Degrees Celcius

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ABSTRACT

Background. The extraordinary variability of HIV poses a major obstacle to HIV vaccine development. The effectiveness of a vaccine is likely to vary in different populations infected with different HIV subtypes, unless innovative vaccine immunogens are developed to protect against the range of HIV diversity. Artificial sequences such as group M consensus have been designed to reduce the genetic distance between a vaccine strain and contemporary circulating viruses. Recently developed vaccine immunogens, mosaic sequences, extend this concept to additionally contain potential T cell epitope (PTE) variants. The degree to which peptides based on these immunogens are recognised in HIV-infected populations with a high degree of viral diversity has not been evaluated. An example of such a country is Cameroon, which is home to multiple HIV subtypes and recombinants. Given the potential impact of HIV diversity on vaccine development, it is particularly important that molecular epidemiological surveillance is continued in HIV diversity hotspots such as Cameroon.

Methods. HIV-1 genetic diversity was characterised in 59 plasma samples from HIV-infected blood donors from Cameroon. HIV *gag* (n=50) and *nef* (n=55) sequences, as well as near full length sequences of virus genomes (n=9) were generated, and phylogenetic analyses were performed. Maximum likelihood phylogenetic trees were constructed from these sequences, following either complete removal of recombinant sequence fragments or the division of recombinant sequences into their constituent fragments by a blinded fully exploratory screen for recombination using RDP3 for *gag* and *nef* sequences. To investigate the extent of the diversity on T cell immune response magnitude, breadth, specificity and immunodominance patterns, peptides based on HIV-1 group M consensus Gag and Nef were used in IFN-γ ELISPOT assays in (i) Cameroonians, where individuals infected with the predominant CRF02_AG subtype (n=24) were compared with those infected with diverse non-CRF02_AG subtypes (n=22); and (ii) in a multiclade epidemic, namely Cameroon, compared with a monoclade C epidemic, South Africa (n=44). In addition, HIV-specific responses to PTE Gag and Nef in HIV-infected individuals from Cameroon (n=32) were investigated to determine whether the detection of responses differed compared to consensus M responses.

Results. All *gag* and *nef* sequences clustered within HIV-1 group M. Thirteen subtypes were identified, with circulating recombinant form CRF02_AG dominating the epidemic,

accounting for 50% of the studied infections. In addition, 22% of the studied viruses had *gag* and *nef* genes from viruses belonging to different subtypes. Interestingly, five *gag* sequences (10%) and three (5%) *nef* sequences were neither obviously recombinant nor easily classifiable into any of the known HIV-1 group M subtypes. Of the full genome sequences generated, three were too divergent to be placed within any existing subtype or CRF grouping and therefore remain unclassified. IFN-γ ELISPOT data showed that there were no differences in the magnitude and breadth of responses to peptides based on HIV-1 group M consensus Gag and Nef for CRF02_AG and non-CRF02_AG-infected individuals. In contrast, the specificity of epitope targeting was markedly different between the two groups, with less than one third of peptides (11/28) commonly recognised. Furthermore, only one peptide was commonly recognised by at least three individuals from both AG and non-CRF02_AG groups, indicating poor immunodominance. For Nef, more than half of all targeted peptides (14/27) were recognised by both groups, and four peptides were commonly targeted by at least three individuals. Similar results were obtained when South Africa, representing a monoclade C epidemic, and Cameroon, a multiclade epidemic, were compared. The magnitude of responses was significantly higher ($p=0.0045$) for Gag when detectable responses to PTE peptides were compared to consensus peptides, although this was not the case for Nef. The breadth of responses was, however, similar when the two set of reagents were compared, both in Gag and Nef. Further analyses in Nef revealed that the magnitude and breadth of responses were similar in CRF02_AG and non-CRF02_AG-infected individuals. Finally, recognition of multiple variants was detected with the PTE peptide set, with up to five variants being recognised for particular peptides.

Conclusion. This study confirms the widespread existence of highly divergent HIV lineages in Cameroon. While the genetic complexity of the Cameroonian HIV-1 epidemic has potentially serious implications for the design of biomedical interventions, detailed analyses of divergent Cameroonian HIV-1 group M lineages could be crucial for dissecting the earliest evolutionary steps in the emergence of HIV-1 group M. In addition, the central nature of HIV-1 consensus M sequences resulted in their broad recognition, but failed to identify highly immunodominant peptides between homogeneous and diverse HIV epidemics. Further refinement of these immunogens may contribute to the development of a globally relevant vaccine. Finally, the use of PTE peptides did not increase the breadth of T cell recognition in

this divergent population when compared to consensus M peptides. This underlies the need to include more mosaic peptides representing the variety of viruses that circulate in the region.

CHAPTER 1: LITERATURE REVIEW

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1.1 INTRODUCTION

The Acquired Immunodeficiency Syndrome (AIDS) pandemic is more than 30 years old. The causative agent, human immunodeficiency virus type one (HIV-1), was discovered in 1983 (Barre-Sinoussi *et al*., 1983) and is responsible of the global epidemic. A second, closely-related virus was identified in 1986 in an individual in west Africa and was termed HIV-2 (Clavel *et al*., 1986). The first antiretroviral (ARV) drug, zidovudine, was used in 1986 (Davtyan *et al*., 1987) and effective combination therapy in 1996 (Hogg *et al*., 1997). According to the UNAIDS 2013 report, the global face of HIV/AIDS has changed in recent years. The number of people living with HIV in late 2012 was estimated at 35.3 million, an increase of 18% compared to 2001, primarily as a result of an increase in the number of infected people taking antiretroviral therapy (ART), and not due to an increased infection rate, since the number of new infections continues to decline. There were 2.3 million new HIV infections in 2012, 33% fewer than in 2001. Much of this success has been recorded in the last four years, and can be attributed to the rapid improvement in access to ART. At the same time the number of AIDS deaths is also declining, with 1.6 million AIDS deaths in 2012, down from 2.3 million in 2005 (UNAIDS 2013). The number of newly-infected people continues to decline also in sub-Saharan Africa (SSA), the worst-affected region, with over 70% of all people living with HIV in 2012, while this region accounts for only 12% of the world's population (UNAIDS, 2013). Whilst differences in HIV prevalence still exist in different African regions, no country saw an increase in the incidence of HIV/AIDS in 2012 (UNAIDS, 2013). Mother to child transmission (MTCT) in developing countries represents about 90% of HIV infections amongst children, and most of them (90%) live in SSA. Despite the overall reduction of HIV infections, AIDS continues to heavily influence maternal and infant mortality (UNAIDS, 2013).

Besides the success in access to ARVs, there have been a number of other successes in the goal to reduce the burden of HIV/AIDS. Several prevention methods have proved efficacious in recent years. The latest data from Kisumu (Kenya) and Orange Farm (South Africa), sites of two randomised trials on voluntary medical male circumcision (MMC), demonstrated long term efficacy of this practice against HIV acquisition (Mehta *et al*., 2013; Lissouba *et al*., 2011). Pre-exposure prophylaxis (PrEP), which involves HIV-uninfected persons taking the same HIV drugs as those used to treat infected people, also showed very

encouraging results for HIV prevention. The first evidence that PrEP might work when applied locally at the site of exposure came from a clinical trial conducted in South Africa, which found that vaginal tenofovir gel was safe, well tolerated, and decreased the risk of contracting HIV by 39% in women (Abdool Karim *et al*., 2010). The efficacy exceeded 50% among participants who used the gel more consistently. The iPrEx trial subsequently revealed that oral tenofovir and emtricitabine, taken daily, reduced the risk of HIV infection among men who have sex with men by 44% (Grant *et al*., 2010). Furthermore, the HPTN052 study showed a 96% prevention of sexual transmission of HIV in heterosexual discordant couples when the infected sexual partner was on ART (Cohen *et al*., 2011). These results are extremely promising, as they showed that oral or local vaginal application of ARVs on a sustained basis, before and after exposure, may reduce the risk of HIV acquisition.

Although full implementation of these preventive measures has the potential to drastically reduce the spread of HIV, they invariably face the huge challenges of cost and social behaviour change, including adherence. Therefore, a highly efficacious vaccine that would prevent HIV infection and/or progression to AIDS is urgently needed. The development of such a vaccine faces many challenges, including the huge genetic diversity the virus exhibits, the incomplete knowledge of immune correlates of an effective immune response against the virus, and how to generate such a response with a vaccine. Understanding all these challenges are of great importance in the design of an effective HIV vaccine. This chapter provides an overview of the key concepts in HIV biology, diversity, immunity and vaccine design and testing pertinent to the studies described in this thesis.

1.2 HIV-1 STRUCTURE AND GENOMIC ORGANISATION

HIV-1 is an RNA virus from the family of retroviridae which uses DNA as an intermediary for its replication (Coffin, 1992). The virus itself belongs to the group lentiviruses. [In this chapter, when mentioning HIV, we will in most cases be referring to HIV-1, the type responsible for the global pandemic. HIV-2 is less pathogenic than HIV-1 and largely restricted to west Africa, with limited spread to other countries.] A number of other lentiviruses have been isolated from primates. For historical reasons, all lentiviruses isolated from humans are HIV and those isolated from simian primates are called simian

immunodeficiency viruses or SIVs. Each viral particle, or virion, is made of a lipid bilayer envelope of 80-120 nm in diameter and contains two copies of the viral genome (Wain-Hobson *et al.*, 1985; Figure 1.1).

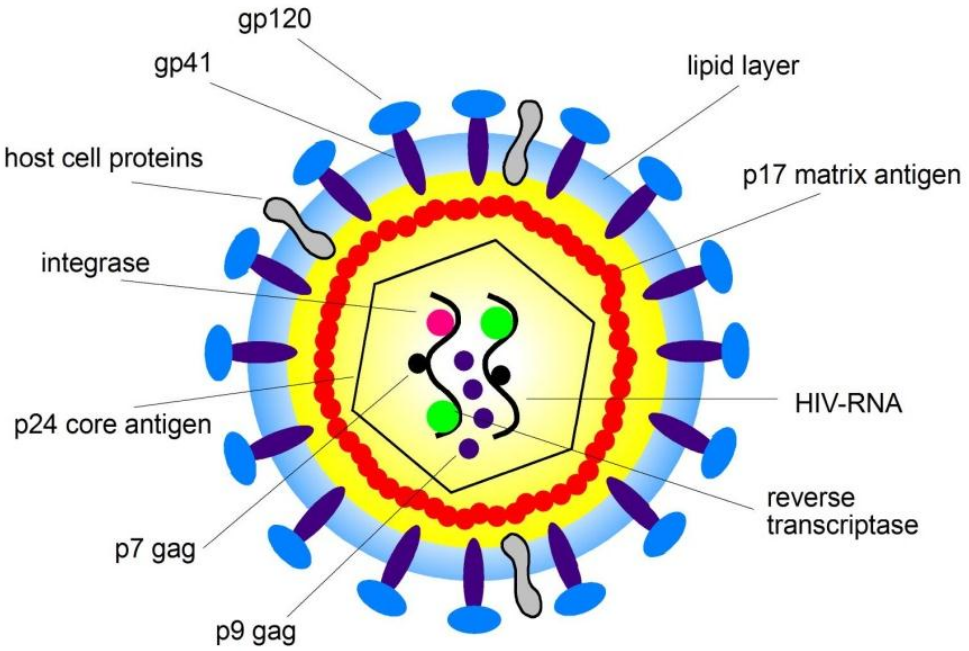

Figure 1.1 Structure of an HIV-1 virion. Envelope glycoproteins gp120 and gp41 are protruded from a lipid bilayer originated from the host cell plasma membrane. The viral core consists of two copies of positive single stranded RNA enclosed by the viral capsid protein p24 together with the reverse transcriptase and integrase enzymes required for the formation of new HIV particles (taken from Rubbert *et al.*, 2011).

The HIV-1 genome consists of nine genes that code for 15 proteins (Figure 1.2). Like other retroviruses, it has three major genes that code for the structural proteins, and in contrast to other retroviruses, HIV encodes six genes that are regulatory and accessory proteins, involved in the regulation of the viral life cycle or virion infectivity.

1.2.1 Structural proteins

1.2.1.1 Gag

The *gag* (group of antigen) gene codes for the 55 kD Gag protein, a molecule that associates with the cell membrane, and during viral assembly recruits all the viral proteins to form the building block of the viral particle core (Bryant *et al*., 1990). After budding, the Gag precursor is cleaved by the viral protease during the maturation of the virus into four small proteins called matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p9), and p6 (Gottlinger *et al*., 1989).

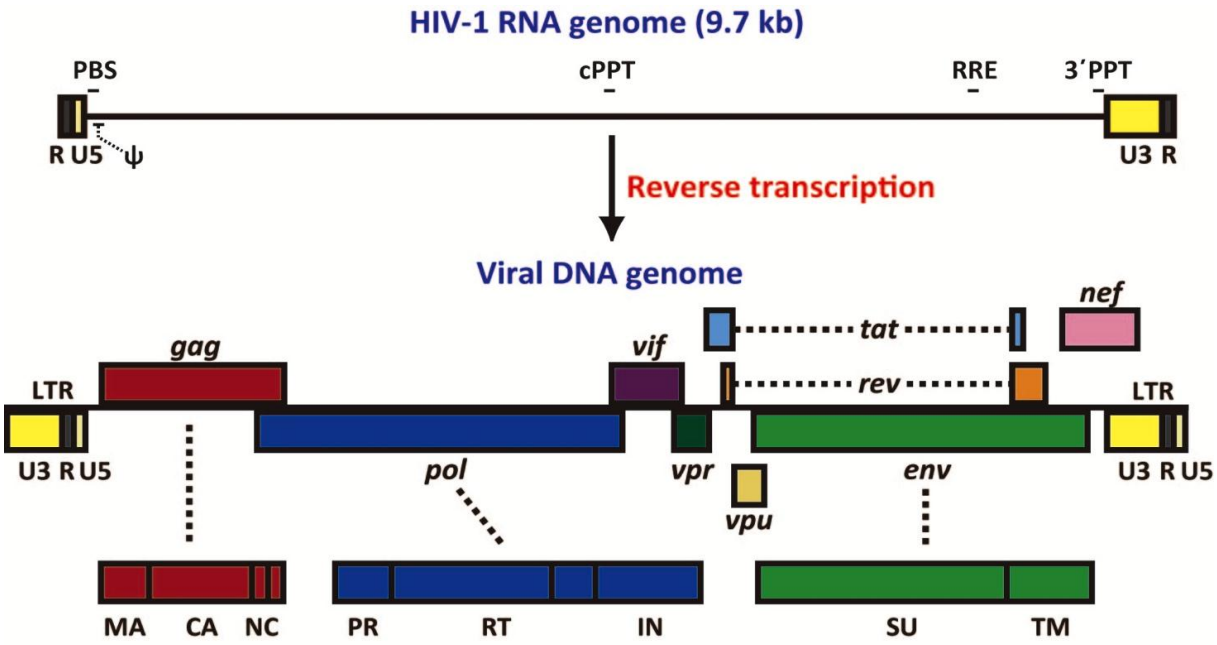

Figure 1.2 A diagrammatic representation of the genetic organisation of HIV genome. The proviral genome is composed of nine genes that are flanked by two long terminal repeats (LTR, 5' and 3'). The nine open reading frames code for at least 15 proteins. The *gag* gene is translated into the structural Gag precursor that is cleaved into matrix (MA), capsid (CA), nucleocapsid (NC). The *pol* gene is translated by a -1 ribosomal frameshift as a Gag-Pol precursor polypeptide that yields the four enzymes Reverse Transcriptase (RT), Integrase (IN), Protease (PR) and Ribonuclease H (RNaseH) upon cleavage. The *env* gene encodes the anchor structural precursor Env, gp160, which is cleaved into the surface SU (gp120) and trans-membrane TM (gp41) glycoproteins. The Gag-Pol mRNAs are spliced to encode the regulatory (Rev, Tat) and accessory (Nef, Vpr, Vpu and Vif) proteins (taken from Yasutsugu and Youichi, 2011).

The matrix is derived from the N-terminal, myristoylated end of p55. The inner surface of the virion lipid bilayer is where most MA molecules remain attached and this aids in stabilising the particle (Gallay *et al*. 1995). This region of Gag also contains a nuclear localisation domain that facilitates the nuclear transport of the viral genome and allows HIV to infect non-dividing cells (Lewis *et al*., 1992, Fassati, 2006).

The CA protein or p24 interacts with cellular cyclophilin A and this leads to its incorporation into HIV particles (Franke et *al*., 1994; Thali *et al*., 1994). Cyclophilin A is a cytosolic human protein involved in protein folding (Zydowsky *et al*., 1992). It has been shown that decreased levels of virion-associated cyclophilin A result in reduced virion infectivity (Thali *et al*., 1994) and decreased HIV replication (Franke and Luban, 1996). CA also plays a role in the incorporation of viral enzymes into the capsid via the Gag-Pol precursor (Bukovsky and Gottlinger, 1996; Huang and Martin, 1997).

The NC region of Gag, also referred to as p9, is important for transmission and pathogenesis of the virus. It is responsible for specifically recognising the packaging signal of the genome, and introducing two copies of the viral genome into progeny virions (Heath *et al*., 2003; Dussupt *et al*., 2011). In addition, NC protein, a highly basic protein with two zinc fingers (Harrison and Lever, 1992), is indispensable for RNA dimerization, encapsidation and the initiation of reverse transcription (Lapadat-Tapolsky *et al*., 1993).

The p6 protein, one of the most variable regions of Gag, is situated at its C-terminal domain; it mediates interactions between Gag and the accessory protein Vpr, leading to the incorporation of Vpr into assembling virions (Paxton *et al*., 1993). It has additional roles in the viral cycle, including binding to Tsg101 and ALIX, cellular factors important for viral budding from infected cells (Heath *et al*., 2003).

The p24 region of Gag is relatively conserved compared to the p17 and nucleocapsid, and targeting of epitopes within p24 region of the HIV Gag protein have been shown to be associated with viral control (Zuniga *et al*., 2006). Overall, CD8+ T cell escape mutations in the Gag protein, particularly p24 epitopes, have been associated with a fitness costs to the virus (Ammaranond *et al*., 2011; Martinez-Picado *et al*.. 2006; Troyer *et al*., 2009), suggesting that Gag is a good target for inclusion in a vaccine.

1.2.1.2 Pol

The *pol* (polymerase) gene codes for the expression of enzymes involved in viral replication (reverse transcriptase or RT), integration (integrase or INT) and maturation (protease or Pro) of the virion. It is expressed as a Gag-Pol precursor (Jacks *et al*., 1988) which is then cleaved to release the Pol polypeptide; the latter is then cleaved into three enzymes. This occurs during viral maturation and is performed by the virally-encoded protease (Parkin *et al*., 1992). Reverse transcriptase is an RNA-dependent polymerase that synthesises DNA from viral RNA (Zack *et al*., 1990). The newly formed DNA is then integrated into the host genome by the viral integrase (Craigie *et al*., 2001). The *pol* gene encodes Pro and RT, which are two major targets of ARVs (Esnouf *et al.*, 1995; Spence *et al*., 1995). In addition, *pol* is one the more conserved genes of HIV and is frequently targeted by the host immune system (Gea-Banacloche *et al*., 2000; Haas *et al*., 1998).

1.2.1.3 Env

The *env* gene encodes a 90 kDa precursor protein that undergoes post-translational modifications including glycosylation, to give rise to a 160 kDa glycoprotein. This protein is cleaved by cellular proteases to give rise to two mature proteins, the transmembrane glycoprotein gp41 and the surface envelope protein gp120 (Capon et *al*., 1991). Env is present on the surface of infected cells and virions as a trimer, a complex of three molecules (Bernstein *et al*., 1995). Gp120, by interacting with CD4 cell receptors and the main co-receptors CCR5 or CXCR4, initiates the fusion process which allows the virus to enter into the cell (Feng *et al*., 1996; Deng *et al*., 1996). Five constant domains (C1-C5) have been identified in the protein, including the CD4 binding site, a target of neutralising antibodies, and five variable domains (V1-V5). These variable regions are the result of errors made by the RT enzyme and are highly immunogenic. The mutations are predominantly single base substitutions (Vartarian *et al*., 1991), suggesting that they arose as a result of misincorporations during RNA or DNA synthesis (Preston *et al*., 1988). One of these regions, the V3 loop, determines when HIV-1 preferentially targets T cells or macrophages (Hwang *et al*., 1991). The V3 loop also contains major neutralisation epitopes (Moore and Nara, 1991; Zolla-Pazner *et al*., 2007), along with the V1-V2 loop (Moore *et al*., 2013). The gp41 protein plays an essential role in the fusion of the virus and target cells; through this action, it facilitates the release of the viral contents into the cytoplasm (Camerini *et al*., 1990). The Env

protein is of particular interest in the design of an effective HIV vaccine as neutralising antibodies target specific regions of Env, and will likely be required to be elicited for an effective HIV vaccine.

1.2.2 Regulatory Proteins

1.2.2.1 Tat

Tat or transactivator (p15) protein is essential for viral replication (Ruben *et al*., 1989). Tat is a transcriptional transactivator and binds to the transactivation response element (TAR) region of the newly formed mRNA LTR and this activates transcription (Roy *et al*., 1990). In the absence of Tat, transcription is prematurely terminated by an interruption of elongation of mRNA (Herrmann and Rice, 1995; Wei, *et al*., 1998). Tat requires a cellular co-factor, cyclin T, which facilitates the binding of Tat to the TAR (Wei *et al*., 1998).

1.2.2.2 Rev

Rev or regulator of viral expression, is a 13 kDa sequence-specific RNA binding protein (reviewed by Zapp, 1989), and functions in the cell nucleus. Like Tat and Nef, Rev is translated during the early phase of viral replication (Malim *et al*., 1989). It facilitates the export of mRNA which codes for Gag, Pol and Env from the nucleus to the cytoplasm (Felber *et al*., 1990), and therefore increases the expression of these proteins. It also down-regulates small mRNAs coding for regulatory proteins, including itself and Tat (Felber *et al*., 1990). Rev is absolutely required for HIV-1 replication; proviruses that lack Rev function are transcriptionally active but do not express viral late genes and thus do not produce viable virions (Hope *et al*., 1992).

1.2.3 Accessory Proteins

1.2.3.1 Vpr

Vpr or viral protein regulatory (p15) is a late-synthesised protein of 96 amino acids that is incorporated into viral particles in approximately the same amount as Gag (Nitahara-Kasahara *et al*., 2007). It plays a role in the ability of HIV to infect target cells, particularly non-dividing cells (Heinzinger *et al*., 1994). Vpr can also stop the viral cycle in G phase when viral replication is very active (Jowett *et al*., 1995).

1.2.3.2 Vpu

Vpu or viral protein unknown (p16) is a membrane protein consisting of 81 amino acids and is not incorporated into viral particles (Federau *et al*., 1996; Willbold *et al*., 1997). Vpu and Env are expressed from the same mRNA, but Vpu is translated much less efficiently than Env (Schwartz *et al*., 1990). Unlike other accessory proteins, Vpu protein is only found in HIV-1 and in SIV (simian immunodeficiency virus) infecting chimpanzees and gorillas (Gao *et al*., 1999). In HIV-2 and other SIVs, it is replaced by the Vpx protein (Courgnaud *et al*., 2003). Vpu forms an oligomeric complex on the membrane of the cell where it downregulates the expression of CD4 (Willey *et al*., 1992; 1992). Unlike Nef, Vpu does not directly downregulate CD4 from the cell surface (rather, it targets newly-made CD4 in the endoplasmic reticulum for degradation). It also inhibits the expression of MHC class I molecules (Derrien *et al*., 2004). Vpu can also help HIV-1 to overcome the restriction imposed by tetherin by antagonising its activities (Neil *et al*., 2008; Van Damme *et al*., 2008). Tetherin is an antiviral protein that can inhibit the cellular release of HIV (Neil *et al*., 2008; Van Damme *et al*., 2008).

1.2.3.3 Vif

Vif or virion infectivity factor is a 23 kDa protein produced late under Rev dependence. It is essential for viral infectivity as a deletion of this gene appears to decrease the virus infectivity (Kishi *et al*., 1992). HIV Vif counteracts the action of the antiviral restriction factor APOBEC3G, and prevents its incorporation into viral particles (Simon *et al*., 1998). APOBEC3G is an intracellular antiviral protein that results in defective virions by deaminating the minus-strand of the viral genome, inducing numerous G to A mutations (Fan *et al*., 2010; Yu *et al*., 2004). Vif-deficient viruses incorporate APOBEC3G, making them non-infectious (Mariani *et al*., 2003). This activity is very specific as SIV Vif does not have the same effect in human cells (Simon *et al*., 1998).

1.2.3.4 Nef

Nef, or negative factor, is a 27 kDa protein that contains a myristol residue at its N-terminal and is associated with the cell membrane (Welker *et al*., 1998). Nef is expressed in the early stage of viral infection and is the first viral protein to accumulate to detectable levels in a cell following HIV-1 infection (Kim *et al*., 1989). Its plays an important role in the

pathogenesis of HIV-1; this has been well established in humans where individuals infected with a virus harbouring deletions in Nef remained asymptomatic for 10-14 years after sero-conversion (Rhodes *et al*., 2000; Salvi *et al*., 1998; Tolstrup *et al*., 2006). The same observations were also seen in macaques where an SIV mutant with a deleted Nef had a very long incubation period before developing AIDS (Kirchhoff *et al*., 1992). Nef has multiple activities, including the downregulation of cell surface expression of CD4 and the activation of T cells, a cellular state required for optimal viral replication and the stimulation of HIV infectivity. Nef increases the rate of CD4 endocytosis and lysosomal degradation and therefore inhibits the expression of CD4 (Aiken *et al*., 1994; Luo *et al*., 1996). In addition to this, Nef also down-regulates CCR5 and CXCR4, to avoid superinfection of cells (Michel *et al*., 2005). Nef also downregulates surface expression of MHC class I and II molecules in infected cells (Schwartz *et al*., 1996) and this hampers antigen presentation. Nef also stimulates the infectivity of HIV virions. HIV-1 particles produced in the presence of Nef can be up to ten times more infectious than virions produced in the absence of Nef (Miller *et al*., 1994). Finally, another major activity of Nef is to induce apoptosis in both infected and uninfected T cells. Nef induces the expression of both Fas (CD95) and the Fas ligand (CD95L) in infected cells, with CD95L aiding immune evasion by inducing the apoptosis of HIV-specific T cells (Peterlin and Trono, 2003).

The HIV genome contains two non-coding regions located at the 5' and 3' end, which form the long terminal repeats (LTR; Verhoef *et al*., 1999; Montano *et al*., 1998). This region plays a role in the replication of the virus by regulating the expression of viral genes. The 5' region contains the initiation site for transcription, and binding sites for several transcription factors, including the TAR which allows Tat to bind to viral RNA (Verhoef *et al*., 1999; Montano *et al*., 1998).

1.3 HIV DIVERSITY

1.3.1 Origin of HIV

SIVs have been identified in many species of non-human primates in Africa. Only African non-human primates are naturally infected with SIV, and no natural SIV infection has

been detected to date in monkeys living in Asia or in the new world (Aghokeng *et al*., 2005; Apetrei *et al*., 2005), with only one exception, isolated from a captive rhesus macaque (Hirsch *et al*., 1989). This macaque is thought to have acquired SIV in captivity by cross-species transmission from a SIV-infected African primate (Hirsch *et al*., 1989). Thus far, 40 different primate species present serological evidence of SIV infection (Klatt *et al*., 2011). Most of the primate hosts harbour a single SIV strain, however cross transmissions have occurred between species (Jin *et al*., 1994; Van Rensburg *et al*., 1998), which has given rise to new circulating SIV lineages (Van Heuverswyn *et al*., 2006). Five equidistant phylogenetic lineages based on phylogenetic analysis of full-length Pol protein sequences have been described (Hahn *et al.*, 2000). These lentiviruses appear to cause no adverse effects in the majority of these primates (Hahn *et al.*, 2000).

The first SIV full length genome observed to have the same genetic structure as HIV-1 was referred to as SIVcpz-gab (Huet *et al*., 1990). The organisation of the genome showed the presence of a *vpu* gene and the absence of the *vpx* gene, common to HIV-2 and most other SIVs. A second complete genome of a chimpanzee SIV, referred to as SIVcpz-ant, was characterised and found to have a similar genetic organisation to SIVcpz-gab (Vanden Haesevelde *et al*., 1996). From protein sequence comparisons, SIVcpz-ant was found to be a closer relation to SIVcpz-gab and to HIV-1 isolates than to members of the other four major phylogenetic lineages of these primate lentiviruses. After isolation of a third full length SIV strain, SIVcpz-us, and determining by mitochondrial DNA analysis the subspecies identity of all known infected chimpanzees, Gao *et al*. (1999) observed that one subspecies of chimpanzee, *P.t. troglodytes*, was the most probable natural ancestral host for HIV-1. Recently, Keele *et al*. (2006) confirmed that *P. t. troglodytes* is in fact the reservoir host of HIV-1 groups M and N (Figure 1.3). Using newly developed sampling techniques, up to 35% of individuals in some communities of wild living *P. t. troglodytes* were infected by the virus (Keele *et al*., 2006).

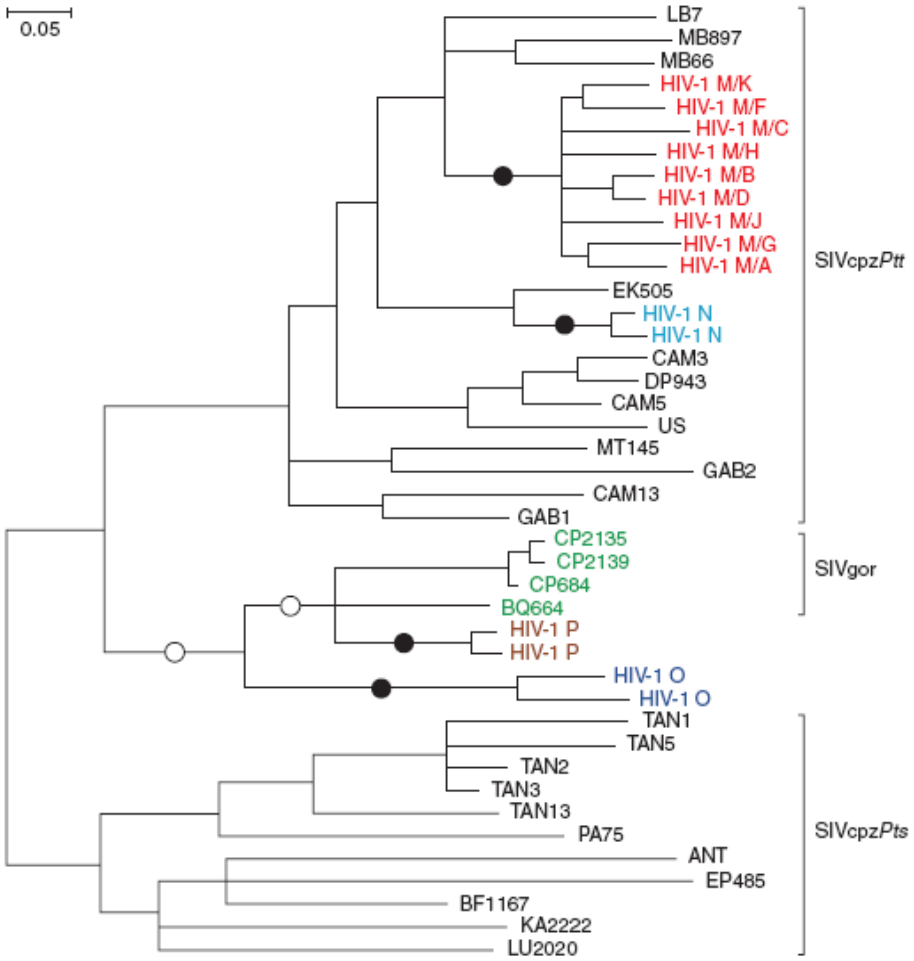

Figure 1.3 Cross species transmission. Maximum likelihood tree displaying the relationship between the four groups of HIV-1 and their SIV relatives across viral *pol* gene of the genome. All SIVcpz and SIVgor sequences are shown in black and green, respectively. The four groups of HIV-1, each of which represents an independent cross-species transmission, are shown in different colours. Black circles indicate the four branches where cross-species transmission-to-humans has occurred. White circles indicate two possible alternative branches on which chimpanzee-to-gorilla transmission occurred (taken from Sharp and Hahn, 2011).

The predecessor to group M (SIVcpz*ptt*) appears to have been a recombinant strain between two primate SIVs, namely red-capped mangabeys (SIVrcm) and greater spot-nosed monkeys (SIVgsn; reviewed by Holmes, 2001; Bailes *et al.*, 2003). Both SIV from red-capped mangabeys and greater spot-nosed monkeys could have infected a single chimpanzee host, as it is known that chimpanzees hunt smaller monkeys for food in overlapping geographical locations within central Africa (reviewed by Mitani and Watts, 1999).

Another SIV was later described in a second species of ape, western gorillas (*Gorilla gorilla*) in Cameroon. Surprisingly, this new virus (SIVgor) formed a monophyletic group closely related to HIV-1 group O and P (Figure 1.3), but chimpanzees were also shown to be the reservoir of SIV found in these gorillas (Van Heuverswyn *et al*., 2006). It is unclear how gorillas became infected, since they are strictly herbivores and are not known to have close contacts with other primates, especially chimpanzees (Van Heuverswyn *et al*., 2006).

HIV-1 group M (Main or Major) is responsible for the global epidemic (Robertson *et al*., 2000; Hemelaar *et al*., 2011). For unknown reasons the group O (Outlier) has largely remained endemic to Cameroon where it is responsible for 1-5% of HIV-1 infections (Mauclere *et al*., 1997; Yamaguchi *et al*., 2004; Ayouba *et al*., 2001). Despite the low prevalence, cases have been isolated in Gabon, Equatorial Guinea, Nigeria, Benin, Ivory Coast, Togo, Senegal, Niger, Chad, Kenya and Zambia, as well as in France, Germany, Belgium, Spain, Norway and the United States, where it is thought that immigration is responsible for its introduction (reviewed by Quinones-Mateu *et al*., 2000; Roques *et al*., 2002; Mauclere *et al*., 1997; Peeters *et al*., 1997). Group N (Non-M and non-O) is only found in Cameroon or in Cameroonian individuals (Roques *et al*., 2004; Simon *et al*., 1998; Bodelle *et al*., 2004). Group P has also only been isolated in two patients from Cameroon (Plantier *et al*., 2009; Vallari *et al*., 2011).

The discovery and characterisation of SIVsmm, a SIV naturally infecting sooty mangabeys in west Africa (Fultz *et al*., 1986; Hirsch *et al*., 1989; Apetrei *et al*., 2005), and its remarkable identity with HIV-2, led to the hypothesis that it was the simian origin of HIV-2. The similarities of the organisation of the genome of these viruses, including the presence of a *vpx* gene, the geographic overlap between the epicentre of the HIV-2 epidemic and the habitation of the sooty mangabey (Gao *et al*., 1994; Hirsch *et al*., 1989), and the fact that these monkeys are regularly hunted or kept as pets (Marx *et al*., 1991), identified SIVsmm as the origin of HIV-2 (Santiago *et al*., 2005).

It was largely believed that SIV was non-pathogenic in its natural host, primarily because the most-studied primates (sooty mangabeys and african green monkey) showed no signs of AIDS-related disease (Paiardini *et al*., 2009). However, it was found that SIVcpz, the progenitor of HIV-1, was pathogenic in wild chimpanzees, from a nine year study in Gombe National Park, Tanzania (Keele *et al*., 2009). In a population of 94 individuals belonging to

two groups, mortality in SIV-infected chimpanzees was 10 to 16 times higher than that of uninfected chimpanzees, with a concomitant loss of CD4 cells in lymphatic tissues. A female who died less than three years after SIVcpz infection had histopathological disorders similar to the terminal stage of AIDS (Keele *et al*., 2009). Although based on limited samples, the results suggest that SIVcpz, like HIV-1 in humans, can cause disease in its host.

1.3.2 Cross-species transmission from monkey to humans

Although we do not know exactly how transmission of SIVcpz and SIVsmm to humans occurred, exposure to blood or secretions from infected monkeys during hunting and handling of bush meat represents the most likely cause of human infection (Hahn *et al*., 2000). Bites from captive monkeys may also have been another possible source of contamination (Hahn *et al*., 2000). The factors that led to the emergence of the virus in humans are likely to be different from those that favoured its epidemic spread, such as behavioural and environmental factors. However, cross-species transmission of other SIVs have not been identified thus far in humans, although the presence of antibodies against SIVmnd and SIVcol (from *Colobus guereza*) have been described in two individuals living in Cameroon, but with no virus isolated (Kalish *et al*., 2005; Souquiere *et al*., 2001). In addition, the transfer from apes to humans of other retroviruses has also been observed, as is the case for simian foamy virus (SFV) or simian T-lymphocyte virus (STLV) (Meertens *et al*., 2001; Switzer *et al*., 2004; Vandamme *et al*., 1997; Wolfe *et al.*, 2005). Therefore, new occurrences of interspecies transmission by other known or even unknown SIV to humans are a distinct possibility (Aghokeng *et al*., 2006; Peeters *et al*., 2002).

1.3.3 Timing of cross-species transmission to humans

Timing of the epidemic to date reveals that HIV-1 group M is the oldest HIV lineage; its common ancestor can be traced to 1930, with a confidence interval ranging from 1915-1941 (Korber *et al*., 2000, Salemi *et al*., 2001). A similar period is also estimated for HIV-1 group O (1920, with a confidence interval from 1891-1940; Lemey *et al*., 2004). It is surprising that group O did not undergo the same spread as group M, despite their progenitors jumping into humans at approximately the same time. Reasons for the success of group M in relation to group O are poorly understood, but initial studies suggest that group O may have a reduced replicative and transmission fitness (Arien *et al*., 2005). The diversity within HIV-1

group N is significantly lower than for groups M and O (Roques *et al*., 2004; Simon *et al*., 1998; Bodelle *et al*., 2004) , suggesting a more recent occurrence of this virus in humans, with its common ancestor traced to 1963 (range 1948-1977; Wertheim *et al.,* 2009). Molecular clock studies traced the emergence of the HIV-2 groups A and B to 1940 and 1945, respectively (Lemey *et al*., 2003). With only two sequences to date, the common ancestor of HIV-1 group P cannot be accurately determined.

1.3.4 Phylogeny of HIV

Group M HIV can be subdivided into subtypes based on their phylogenetic relatedness. Currently there are nine described subtypes named A, B, C, D, F, G, H, J and K (Robertson *et al*., 2000). Two of these, subtypes A and F, have been further subdivided into sub – subtypes (referred to as A1, A2 and F1, F2 respectively; Robertson *et al*., 2000). They are consistent in their phylogenetic topology in relation to each other, regardless of the section of their genome being compared (Archer and Robertson, 2007). Other significant clusters are formed by circulating recombinant forms (CRFs). These have arisen as a result of recombination events between divergent HIV strains within individual hosts. To qualify as a CRF, three epidemiologically unlinked viruses with the same mosaic genome and consistent phylogenetic clustering must be characterised (Robertson *et al*., 2000). Their number is constantly changing, and there are currently more than 50 CRFs (*http://hiv-web.lanl.gov*). When CRFs are found in isolated individuals, they are termed unique recombinant forms (URFs).

The nine HIV group M subtypes are often referred to as being pure, recombinant-free lineages. However, this is misleading, as recombination is an important part of the life cycle of HIV (Jetzt *et al*., 2000; Robertson *et al*., 1995). The subtypes have emerged through a unique epidemiological history (Archer and Robertson, 2007), and their classification has been largely based on global sampling bias. Recently, one study observed that subtype G is actually a recombinant lineage involving subtypes A and J, as well as the designated recombinant lineage CRF02_AG (Abecasis *et al*., 2007). Thus, despite the rigid classification system, the phylogeny of HIV should be viewed as being highly dynamic in nature. The currently classified HIV subtypes and CRFs are representative of the viruses primarily responsible for the AIDS epidemic. They do not give a complete view of HIV diversity. There are presently many fully sequenced group M HIVs that are too divergent to be placed within

any existing subtype or CRF grouping, and these have remained unclassified (Carr *et al*., 2010). In addition, certain inter-subtype recombinant viruses contain sequences that are of indeterminate origin, providing further evidence that HIV diversity is not fully represented under the current classification system (Carr *et al.,* 2010). This implies that there is potentially far more diverse pool of HIV sequences circulating amongst humans than the classified subtypes and CRFs might suggest.

1.3.5 Mechanisms of HIV diversification

One of the most important characteristics of HIV-1 is its extreme genetic diversity. There are two viral mechanisms that generate HIV diversity: (1) mutations are introduced into viral genomes during replication, and (2) mutations are introduced by recombination between viral genomes.

Mutations are introduced into the viral genome primarily due to the error prone nature of the viral replication enzyme, reverse transcriptase (Roberts *et al*., 1988). This enzyme introduces approximately one error for every 2000 nucleotides it incorporates (Preston *et al*., 1988). In addition, unlike the human polymerases, reverse transcriptase has no proof-reading activity and is therefore unable to correct these errors. As a consequence, within an infected individual, no two viruses are identical. This assemblage of unique individual mutants is referred to as a quasispecies.

In addition, mutations occur in HIV as a result of recombination. Recombination occurs during reverse transcription where the reverse transcriptase enzyme frequently switches between any HIV RNAs that are in close proximity (reviewed by Temin, 1993). The resulting proviral DNAs that are generated, and eventually integrated, will almost invariably be recombinant.

The most visible result of recombination is the high number of recombinant and CRFs of HIV (reviewed by Peeters and Sharp, 2000). These CRFs are the results of co- and/or super-infections, particularly in areas where several HIV subtypes co-circulate (Hoelscher *et al*., 2002; Peeters and Sharp, 2000). Analyses of recombinant viruses had helped in determining the frequency of occurrence of recombination breakthrough points in different genes. *Env* has the highest frequency (34.6%), followed by *gag* (24.7%), *pol* (19.7%), accessory genes (11.1%), *LTR* (7.4%) and *nef* (2.5%; Robertson *et al*., 2000). Examples of

CRF structures are shown in Figure 1.4. This large number of recombinant viruses implies that co-infection involving divergent HIV strains is a more common phenomenon than previously thought (Apetrei *et al*., 2004; Spira *et al*., 2003). Several studies have reported that super-infections are often followed by recombination (Altfeld *et al*., 2002; Blackard *et al*., 2002; Fang *et al.*, 2004). However, this is usually observed in areas where several subtypes co-circulate, particularly in west central Africa (Gao *et al*., 1998; Janssens *et al*., 1997; Kanki *et al*., 1997; Renjifo *et al*., 1998). Cases of HIV-1/HIV-2 dual infection have also been reported, but there has been no reported case of recombination between these two viruses (Curlin *et al*., 2004; Kanki *et al*., 1997; Nkengasong *et al*., 2000). Recombination in HIV has also been shown to occur between isolates of different groups, or between the same subtypes (McVean *et al*., 2002). In line of this, co-infections and group M and O recombinant lineages have been identified, especially in Cameroon where the two groups of viruses co-circulate (Peeters *et al*., 1999; Takehisa *et al*., 1999; Vergne *et al*., 2003). The low number of full genomes sequenced in some areas suggests that the impact of recombination in the HIV epidemic is still underestimated.

1.3.6 Origin of the HIV pandemic

After SIV crossed species into humans, early viruses likely circulated in a very small population for several years before wider spread. This has been attributed to environmental, social and demographic factors like migrations, massive urbanisation, and mass medicine practices, such as non-sterile needles in vaccination campaigns (Hahn *et al*., 2000).

Given that all HIV-1 viruses infecting humans fit into the same phylogenetic HIV-1/SIVcpz/SIVgor radiation, it is likely that cross-species transmission took place in equatorial west Africa, and especially in south Cameroon, habitat of western gorillas and chimpanzees *P. t. troglodytes*. (Keele *et al*., 2006; Van Heuverswyn *et al*., 2007). After transmission to humans, HIV-1 group M began to diversify. The greatest genetic diversity of HIV-1 M in terms of number of subtypes and genetic diversity within subtypes has been observed in the western region of the Democratic Republic of Congo (DRC), suggesting that this was the epicentre of the epidemic (Vidal *et al*., 2005; Kalish *et al*., 2004).

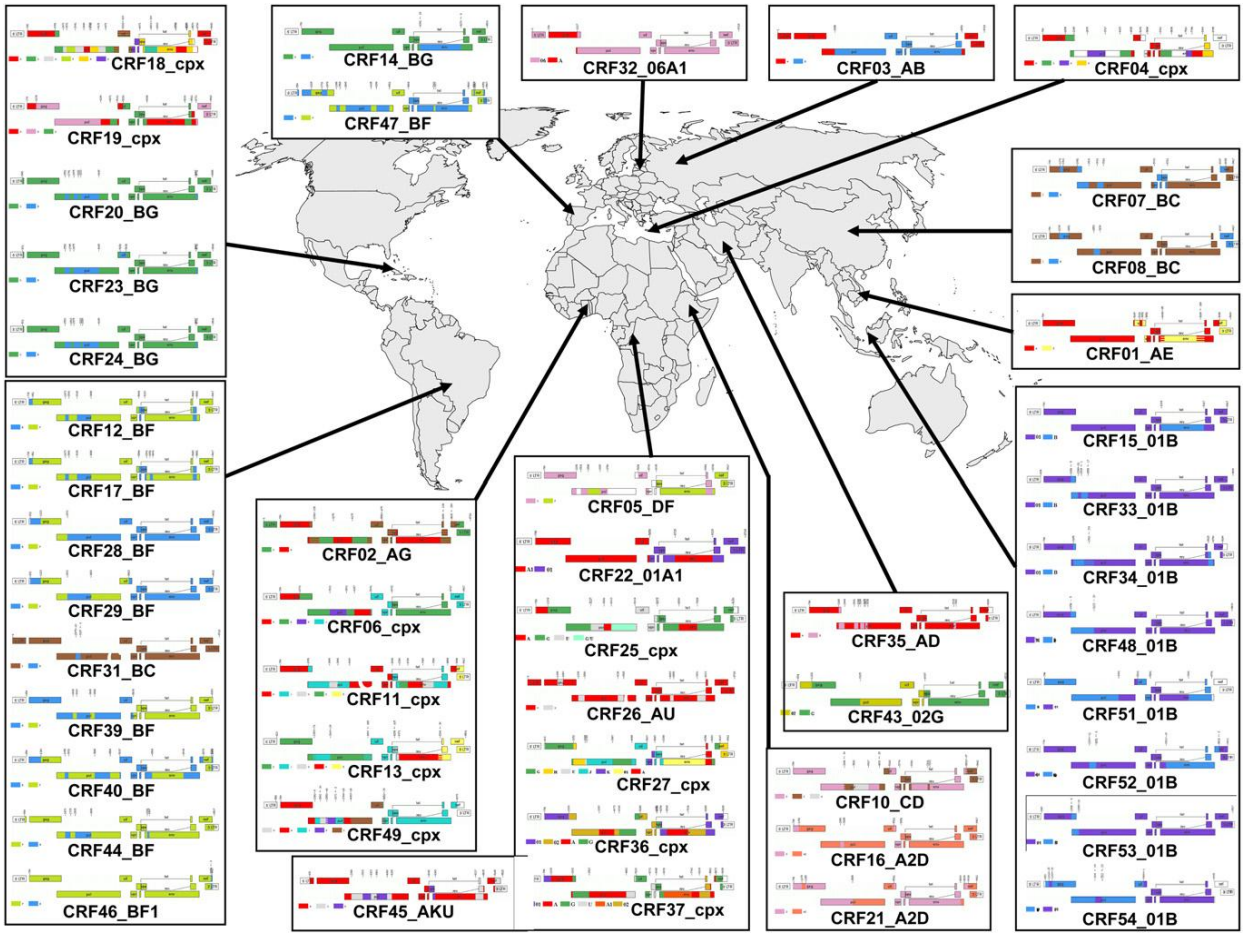

Figure 1.4 Mosaic structures of the most dominant circulating recombinant forms in the HIV pandemic. Each colour represents a specific HIV-1 subtype and arrows indicate where the CRFs are most prevalent (taken from Hemelaar *et al*., 2011).

Different variants of HIV-1 began to spread around the world from this region, and the heterogeneous spatial distribution of subtypes/CRFs is the result of different founder effects (a single introduction followed by a rapid spread), demographic factors and the displacement and migration of populations (Figure 1.5; Tebit and Arts, 2011).

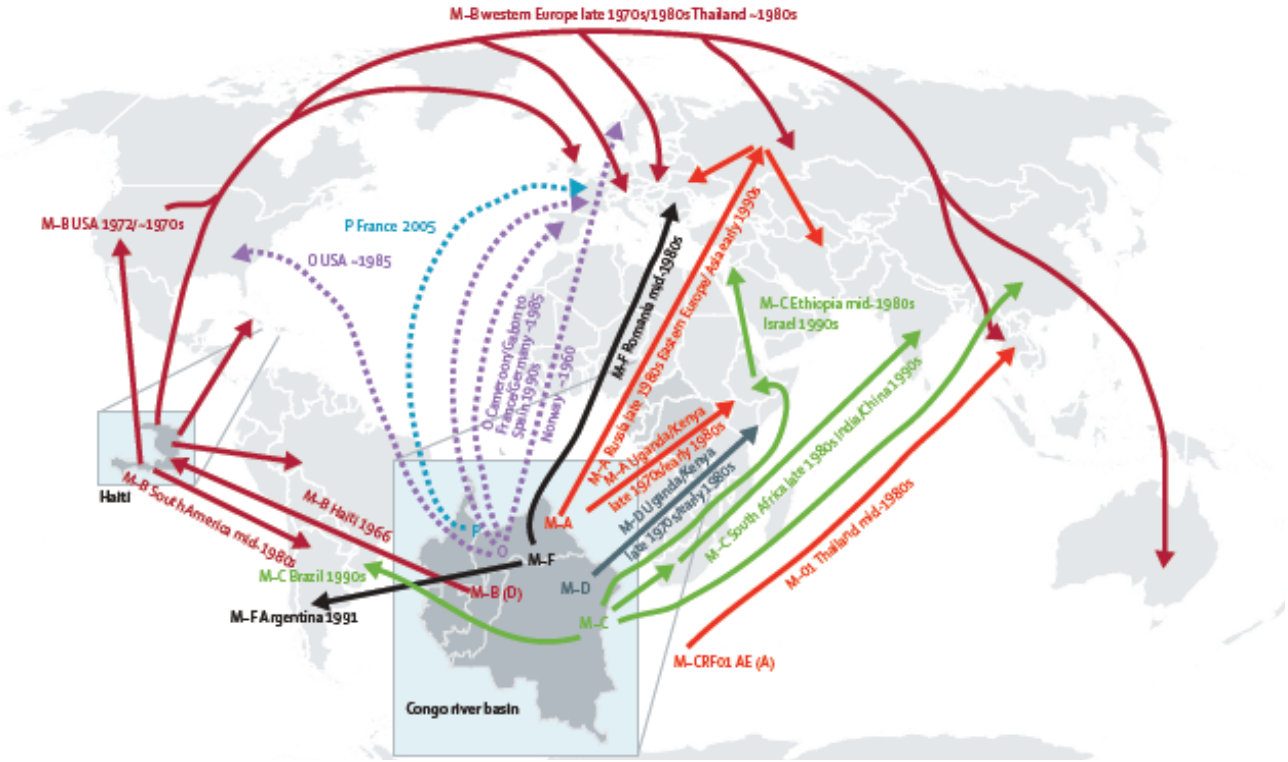

Figure 1.5 Global spread of HIV groups and subtypes from the Congo basin epicenter region. (taken from Tebit and Arts, 2011)

1.3.7 HIV-1 molecular epidemiology

The geographical distribution of HIV-1 subtypes/CRFs is variable (Figure 1.6) (*http://www.hiv.lanl.gov*). Different HIV-1 subtypes predominate in different regions of the world. For example, subtype B is predominant in Europe, Australia and North America (Hemelaar *et al*., 2011). In east Africa, subtypes A, D and C predominate, while subtype C is the major clade in southern Africa. Recombinant subtype CRF02_AG predominates in west central Africa. In India, subtype C is the major circulating virus, while in southeast Asia the main subtypes include B and CRF01_AE. In Latin America, three main subtypes predominate, namely B, F and C. The greatest diversity had been observed in DRC, where almost all subtypes and CRFs are found (Figure 1.6; Hemelaar *et al*., 2011). Overall, the predominant viral forms in the world are subtype C (48%), A (12%), B (11%), followed by recombinant CRF02_AG (8%) and CRF01_AE (5%).

The geographical distribution of subtypes is a dynamic process; an increasing frequency of recombinant viruses may arise as different HIV subtypes spread across all continents and recombinant viruses continue to recombine (Hemelaar *et al*., 2011).

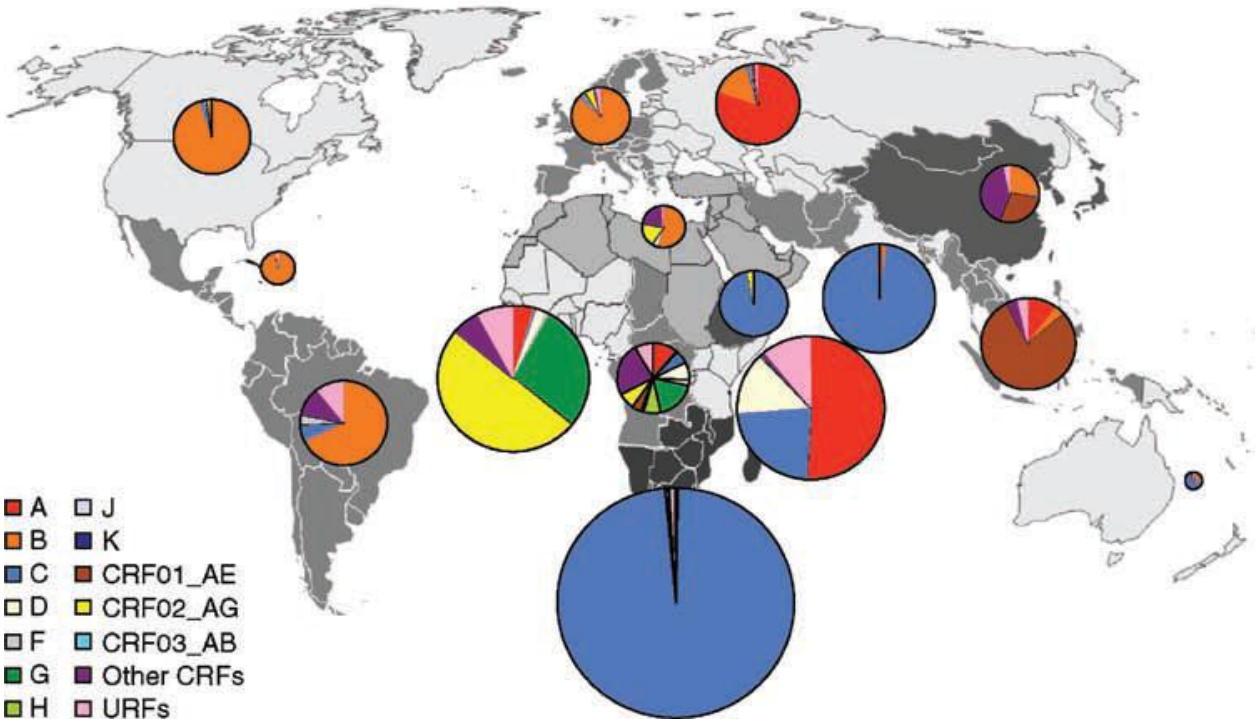

Figure 1.6 Global distribution of HIV-1 subtypes and CRFs. Each pie represents the contribution of the particular subtype or CRF to the each world region (taken from Hemelaar *et al*., 2011)

1.3.8 HIV diversity in Cameroon

HIV infection in Cameroon is the main focus of this thesis. By the end of 2012, the prevalence of HIV infection in the adult population in Cameroon was estimated at 4.5% (4.1% to 4.9%), down from 5.2% in 2001 (UNAIDS, 2013). Although the prevalence is decreasing, it remains one of the highest in the region (UNAIDS, 2013). Cameroon is located in west central Africa, where the HIV-1 pandemic originated from cross-species transmission. Likely as a consequence of this, it has one of the most genetically diverse HIV epidemics in the world. This has led to Cameroon being termed the 'Ground Zero' of the epidemic (Carr *et al*., 2010). A number of studies have characterised HIV-1 genetic diversity in Cameroon both

in urban and rural areas (Zhong *et al*., 2002; Ndongmo *et al*., 2006; Njai *et al*., 2006; Konings *et al*., 2006; Ndembi *et al*., 2008; Torimiro *et al*., 2009). From these studies, it appears that HIV-1 distribution exhibits extensive diversity not only in urban settings, but also in rural regions and isolated remote areas. In the most extensive study to date, Carr *et al*. (2010) investigated HIV diversity in rural villages in Cameroon, demonstrated that while the majority (66 %) of isolates were CRF02_AG, the remainder were pure subtypes including A, A1, A2, F (F2 only), B, D, G, H and J and other recombinant forms such as CRF01_AE, CRF06_cpx, CRF11_cpx and CRF13_cpx. Another particularity of the HIV epidemic in Cameroon is the presence of about 10% of URFs or mosaic viruses, likely to be extant examples of early diverging lineages, possibly resembling the pre-epidemic viruses or evolutionary relics (Carr *et al*., 2010). The second most prevalent HIV-1 group is O, which accounts for 1-5% of all HIV infection in Cameroon. Furthermore, groups N and P are only found in Cameroon or in Cameroonian individuals (Roques *et al*., 2004; Simon *et al*., 1998; Bodelle *et al*., 2004). Inter-group recombinants have also been described in Cameroon, for example M/O recombinants (Peeters *et al*., 1999; Takehisa *et al*., 1999; Vergne *et al*., 2003). Although CRF02_AG is the predominant group M subtype in Cameroon, it is thought to have originated from DRC, where it migrated to Cameroon and the rest of the region (Faria *et al*., 2011). This subtype is also characterised by its remarkable diversity, and it is believed that at least two lineages circulate in the country (Faria *et al*., 2011).

1.3.9 Consequences of HIV-1 genetic diversity

HIV genetic diversity has consequences for virulence, transmission, diagnosis, antiretroviral therapy and vaccine development.

Several independent studies have shown that progression to AIDS may depend on genetic variant; For example, a large study in Uganda showed that individuals infected with subtype D progressed faster to AIDS than those infected with subtype A (Kaleebu *et al*., 2002). Meanwhile, there was no difference in the rate of mother to child transmission between these two subtypes (Eshleman *et al*., 2001). In another study of a large Kenyan cohort, higher viral load and lower CD4 counts were observed in individuals infected with subtype C compared to subtypes A or D (Alaeus *et al*., 1999); however, the biological mechanism for this has not yet been determined (Wright *et al*., 2011). Contradictory results regarding the pathogenesis within the group M have been reported. Laurent *et al*. (2002) compared

individuals infected with CRF02_AG and non-AG subtypes, and found no difference in CD4 decline and viral loads over time. A prospective study in Thailand showed no difference between the progression to disease in patients infected with CRF01_AE and those infected with subtype B (Amornkul *et al*., 1999).

Diagnostic tests are essential for monitoring HIV infection, and need to be extremely sensitive. Although current tests detect all known genetic variants (type, subtype or group), this was not the case early in the pandemic. The first commercial tests were developed based on subtype B, and did not detect genetic variants from group O (Loussert-Ajaka *et al*., 1994). This suggests that monitoring of genetic variants is important, especially in west central African countries where there is a high genetic diversity and where new variants may easily emerge as a result of other zoonotic transmissions. The failure to detect the emergence of a new variant can have dramatic consequences, such as a breakthrough of a new epidemic.

Another tool, quantification of viral load, is indispensable to monitor infection and the impact of ART. Genetic diversity may also affect this method, since it is based on molecular detection of viral genomes. Tests used to quantify viral load have been optimized and are now able to identify many genetic variants (Peeters *et al*., 2010; Rouet and Rouzioux, 2007). However, several studies have reported that commercial tests used for the detection of viral load are unable to properly detect or quantify viral RNA in patients infected with group O strains (Gueudin *et al*., 2003; Rouet and Rouzioux, 2007). Given the diversity of HIV-1, these tests require continuous updating to assess the effectiveness of primers and probes to detect the full spectrum of viruses.

ART is now widely used in developing countries where non-B HIV-1 subtypes are predominant. HIV-1 group O and HIV-2 are naturally resistant to non-nucleoside reverse transcriptase inhibitors (NNRTIs) (Lal *et al*., 2005; Quiñones-Mateu *et al*., 1998). There is also a differing sensitivity within group M. For example, subtype F is less sensitive to certain NNRTIs such as the tetrahydroimidazo[4,5,1-jk] [1,4]-benzodiazepin-2-(1H)-one and -thione (TIBO) derivate R82913 (Apetrei *et al*., 1998), and some subtype G viruses are less sensitive to protease inhibitors such as ritonavir and saquinavir (Descamps *et al*., 1998). Under the action of ART, resistance mutations can occur, allowing virus to escape drug pressure. The speed at which these resistant strains emerge appears to vary amongst subtypes. For example,

it has been shown that subtype C viruses generated NNRTI-resistance faster than subtype B (Loemba *et al*., 2002).

HIV genetic diversity and the ability of the virus to evade immune responses through genetic variation are among the major obstacles to the development of an effective HIV vaccine (Esparza and Osmanov, 2003; Stratov *et al*., 2004; Thomson *et al*., 2002). This obstacle was clearly illustrated in Altfeld *et al.* (2002), where it was shown that superinfection with a second subtype B virus was coincident with the loss of immune control. The sudden increase in viraemia was associated with a decline in half of the CD8+ T cell responses and that the declining of these responses were coupled with sequence changes relative to the initial virus that resulted in impaired recognition (Altfed *et al*., 2002). The issue of viral diversity leading to immune escape will be dealt with in more detail in the next section.

1.4 ADAPTIVE IMMUNITY TO HIV

1.4.1 T cell responses to HIV

CD8+ T cells are the main effector cells during HIV infection. Their mechanisms of action include production of cytokines, and lysis of infected cells through perforin and granzyme secretion (Schmitz *et al*., 1999; Moore *et al*., 2002; Hersperger *et al*., 2010). Early studies demonstrated that HIV-infected people mount vigorous CD8+ T cell responses to the virus (reviewed by Mills *et al*., 1989; Walker and Plata, 1990), and these responses were considered as potential effectors for future HIV vaccines (reviewed by Berzofsky, 1991; Johnson and Walker, 1991). Understanding the dynamics of cellular immune responses in natural HIV infection in humans and SIV infection in animal models has been the topic of much study over the last 20 years (McMichael *et al*., 2000; McMichael and Rowland-Jones, 2001; Mudd *et al*., 2012). These studies have provided strong evidence that CD8+ T cells are important in controlling virus replication during HIV infection.

HIV-specific CD8+ T cells become detectable around 2 to 3 weeks after infection, and their frequency increases gradually and reaches a peak when viraemia levels decline (reviewed by McMichael *et al*., 2010). This initial CD8+ T cell response is narrow, and it is critical in the reduction of viral replication during primary infection (Lichterfeld *et al*., 2004).

In response to CD8+ T cell pressure, HIV mutates to escape recognition, and this has been observed to happen approximately 20 days post-infection, at the time of peak CD8+ T cell responses (Goonetilleke *et al*., 2009). Therefore, CD8+ T cells corresponding to the initial viral epitopes decrease rapidly; new CD8+ T cells recognising the mutated sequences may arise, leading to a new selection pressure and promoting new mutations (Soudeyns *et al*., 1999). The race between the CD8+ T cell responses and the virus takes place throughout the asymptomatic phase of infection and leads to the exhaustion of the T cell response (Streeck *et al*., 2009). During chronic infection, HIV-specific CD8+ T cells persist at high frequencies and the number of epitopes targeted broadens (Addo *et al*., 2003), especially in people with progressive infection compared to those who control the infection (Pereyra *et al*., 2008). The functions of most of these cells are seriously impaired and this limits their efficacy in the control of HIV replication. Day *et al.* (2006) reported that PD-1, a negative immune-regulatory molecule present at the surface of CD8+ T cells, is significantly upregulated. PD-1 expression correlated with impaired HIV-specific CD8+ T cell function, as well as higher plasma viral load and an inverse correlation with CD4+ T cell count (Day *et al*., 2006). A small percentage (1–5%) of HIV-infected individuals experience a lack of decline of CD4+ T cell counts, and control of virus replication below 1000 HIV RNA copies/ml for an extended period of time (at least 7–10 years) in the absence of ART (reviewed by Walker and Korber, 2001). These individuals are called long-term nonprogressors (LTNPs) (Pantaleo *et al*., 1995). Furthermore, an even smaller percentage (<1%) of individuals show control of virus replication below 50 HIV RNA copies/ml regardless of the length of the time of control and are called elite controllers (EC) (reviewed by Deeks and Walker, 2007). Study of these individuals has led to important insights into T cell correlates of viral control.

1.4.2 The role of T cells in the control of HIV

Several arguments underscore the essential role of the CD8+ T cell response in controlling viral replication during HIV infection. These include the parallel decrease of HIV viral load with the peak of CD8+ T cell response during the acute phase (Koup *et al*., 1994), the loss of control of SIV infection in macaques after removal of their CD8+ T cells (Jin *et al*., 1999; Schmitz *et al*., 1999) and the correlation between certain HLA class I alleles and better control of the infection (Pereyra *et al*., 2010; Fellay *et al*., 2007). Thus, the search for the characteristics of HIV-specific CD8+ T cell responses that are associated with viral

control, including the quantity, specificity and functional and phenotypic nature of the response, would certainly help with designing an effective HIV vaccine.

The first studies on the quantity of HIV-specific T cell responses examined the magnitude and breadth, in an attempt to determine how these parameters were associated with clinical measures of disease. Addo *et al*. (2003), using an interferon-gamma enzyme-linked immunospot (IFN-γ ELISPOT) assay, screened PBMC from HIV-infected individuals from the US for virus-specific T cell responses using peptides spanning all HIV proteins. Despite broad and strong HIV-specific responses amongst these individuals, neither the breadth nor the magnitude of the total HIV-specific CD8+ T cell response was associated with plasma viral load (Addo *et al*., 2003). These findings were replicated in many other studies, including those infected with subtype C virus from southern Africa (Masemola *et al*., 2004). These studies were performed at a single time point with infected individuals at different stages of infection. Follow up studies using the same methodology on longitudinal samples, even those from early infection, also found no association with the rate of CD4 T cell decline or the course of disease in the first year of infection (Peretz *et al*., 2005; Gray *et al*., 2009). Collectively, the conclusion drown from these studies were that most HIV-infected individuals can mount robust CD8+ T cell IFN-γ responses and that the overall breadth and magnitude of these responses was not a correlate of viral control. These studies also suggested that other features, including the specificity (*i.e.* which regions of HIV were targeted) or the quality (*eg* other functions of immune cells) of the response may be important for viral control.

Edwards *et al*. (2002) demonstrated that targeting of the HIV Gag protein was associated with reduced viral load. They measured T cell responses in PBMC from individuals in chronic HIV-1 infection after stimulation with overlapping HIV-1 peptides spanning the Gag, Pol, Env, and Nef proteins using the IFN-γ ELISPOT assay. The magnitude and breadth of T cell responses directed to Gag correlated inversely with viral load and directly with absolute CD4+ T cell counts (Edwards *et al*., 2002). No such associations were found with other HIV proteins. Consistent with this finding, Masemola *et al*. (2004) showed that the hierarchical targeting of the HIV Gag protein, rather than the overall magnitude of T cell responses to HIV proteins, was associated with viral control. In a more extensive study to define the relationship between the specificity of the cellular immune

response and viral control, Kiepiela *et al*. (2007) showed that a greater breadth of Gag-specific CD8+ responses were associated with reduced viral load, while Env-specific responses were associated with higher viraemia. This association was independent of major histocompatibility complex class one (MHC-1) type and unrelated to epitope sequence conservation. These data serve as strong evidence that major MHC class I presentation of Gag peptides is an essential feature for any HIV vaccine designed to elicit optimal CD8+ T cell responses. Recently, these observations were extended to other HIV proteins. Budde *et al*. (2012) showed that macaques that controlled SIV replication shared durable T cell responses against similar regions of Nef. In addition, vaccinated groups of macaques controlled replication of the highly pathogenic SIV mac239 virus by means of high frequencies of CD8+ T cells against Vif and Nef epitopes (Mudd *et al*., 2012). These studies therefore suggest that other proteins besides Gag can also play a role in the control of viral replication, and that this should guide in the design of vaccine immunogens.

All the studies mentioned above employed the IFN-γ ELISPOT assay as a general screening method for the quantification of the HIV-specific T cell responses. The utility of this assay was challenged due to the lack of efficacy of a recently conducted HIV vaccine trial (the STEP trial, discussed later), where despite induction of robust ELISPOT responses (Buchbinder *et al*., 2008), no efficacy was observed. This finding, plus advances in multiparameter flow cytometry, directed researchers to examine the immune response to HIV in more depth, by assessing the quality rather than the quantity of virus-specific T cell responses. Efforts at elucidating qualitative features of T cell responses that control viral replication have been done mainly by studying of individuals who control virus without ARV treatment. Betts *et al*. (2006) compared the capacity of CD8+ T cells from HIV progressors and nonprogressors to produce different cytokines by measuring five different functions of CD8+ T cells (secretion of IFN-γ, IL-2, TNF-α, MIP1β, and expression of CD107a as a surrogate for degranulation). Polyfunctionality, or the capacity to produce three or more cytokines simultaneously, was associated with control of viral replication, and nonprogressors consistently maintained highly functional CD8+ T cells (Betts *et al*., 2006). Beyond polyfunctionality, cytotoxicity appears to be vital to the control of HIV replication. Hersperger *et al*. (2010) measured perforin upregulation, cytokine production, and degranulation after stimulation with peptide pools to all HIV proteins in various HIV infected groups, including those who controlled and those who did not control viral replication. They

observed that CD8+ T cells from elite controllers produced significantly higher perforin and granzymes than progressors (Hersperger *et al*., 2010). Dissection and characterisation of different combination of functions in these HIV-specific T cells generated suggested that highly functional HIV-specific T cells were distinguished by perforin and IL-2 up regulation (Hersperger *et al*., 2010). Another qualitative feature of CD8+ T cells, namely their proliferative capacity, is inversely associated with viral load (Day *et al*., 2007). A recent study demonstrated that the proliferative capacity of HIV-specific CD8+ T cells correlated with delayed disease progression while the magnitude of IFN-γ responses did not (McKinnon *et al*., 2011). Very recently, in an effort to combine quantitative and qualitative measures of immunity, Riou *et al*. (2013) evaluated the impact of both the quantity and quality of CD8+ T cell responses at approximately 6 months post-infection on the viral set point at 12 months in a cohort of HIV-infected individuals in South Africa. A high frequency of highly functional Gag- and Nef-specific CD8+ T cell responses was the best predictors of a low viral set point (Riou *et al*., 2013). These findings further highlight the importance of eliciting such cells by vaccination to control HIV replication. Functional avidity, the capacity of T cells to respond to low concentrations of peptide, and differentiation phenotype of HIV-specific T cells has also been examined. Mothe *et al*. (2012) analysed HIV Gag p24-specific T cell responses in controllers and non-controllers to determine the functional avidity of these responses. Although responses to p24 were of comparable breadth and magnitude, significantly higher avidity responses were observed in controllers compared to non-controllers (Mothe *et al*., 2012). The phenotype of CD8+ T cells is likely to be a key parameter of viral control. Fully differentiated HIV-specific CD8+ effector cells were seen to be more frequently detectable in controlled than in progressive HIV infection (Addo *et al*., 2007; Northfield *et al*., 2007), whilst central memory CD8+ T cells were associated with lower viral set points in early infection in another study (Burgers *et al*., 2009).

There are strong data to suggest that not all HLA alleles have a similar impact on HIV-1 control. Certain HLAs have been associated with better diseases outcomes, including HLA B*57, B*27, B*58:01and B63 (reviewed by Kaslow *et al*., 2005). On the other hand, HLA B*35, B*58:02 and Cw*04 have been shown to negatively affect the outcome of disease (reviewed by Kaslow *et al*., 2005). Mutations in viral epitopes may result in abrogation of the peptide presentation by HLA. A major concern regarding this may be that escape mutants may accumulate at the population level, leading to a loss of recognized epitopes and vaccine-

induced T cell responses with reduced effectiveness over time. Indeed, HIV has been shown to adapt at the population level in the face of immune pressure (Kawashima *et al*., 2009). Therefore, knowledge of the distribution of HLA alleles within local populations is essential for the development of peptide-based vaccines with large population coverage.

1.4.3 The B cell response to HIV

Seroconversion usually occurs 3-12 weeks after HIV infection, and the first antibodies to be produced are IgG targeting the gp41 region (reviewed by Liu *et al*., 2011). After peak viraemia, when viral load starts to decline, antibodies against other HIV proteins begin to appear, such as antibodies against gp120 in chronic infection. However, these binding antibodies have no detectable effect on viremia (Tomaras *et al*. 2008), and apparently do not exert any selective immune pressure on the envelope (Keele *et al*. 2008). It is specifically neutralizing antibody (NAbs) that can potentially protect against infection by blocking viral entry into host cells (Gallarda *et al*., 1992).

NAb responses to HIV usually arise only several weeks after infection (Tomaras *et al*., 2008), most are strain-specific (or autologous), and are not able to neutralise more divergent viruses (heterologous viruses) isolated from other individuals (Moore *et al*., 1994; Legrand *et al*. 1997; Moog *et al*., 1997; Gray *et al*., 2007). These autologous NAbs drive neutralisation escape, as evidenced by the fact that contemporaneous viruses are less sensitive to autologous neutralisation than earlier viruses, although later viruses remain sensitive to new NAb responses. Escape occurs mostly through single amino acid substitutions, insertions and deletions (Overbaugh and Rudensey 1992; Chackerian *et al*., 1997; Herrera *et al*., 2003; Bunnik *et al*., 2008). Some infected individuals, however, develop broadly neutralising antibodies (bNAb) in their serum that can neutralize primary virus isolates in a cross-clade manner (Gray *et al*., 2007; Shen *et al*., 2009; Scheid *et al*., 2009; Moore *et al*, 2012). Their development seems to correlate with greater time after infection, implying a need for antibody affinity maturation, as well as higher viral plasma load, suggesting a need for chronic antigen exposure (Sather *et al*., 2009; Stamatatos *et al*., 2009; Doria-Rose *et al*., 2009). Moore *et al*. (2012) analysed sequence and neutralisation data from 101 transmitted/founder subtype C viruses and they found that glycan at residue 332 of the Gp120 is underrepresented in transmitted subtype C viruses compared to chronic viruses. They subsequently identified two HIV-1–infected individuals who developed BCN antibodies targeting the glycan at Asn332

and that this glycan was absent in the initial infecting virus. Only 15 to 25% of HIV-infected individuals develop these bNAbs (Binley *et al*., 2008; Euler *et al*., 2010). A recent study demonstrated that the frequency of a subset of circulating follicular helper CD4+ T cells was greater in individuals that developed bNAbs (Locci *et al*., 2013).

Antibodies can also mediate the clearing of the virus through their Fab fragment and in this case, they bind specifically to HIV antigens on the surface of HIV-infected cells and recruit innate immune cells such as natural killers (NK) cells by their Fc fragment (reviewed by Forthal *et al*., 2009; 2001). These cells can bind through their Fc receptor and kill the infected cell, and this is known as antibody dependent cell-mediated cytotoxicity (ADCC). If it is rather inhibition of viral replication that occurs, then this is referred to as antibody-dependent cell-mediated virus inhibition (ADCVI). Such interactions are likely to play an important role in preventing or modulating HIV infections.

1.4.4 The role of broadly neutralizing antibodies in the control of HIV

For several years, the isolation of bNAbs has focused the attention of the scientific community. The first generation of these antibodies derived from individuals infected by HIV-1 subtype B and was limited in their breadth (Binley *et al*., 2004; Pantophlet *et al*., 2007; Conley *et al*., 1994). They targeted the CD4-binding site (CD4bs; Burton *et al*., 1991; 1994), the gp120-glycan complex (Kunert *et al*., 1998; Calarese *et al*., 2003) and the membrane-proximal external region (MPER) of gp41 (Buchache *et al*., 1994; Binley *et al*., 2004). Following this, many second-generation human monoclonal antibodies, also targeting the glycan and MPER region but with a far greater ability to neutralise viruses from different subtypes have been isolated (Walker *et al*., 2009; Gorny *et al*. 2005; Wu *et al*., 2011; Moore *et al*., 2012). Some of these new bNAbs contain highly-mutated VH chains, underscoring the importance of the affinity maturation process that takes place in the development of these antibodies (Scheid *et al*., 2009; Wu *et al*., 2010). Naturally occuring bNAbs do not prevent or slow disease progression (Binley *et al*., 2008; Euler *et al*., 2010), most likely because they appear late during the course of infection and viral escape occurs rapidly. In addition, they have been difficult to induce by vaccination, largely as a result of our inability to design appropriate immunogens that induce a bNAb response (Wyatt *et al*., 1998). Current efforts to identify protective HIV vaccine immunogens are inspired by the results of recent passive immunisation studies with bNAbs, demonstrating a profound therapeutic effect in SHIV-

infected rhesus monkeys as well as an impact on host immune responses (Barouch *et al*., 2013; Shingai *et al*., 2013).

1.4.5 Role of non-neutralizing antibodies in the control of HIV

Several studies have underscored the role of ADCC/ADCVI in the prevention of SIV infections in the rhesus macaque model. Van Rompay *et al*. (1998) showed that new-born macaques were prevented from acquiring oral SIV after administration of non-neutralizing anti-SIV serum. In addition, rhesus macaques were partially protected against SIV after vaccination in the absence of a neutralizing antibody response (Patterson *et al*., 2004). More importantly, ADCC and ADCVI have been found to reduce viraemia both at the acute and chronic phases of the infection in rhesus macaques after vaccination (Gomez-Roman *et al*., 2005; Hidajat *et al*., 2009; Xiao *et al*., 2010). In humans, ADCVI developed before the appearance of the neutralising antibody response (Tomaras *et al*., 2008; Forthal *et al*., 2001). Furthermore, ADCC activity was shown to be more potent in elite controllers compared to viraemic individuals (Lambotte *et al*., 2009). Finally, non-neutralizing together with vaccine-elicited neutralizing antibodies were associated with protection against acquisition of infection in macaques immunised with an HIV Env mosaic vaccine (Barouch *et al*., 2013).

1.5 HIV VACCINES

1.5.1 HIV vaccine development

The development of an HIV vaccine is an enormous task. Immunisation has been successful in the eradication or elimination of infections of some viral diseases, such as smallpox, polio and measles. Many traditional vaccines have been developed using live attenuated forms of virus, inactivated (killed) virus or protein subunits (reviewed by Girard, 2006). These approaches are not suitable for developing a vaccine against HIV. Attenuated HIV may mutate and regain its pathogenicity after inoculation (reviewed by Letvin, 2006). Inactive HIV on the other hand may still contain enough live viruses to pose a risk (reviewed by Burgers and Williamson, 2005). Finally, subunit vaccines to HIV envelope monomers were shown not to be protective in two large Phase III efficacy trials in humans, though protein boosts were used in the only "successful" trial to date (Gilbert *et al*., 2005; Flynn *et*

al., 2005). Thus, challenges to developing an HIV vaccine are complex and immense, and include the high genetic variability of the virus, the difficulty in generating broadly neutralising antibody responses, unknown correlates of protection, limitations of animal models, and the difficulty in performing large clinical trials.

1.5.1.1 HIV latent reservoir

To date, there have been no spontaneous cures of HIV infection and no documented cases of immune-mediated clearance of HIV from an infected individual. This is a result of a major property of the virus, that it rapidly establishes a persistent reservoir of latently infected memory T cells (reviewed by Haase *et al*., 2010). Latency occurs within days to week after infection and once established, cannot be eradicated (Finzi *et al*., 1997). This is clearly seen in patients on antiretroviral therapy, where the virus persists in a latent state and replication resumes upon treatment interruption. This viral reservoir poses a serious challenge to the development of an effective HIV vaccine, as such a vaccine would have to generate effective immune responses very quickly after infection, at relevant sites of exposure such as genital tissue.

1.5.1.2 HIV genetic diversity

Another major obstacle to the development of an effective HIV vaccine is the enormous genetic variability of the virus, with high numbers of subtypes and CRFs. In addition, co-infections and super-infections which can result in recombination between subtypes increase the genetic diversity. The overall variability of the virus is further complicated by a complex mixture of viral populations or quasispecies, closely related but not identical, which vary continuously under immune pressure. For example, Korber *et al.* (2001) have demonstrated that the variability of HIV within one host is comparable to the global variation of influenza A. The mutability of HIV readily allows it to escape the neutralising antibody and T cell responses of the host during the course of infection (Boutwell *et al*., 2010). This phenomenon has been well documented in SIV-vaccinated macaques, where CD8+ T cell escape variants have led to the vaccine failure (Allen *et al*., 2000; Barouch *et al*., 2002). More recently, to test the causal role of the vaccines tested in the RV144 trial in preventing certain HIV variants from infecting vaccinated individuals, Rolland *et al*. (2012) performed a sieve analysis, which compares breakthrough viruses between vaccinees and

individuals who received the placebo during the trial. The vaccine was 48% effective (higher than the overall efficacy of 31%) at preventing infection with viruses that matched the vaccine at amino acid position K169 (lysine) in V2. These results suggest that RV144 preferentially blocked viruses matching the vaccine immunogen at this residue and that responses to the Env V2 may not have blocked infections with viruses not matching the vaccine at K169 (Rolland *et al*., 2012). This can complicate the development of an effective vaccine in humans.

1.5.1.3 Unknown correlates of protection

In addition to all these obstacles, we do not know exactly what type of immune response should be induced to protect from HIV infection or control viral replication (Pantaleo *et al*., 2004; Friedrich *et al*., 2008). In the absence of natural immune clearance of HIV, the search for immune correlates of viral control has largely been performed in the SIV macaque model and in individuals who control HIV replication (reviewed by Koup *et al*., 2011; Blankson *et al*., 2011). This does not imply that immune characteristics identified in these studies would provide protection if elicited by a vaccine. For example, polyfunctional cells were found to be associated with control of viral replication in HIV-infected individuals, but at the same time these polyfunctional cells are just a small fraction of the total CD8+ T cell compartment and are absent in many individuals who control HIV replication (reviewed by Makedonas and Betts, 2011).

1.5.1.4 Animal models

The use of SIV vaccines to protect macaque monkeys against experimental SIV infection provides the most reliable animal model for the testing of candidate vaccines against HIV available today. The pathophysiology of SIV infection in monkeys closely mimics that of HIV in humans (Brenchley *et al*., 2010; reviewed by Hartigan-O'Connor and Hirao, 2011). Plasma viral loads at the peak of primary SIV infection and at the set point during chronic SIV infection in macaques parallel those observed in HIV-infected humans (Parker *et al*., 2001). However, there are features of the SIV macaque model that limit its utility. The challenge virus used is often homogenous, using a virus containing sequences very similar to the vaccine sequence, which may be more easily controlled than a heterologous challenge, which is more relevant to natural human HIV infection (reviewed by Wilson and Watkins, 2009). A good illustration of the caution in the use of this model came from the the STEP

study (to be discussed later), where the vaccine failed in human trials, whilst it showed reduced viral loads in challenged macaques (McElrath *et al*., 2008; Buchbinder *et al*., 2008; Shiver *et al*., 2002). Therefore, improvement and standardisation should be made in terms of challenge virus, route and dose and endpoint measurement (Morgan *et al*., 2008) to provide meaningful information on whether a candidate vaccine should be advanced to human trials.

Table 1.1 Summary of phase IIb/III HIV vaccine trials performed in humans for HIV (adapted from Girard *et al.*, 2011)

Type of vaccine	Code name and place of trial	Results
Gp120 (B/B or B/E) + alum	VAX003 (Americas)	No protective efficacy observed
	VAX004 (Thailand	
Ad5-HIV-1 trivalent vaccine (Gag, Pol and Nef)	STEP (Americas + Australia)	No efficacy observed
	Phambili (South Africa)	Enhanced rate of infection in uncircumcised Ad5-sero-positive male volunteers
Canarypox (ALVAC)-Gag, Pol and Env-E + gp120 B/E + alum	RV144 (Thailand)	31% protective efficacy against HIV acquisition
DNA-Gag, Pol and Env priming + Ad5-Gag, Pol and Env (A, B and C) boost	HVTN505 (America)	No protective efficacy observed

1.5.1.5 Human efficacy trials

Under these conditions, HIV vaccine development and testing proceeds in an empirical manner. In addition, testing vaccines in humans remains the best way to evaluate protection against infection or control of viral replication. Since the start of HIV vaccine

development, more than 40 candidate vaccines have been tested in over 80 Phase I/II clinical trials, involving more than 10 000 healthy human volunteers (reviewed by Mascola and Montefiori, 2010; Ross *et al*., 2010). HIV vaccines that were tested in humans were first developed to elicit antibodies, then later to induce cellular immune responses (reviewed by Bansal *et al*., 2010). Five vaccine regimens have advanced to Phase III or IIb in humans to date (Table 1.1).

The first two phase III clinical trials of an HIV vaccine were initiated in 1998. The two vaccines consisted of a recombinant form of the gp120 and were administrated in the attempt to generate neutralising antibodies. The first vaccine, AIDSVax B/B (VAX003) was trialled in North America and the Netherlands in men who sex with men and showed no efficacy (Flynn *et al*., 2005; Girard *et al*., 2005). The second vaccine, AIDSVax E/B (VAX004), (subtypes CRF01_AE and B) was trialled in injection drug users in Thailand. Similarly, it showed no protection (Pitisuttithum *et al*., 2006). This was likely because gp120 monomers fail to elicit neutralising antibodies.

The next large clinical trial, the STEP trial, was initiated in the late 2004 and was the first vaccine focusing on producing T cell immune responses. A sister trial, Phambili, was performed in South Africa. This will be discussed more in the next section.

Following this, the RV144 trial was designed to test the efficacy of a prime-boost vaccine against HIV. This trial, which involved a canarypox vector (ALVAC) expressing HIV genes including *env* as a prime, accompanied by boosts with the same gp120 used in the VAX004 trial, was performed in Thailand in heterosexual men and women and resulted in a modest but significant 31% protection against infection in low-risk volunteers (Rerks-Ngarm *et al*., 2009). This protective effect was seen in the first year following vaccination (Rerks-Ngarm *et al*., 2009). Although the vaccine provided modest protection against infection, it had no impact on disease progression (viral load or CD4 cell count) in vaccinated individuals who became infected (Haynes *et al*., 2012). Subsequent analyses revealed that non-neutralising antibodies specific to the V1V2 region of Env were associated with a reduced risk of acquisition in vaccinated participants (Haynes *et al*., 2012).

The last efficacy trial was a Phase III trial, HVTN 505. It was initiated in the USA in individuals at risk for HIV infection (Hammer *et al*., 2013). It consisted of a prime-boost

regimen involving three vaccinations with DNA encoding HIV subtype B *gag*, *pol*, and *nef* genes, and *env* gene from HIV subtypes A, B, and C, followed by an Ad5 vector-based vaccine candidate encoding subtype B *gag* and *pol* genes, and *env* from subtypes A, B, and C (Catanzaro *et al*., 2006; 2007). Vaccine and placebo were administered to 1253 and 1251 participants respectively in a population of men or transgender women who have sex with men. At week 28 post administration, 27 participants in the vaccine group and 21 in the placebo group were diagnosed positive. This DNA/rAd5 vaccine regimen also failed to reduce either the rate of HIV acquisition or the viral load set point in the population studied (Hammer *et al*., 2013).

These trials have shown that testing a vaccine candidate in humans is the best way of determining vaccine efficacy, as well as highlighted the difficulties in achieving such a task.

1.5.2 T cell vaccines against HIV

In view of the lack of success of the early development of vaccines that would elicit a protective neutralising antibody response, HIV vaccine development proceeded to attempt to induce T cell responses for protection Vaccines that elicit cellular immunity against HIV could be partially effective, resulting in lower virus levels, less destruction of CD4+ T cells, and a delay in disease progression (reviewed by Barouch, 2008; Wilson and Watkins, 2009). This could also lower the probability of virus transmission (Figure 1.7). The induction of CD8+ T cell responses could be achieved through the use of vectors, and based on this hypothesis, several types of HIV vaccine candidates capable of inducing T cell responses have been developed over the past decade.

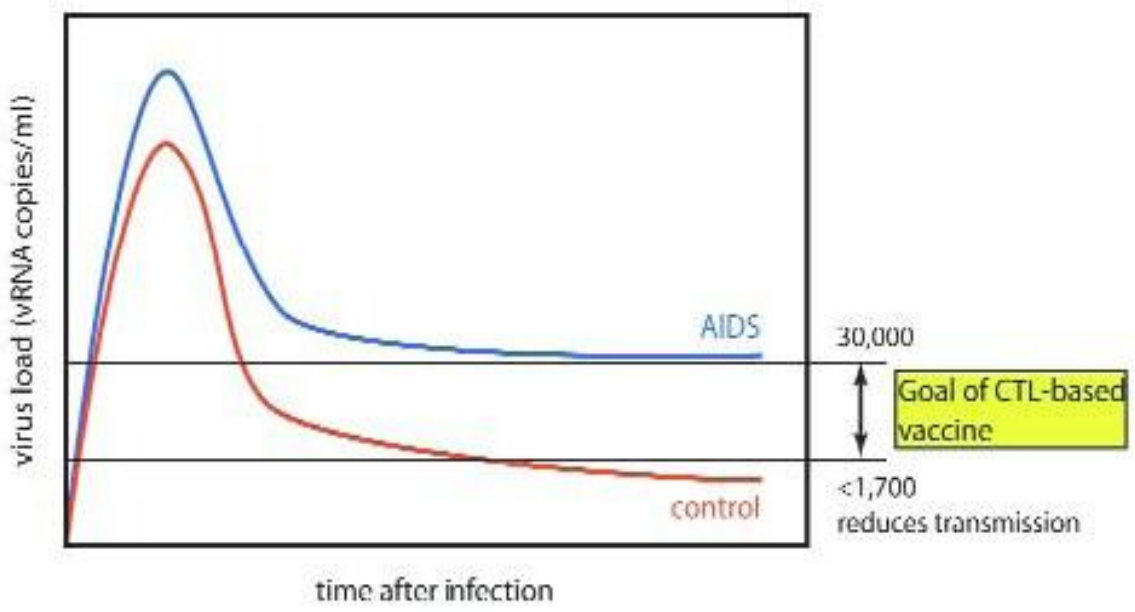

Figure 1.7 Goal of an HIV vaccine. After infection with HIV, replication as determined by viral load continues to a point, then drops to set point and is partially maintained (dark blue). An ideal T cell vaccine (red) would reduce peak viral replication during the acute phase and results in lower or undetectable set point viral load during chronic infection (taken from Wilson and Watkins, 2009).

1.5.2.1 T cell vaccine vectors

DNA plasmid vaccines were the first to be developed (reviewed by Lu, 1998). They are referred to as genetic vaccines and are based on bacterial plasmids that, once injected into a host, express the desired antigen *in situ*, which leads to induction of an immune response (Wang *et al*., 1995; 1993). Because they allow the immune system to focus only on the immunogen, they are safe and have no risk of virulence (MacGregor *et al*., 1998). They generally elicit low magnitude of responses of short duration (reviewed by Lu *et al*., 1998; MacGregor *et al*., 1998; Graham *et al*., 2006). To address that problem, different approaches were explored to enhance the immunogenicity of DNA vaccines, including co-expression of adjuvants and different ways of administration (reviewed by Webster and Robinson, 1997; Hutnick *et al*., 2011). DNA vaccines have a great capacity to drive a CD8+ T cell responses when used in a prime-boost regimen, as they were found to prime cellular responses to viral candidate vectors well (reviewed by Paris *et al*., 2010; Harari *et al*., 2008; Goepfert *et al*., 2011).

Live vector (viral or attenuated bacteria) recombinant vaccines were another type of T cell vaccine to be developed. Two viral vectors have been widely used for the development of HIV vaccines, namely poxviruses and adenoviruses. Live recombinant vaccine candidates for HIV were constructed using poxviruses as vectors, especially human non-replicating poxviruses such as canarypox (ALVAC; Belshe *et al*., 2001), the avian fowlpoxvirus (FPV; Kent *et al*., 1998), or attenuated strains of vaccinia virus, NYVAC (Tartaglia *et al*., 1992) and MVA (modified vaccinia Ankara; Amara *et al*., 2001; Im *et al*., 2004). These candidate vaccines were unfortunately not capable of inducing high magnitude and long lasting CD8+ T cell responses in humans (reviewed by Robinson, 2002). However, their immunogenicity was improved when used in a prime-boost strategy, when given after a DNA prime (reviewed by Robinson, 2002). Many other live recombinant vaccines have been developed in the past decade (reviewed by Excler, 2005; Sauter *et al*., 2005). Adenovirus (or Ad) vectors have been intensively explored as a vector for HIV vaccines, mostly with the goal of eliciting HIV-specific cellular immune responses (reviewed by Lasaro and Ertl, 2009; Barouch and Nabel, 2005). A candidate vaccine using a mixture of recombinant Ad5 vectors expressing the HIV *gag*, *pol* and *nef* genes from subtype B was tested in phase I clinical trials and found to be safe and immunogenic (Priddy *et al*., 2008). This was the vaccine used in the STEP and Phambili trials (see below), which unfortunately turned out to raise the risk of HIV infection Other alternative Ad vectors are now being developed based on less prevalent human adenovirus types, or on simian adenoviruses for which no pre-existing immunity exists in human populations (reviewed by Barouch, 2010; Dicks *et al*., 2012; Barouch *et al*., 2011; O'Hara *et al*., 2012). Ad26 vector with an EnvA insert was found to be well tolerated in a phase 1 study (Baden *et al*., 2013), and elicited broad immune responses, including humoral and CD4+ and CD8+ T cell responses (Barouch *et al*., 2013). Similar results were obtained with Ad35 (Ratto-Kim *et al*., 2012). Nonhuman Ad vectors were also shown to be well tolerated in chimpanzees as an alternative to Ad5 (Dicks *et al*., 2012). Additional live recombinant vaccine candidates have also been developed using bacterial vectors, such as BCG (Lagranderie *et al*., 1997; Ami *et al*., 2005) and attenuated Salmonella strains (Devico *et al*., 2002).

A replication-competent viral vector that has attracted much attention recently is cytomegalovirus (CMV), as it can elicit potent and long lasting CD4+ and CD8+ T effector memory (Tem) responses (Remmerswaa *et al*., 2012). Tem reside in tissues and can rapidly

proliferate and control early virus replication (reviewed by Ahlers and Belyakov, 2010). Remarkable vaccine efficacy in rhesus macaques was obtained with a replicating simian CMV vector expressing the SIV *gag*, *rev*, *tat*, *nef* and *env* genes (Hansen *et al*., 2011). The vaccine efficiently induced effector memory CD4+ and CD8+ tissue-resident T cells, which did not protect the animals against infection following low-dose rectal challenge, but elicited early and profound control of virus replication, with half of the monkeys showing viral loads below detection level (Hansen *et al*., 2011). These findings were extended and clearance of infection was confirmed in additional vaccinated and challenged cohorts (Hansen *et al*., 2013). These results have paved the way for development of a well-tolerated recombinant CMV candidate vaccine for humans.

1.5.2.2 The Step and HVTN505 trials

The Step trial was a Phase IIb study which involved 3 000 volunteers at risk of HIV infection, from Australia, Brazil, Canada, the Dominican Republic, Haiti, Jamaica, Peru, Puerto Rico and the United States (Buchbinder *et al*., 2008). In all these countries HIV subtype B is prevalent, matching the subtype of the vaccine-delivered antigens. The goal of the trial was to see if the vaccine could prevent HIV infection or reduce viral load in those who became infected. The vaccine regimen (MRKAd5 HIV-1) consisted of an Ad5 vector expressing the *gag, pol* and *nef* genes of HIV-1 subtype B. The Phambili trial of the same vaccine also entered phase IIb evaluation in South Africa, to explore whether it would also be effective at preventing infection from HIV subtype C, which is prevalent in southern Africa, and was halted prematurely after the immunisation in the Step study was stopped, due to lack of efficacy and a higher incidence of infections in the vaccine arm. The two trials showed that the vaccine failed to prevent HIV infection or lower viral load set point (Buchbinder *et al*., 2008; Gray *et al*., 2011). However, 77% of vaccinees mounted CD8+ T cell responses to the vaccine, detected by ELISPOT. Intracellular cytokine staining revealed similar results, with 73% of vaccinated individuals mounting CD8+ T cell responses. Lower magnitude CD4+ T cell responses were detected (Buchbinder *et al*., 2008). It is important to note that vaccinees only mounted responses to two to three epitopes and this response may not have been broad enough to recognise epitopes in the infecting virus (reviewed by Wilson and Watkins, 2009). Thus, despite the generation of T cell responses in these trials, no protection from infection or effect on viral load was demonstrated. However, it may be possible that greater breadth,

different targeting, higher magnitude, and eliciting T cells at the local site of exposure remain challenges to overcome for T cell-inducing vaccines.

1.5.3 T cell vaccine design in the context of HIV diversity

Selection of immunogens in vaccine development remains a crucial issue as we do not know the true extent of HIV diversity on immune responses. Various approaches have been taken, including the development of vaccine candidates with single natural sequences or sequences from multiple subtypes, centralised and mosaic sequences, and conserved epitopes.

1.5.3.1 Single or multi-subtype vaccines

Many vaccines have been developed with sequences that match circulating HIV subtypes with the hope that if effective, they can generate protection against heterologous subtypes (cross-clade protection). In fact, cross-clade responses or immune responses generated by infected individuals to heterologous HIV subtypes have been largely demonstrated (Zembe *et al*., 2011; Aidoo *et al*., 2008; Currier *et al*., 2003; Gillespie *et al*., 2002; Keating *et al*., 2002). In an extensive study on cross-clade immune responses, Coplan *et al*. (2005) determined cellular immune responses to HIV subtypes A, B and C peptides as measures by IFN-γ ELISPOT assays. In a large cohort of HIV-infected individual from four continents, with subtype A, B and C epidemics. Cross-reactivity of cellular immune responses measured as the magnitude of responses to pools of peptides was observed in all HIV proteins tested, with Gag and Nef contributing most of the responses (Coplan *et al*., 2005). Further analyses from the same cohort extended to more HIV-infected individuals demonstrated similar extensive cross-reactivity among the three HIV-1 subtypes tested for Gag and Nef. In this larger cohort, cross-reactivity measured by the number of infected individuals who reacted to heterologous peptides was 99.1% between subtype C-infected individuals and subtype B Gag proteins, and 97.8% between subtype A infected individuals and subtype B Gag proteins (Gupta *et al*., 2006). In another study involving 39 subtype C-infected individuals, the magnitude and breadth of IFN-γ Gag-specific T cell responses were assessed for reactivity to five sets of overlapping peptides, two sets matching subtype C strains from South Africa and China, and three peptide sets corresponding to consensus subtypes A, B, and D sequences. A total of 84 peptides were recognized, of which 19 were exclusively from subtype C, 8 exclusively from subtype B, one peptide each from A and D and 17 were

commonly recognized by subtypes A, B, C and D (Zembe *et al*., 2011). When taken together, these studies demonstrate that HIV-specific T cells from individuals infected with a particular HIV subtype can cross-recognise HIV proteins or peptides based on others HIV subtypes. Although these kinds of studies have their limitations, and since those immune responses generated in natural infection might differ from those generated by a vaccine, they may nevertheless reveal important insights into whether vaccine immunogens may be sufficiently cross-reactive in a given population with particular HIV subtypes circulating.

The phase III/IIb STEP and Phambili trials used a vaccine based on genes from HIV-1 subtype B (Buchbinder *et al*., 2008). These trials were performed in the Americas and Australia with a subtype B epidemic and in South Africa with a subtype C epidemic, to study cross-protection (Buchbinder *et al*., 2008; Gray *et al*., 2010; 2011). Unfortunately the vaccine was not effective, so no insights could be gained on cross-protection. Another example of a subtype-specific vaccine was the RV144 trial, which used in a prime-boost strategy utilising subtype B and CRF01_AE genes, the infecting subtypes in Thailand where the trial was conducted (Rerks-Ngarm *et al*., 2009). The latest vaccine formulation to enter a phase III/IIb trial contained gene sequences from subtypes A, B and C in the HVTN505 trial in the US (Hammer *et al*., 2013). The trial showed no efficacy against HIV.

1.5.3.2 Centralised sequences

Different strategies have been proposed to tackle the huge genetic variability of the HIV in vaccine immunogen design. One of these approaches is the design of centralised HIV sequences that are based on a subtype or on all group M viruses. This includes the most recent common ancestor (MCRA) sequence, which represents the ancestor from which a given group of sequences have descended (Doria-Rose *et al*., 2005; Kothe *et al*., 2006), the centre-of-tree sequence (COT), which is a sequence whereby the average evolutionary distance to each tip of a phylogeny tree is minimised (Nickle *et al*., 2003; Nickle *et al*., 2007; Rolland *et al*., 2007), and a consensus sequence, which is a sequence that consists of the most common amino acid found at a given position in the group of sequences (Gaschen *et al*., 2002; Kothe *et al*., 2006).

To date, there have been no vaccine trials in humans that have tested and compared the efficacy of these centralised immunogens in different regions of the world (IAVI, 2013).

However, these sequences have been tested as candidate vaccines in preclinical studies in animal models, and most of these data are generated with Env protein sequences. It was shown that BALB/c mice, when vaccinated with a DNA vaccine followed by recombinant vaccinia virus boost expressing consensus M gp120 and gp140, elicited T cell responses that targeted epitopes not only from group M Env proteins, but also from subtype B and C (Gao *et al*., 2005). In addition, Weaver *et al*. (2010) found a similar magnitude and breadth of immune responses when comparing group M consensus Env immunogens with a B/C recombinant virus isolate in mice. These studies and others (Kothe *et al*., 2006; Weaver *et al*., 2006) have clearly demonstrated at least similar HIV-specific T cell responses are elicited compared to immunogens based on a single or multiple subtypes in mice.

In humans, in the absence of clinical trials testing centralised sequences, numerous studies have characterised the immunological recognition of HIV centralised peptides in HIV-infected individuals. In a study involving HIV subtypes B and C chronically-infected individuals from the US and South Africa respectively, Bansal *et al*. (2006) measured T cell immune responses to consensus Gag from subtype A, B, C and group M peptides and to ancestral subtype B and group M peptides using the IFN-γ ELISPOT assay. They demonstrated a similar broad cross-reactivity among the different peptides tested in both B and C epidemics. Additionally, Malhotra *et al*. (2007) also showed a comparable magnitude and breadth of immune responses among subtype-specific peptides and group M peptides, all based on centralised sequences. On the other hand, Frahm *et al*. (2008) found that group M peptides were less frequently targeted compared to subtype B peptides in a subtype B epidemic, whereas there was a similarity in the frequency of responses between group M reagents and subtypes C peptides in the same study involving subtype C-infected individuals. Similarly, a higher magnitude of HIV-specific T cell responses to consensus M Gag and Nef peptides was observed in Brazil in a multi-subtype epidemic (subtypes B, F1 than C; Côrtes *et al*., 2013). Overall, these studies suggest that centralised sequences, and especially group M peptides, were able to detect broad T cell responses. At the same time, these studies also emphasise the need to test centralised reagents in other epidemics, as the results generated from these studies may not be directly applicable to areas with different infecting subtypes or with a large degree of HIV diversity, such as west central Africa. One limitation of this approach is that centralised sequences are generated with available sequences and need permanent updating; many regions of the world have few sequences available.

1.5.3.3 Mosaic sequences

HIV mosaic antigens are bioinformatically-optimised immunogens that maximise the coverage of natural variation of the virus, and also take into account the diverse major histocompatibility complex class haplotypes (Korber *et al*., 2009). They were developed by combining sequences from different HIV subtypes using artificial recombination methods designed to mimic the recombination process that occurs during natural HIV evolution (Fischer *et al*., 2007). This can be performed for a single subtype of for all the group M variants. Vaccines based on this approach utilise computerised algorithms to generate optimised sequences similar to naturally circulating HIV sequences (Fischer *et al*., 2007). They are intended to demonstrate a greater depth or coverage of HIV potential T cell epitopes (PTEs) for different HIV proteins (Fischer *et al*., 2008). Preliminary data on this approach suggested that mosaic approach provides enhanced coverage of 9mer peptides compared to the COT approach using the same data set (Fischer *et al*., 2008).

Several vaccine candidates using the mosaic approach have been tested in mice and macaques. In mice, DNA vaccines expressing mosaic Env antigens were compared to natural Env and mutated Env (gp120 without V regions and gp41 deletion) strains. Mosaic candidates elicited responses to an average of eight peptide pools, compared to two pools for a set of natural Envs (Kong *et al*., 2009). Similar observations were made in rhesus macaques. Barouch *et al*. (2010) immunised macaques with a recombinant, replication-incompetent Ad26 expressing mosaic HIV Gag, Pol and Env antigens, M consensus, combined subtypes B and C, or natural subtype C. The total number of Gag-, Pol- and Env-specific cellular immune responses to PTE peptides elicited by the mosaic antigens was higher than the number of responses induced by the consensus or natural sequence antigens (Barouch *et al.*, 2010). Santra *et al*. (2010) also demonstrated an increase in both the breadth and depth of mosaic HIV Gag and Nef antigens expressed by a DNA prime-recombinant vaccinia regimen, compared with consensus or natural sequence HIV antigens in macaques. Barouch *et al*. (2013) evaluated for the first time the protective efficacy of an HIV mosaic vaccine. They immunised macaques with Ad/poxvirus and Ad/Ad vector-based vaccines expressing HIV mosaic Env, Gag, and Pol and challenged the monkeys with the difficult-to-neutralise SHIV-SF162P3. HIV mosaic antigens protected rhesus monkeys against acquisition of infection

following heterologous challenge, and this protection correlated with vaccine-elicited neutralising and non-neutralising antibodies (Barouch *et al*., 2013).

No vaccine candidates expressing mosaic peptides have been tested in humans to date. However, one study tested the ability of mosaic HIV immunogens expressed by recombinant, replication-incompetent Ad26 vectors to process and present major HIV subtypes B and C CD8+ T cell epitopes in human cells in an *in vitro* immunisation (Ndhlovu *et al*., 2011). A bivalent mosaic vaccine expressing HIV Gag sequences was used to transduce PBMC from 12 HIV-infected individuals from the US and 10 HIV-infected individuals from South Africa. Mosaic Gag expressed all eight subtype B epitopes tested in all US samples, and all five subtype C epitopes tested in all South Africans. In addition, the magnitude of responses induced by stimulation with mosaic immunogens was comparable to subtypes B and C immunogens tested, but the mosaic elicited greater cross-clade recognition (Ndhlovu *et al*., 2011). Polyvalent mosaic antigens therefore represent a promising strategy to expand cellular immunologic vaccine coverage for genetically diverse pathogens such as HIV.

1.6.3.4 Conserved epitopes

Certain regions of HIV are less variable than others due to a need for functional or structural conservation, and changes in these regions result in a cost to the viral fitness, or the capacity of the virus to replicate (Fernandez *et al*., 2005; Martinez-Picado *et al*., 2006). Therefore, targeting conserved regions may be beneficial in the design of HIV vaccines, to generate both cross-reactive and highly effective immune responses. This strategy was applied by Letourneau *et al*. (2007) in designing a T cell immunogen, HIV_{CONSV}. They made a chimeric protein with the 14 most conserved regions of the HIV proteome derived from a consensus sequence from subtypes A, B, C and D, and used three most studied vaccine vectors, namely DNA, Ad5 and MVA expressing HIV_{CONSV} to deliver the protein in mice. HIV_{CONSV} was found to be immunogenic in these animals, inducing HIV-specific T cell responses that produced cytokines and were capable of killing target cells (Letourneau *et al*., 2007). In addition, using PBMC from healthy donors and from an HIV vaccine trial, they found that these conserved regions primed CD8+ and CD4+ T cell to highly conserved epitopes in human PBMC (Letourneau *et al*., 2007). In a more recent study, Borthwick *et al*. (2013) provided a proof of concept of this approach in human. They used a vaccine to target T cell conserved epitopes. A DNA, simian adenovirus and modified vaccinia virus Ankara

vaccine, delivered in a prime-boost strategy, was administered in a Phase I trial in the UK. The vaccine induced high levels of T cells that recognised virus-infected autologous CD4+ cells and with strong viral inhibition capacity. The virus inhibition was mediated by both Gag- and Pol- specific effector CD8+ T cells targeting epitopes that are typically subdominant in natural infection (Borthwick *et al*., 2013). One group (Rolland *et al*., 2007) also proposed the use of conserved regions for an HIV vaccine referred to as conserved elements (CE)-vaccine, that is composed of 45 discrete viral segments of at least eight amino acids (resembling the minimum length of a CD8+ T cell epitope) that fulfil a certain conservation criteria. This analysis resulted in identification of first tier segments in which there was 98% conservation of a single amino acid at a single position across the group M sequences, and second tier segments in which there was 99% conservation of two variant amino acids at a single position across group M sequences (Rolland *et al*., 2007). These results provide evidence that vaccines targeting conserved elements in the HIV proteasome may be able to achieve control of HIV replication. Which approach is superior can only being determined in human trials. Since these trials are very difficult to implement, costly and lengthy, other strategies should be employed to understand the potentially relevant responses that T cell-based vaccines to peptides based on these candidate immunogens should elicit.

1.7 AIMS AND OBJECTIVES OF THE THESIS

Consideration of global HIV diversity has been a central focus of world-wide efforts to develop HIV vaccines. The question of whether the global burden of viral diversity can be controlled by an effective HIV vaccine has quite appropriately become somewhat of a huge challenge amongst researchers hoping to develop HIV vaccines. To date, the question of whether region-specific vaccines are necessary, or a universal vaccine is feasible, still needs to be answered. Tremendous efforts have been made to generate immunogens that can cope with HIV diversity. These vaccine projects derive hope from the fact that despite the incredible rate at which HIV sequences can evolve, it is apparent that there are regions of the genome where sequences are quite significantly conserved. With the disappointing results from vaccine trials around the world (apart from the modest protection achieved in the RV144 trial), the search for sequences or groups of sequences capable of eliciting immune responses that would be cross-reactive against all HIV-1 group M viruses is still an important focus.

Artificial sequences have been proposed to reduce the genetic distance amongst circulating subtypes, and thus addressing the issue of HIV diversity. It is therefore very important to understand the potentially relevant responses that T cell-based vaccines to these new immunogens should be elicited. This can be done using blood from HIV-1 infected individuals and identify highly targeted epitopes that can be used in vaccines formulation even though responses seen in infected individuals may not necessarily be the ones that should be induced by a vaccine.

Objectives

This study aimed to evaluate the influence of HIV diversity on the patterns of T cell recognition to peptides based on proposed HIV-1 immunogens (consensus and mosaic), mounted by HIV-infected individuals from Cameroon, a country with a highly diverse HIV epidemic. The specific objectives of the study were as follows:

Aim 1: To characterise HIV-1 genetic diversity in Cameroon
Aim 1a: To characterise HIV-1 *gag* and *nef* gene from Cameroon
Aim 1b: To characterise new HIV near full length viruses from Cameroon

Rationale

Cameroon is located in the Congo basin in west central Africa and is likely the country within which HIV-1 group M originated. Possibly as a consequence of this, it has one of the most genetically diverse HIV epidemics in the world. Alongside CRF02_AG, which accounts for more than half of infections in the country, circulating virus lineages include every known HIV-1 group M subtype, numerous CRFs, and a variety of URFs or apparently mosaic viruses which are not easily classifiable into any existing subtypes. The range of subtypes and the amount of intra-subtype diversity within any geographical region will very likely significantly impact HIV vaccine efficacy in that region. It is therefore imperative to constantly monitor and characterise HIV in different regions of the world, and more specifically in HIV diversity hot spots like Cameroon.

Aim 2: To measure immune responses to HIV-1 group M consensus Gag and Nef peptides using the IFN-γ ELISPOT assay, in order to assess their recognition by HIV-infected

individuals from Cameroon harbouring a large degree of HIV diversity, and compare this to a low diversity subtype C epidemic from South Africa.

Rationale

Numerous studies have described broadly cross-reactive T cell immune responses in several African populations, namely from Uganda, Kenya, Cote d'Ivoire and South Africa, using the IFN-γ ELISPOT assay. Predictably, these studies have shown that the majority of cross-reactive responses were directed towards regions of low intra-subtype diversity and high inter-subtype homology. In addition, the majority of these studies have focused on measuring responses to immunodominant regions of HIV-1 subtypes A, B or C, the three subtypes that dominate the epidemic globally, since testing responses to all isolates on the African continent is not feasible. The results generated from such studies may not be directly applicable to areas with a large degree of HIV diversity, such as west central Africa. One of the simplest ways to reduce the genetic distance between a vaccine strain and contemporary circulating viruses is to create artificial sequences such as the consensus Group M sequence. There is a need to evaluate cross-reactive responses to reagent sets based on these new sequences, particularly in HIV-infected populations in areas where multiple subtypes and recombinant forms circulate, such as West Central Africa. In fact, there is a paucity of immunological studies on HIV infection from this region, with only a single published study to date on T cell responses in HIV-infected individuals from Cameroon.

Aim 3: To measure immune responses to HIV-1 group M potential T cell epitope (PTE) Gag and Nef peptides using the IFN-γ ELISPOT assay in HIV-infected individuals from Cameroon, and compare these to consensus peptide responses, in order to determine whether reagents based on immunogens designed to encompass greater HIV variability would lead to detection of broader immune responses than consensus reagents.

Rationale

New candidate HIV vaccine immunogens have recently been designed that attempt to take into account the large degree of genetic diversity of HIV, by including a vast array of

potential T cell epitopes from multiple subtypes. These are known as ‘mosaic’ constructs and preclinical studies in rhesus macaques have demonstrated that they augment both the breadth and depth of antigen-specific T cell responses compared to both natural and central (e.g. consensus) sequences. PTE peptides based on the mosaic constructs will be tested in order to identify T cell epitope reactivity in PBMC from HIV-infected Cameroonians, and determine whether this approach leads to broader detection of T cell responses than consensus M reagents.

CHAPTER 2: CHARACTERISATION OF HIV-1 GENETIC DIVERSITY IN CAMEROON

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2.1 INTRODUCTION

The Congo basin in west central Africa is thought to be the origin of HIV, where several cross-species transmission events from chimpanzees to humans occurred (Keele *et al*., 2006; Sharp and Hahn, 2011). Cameroon, located in this region, has one of the most genetically diverse HIV epidemics in the world (Brennan *et al*., 2008; Ragupathy *et al*., 2011; Machuca *et al*., 2007; Ndembi *et al*., 2008). Alongside CRF02_AG, which accounts for more than half of infections in the country, circulating virus lineages include every known HIV-1 group M subtype, numerous CRFs and a variety of apparently URFs (Carr *et al*., 2010). The prevalence of HIV-1 in Cameroon is 4.5%, which is one of the highest in west central Africa (UNAIDS, 2013). This, together with the co-circulation of divergent variants of multiple subtypes, has created the conditions for frequent mixed infections and inter-subtype recombination.

Despite the high diversity of HIV in Cameroon and the west central African region as a whole, HIV-1 group M CRF02_AG dominates the epidemic throughout this part of Africa. CRF02_AG is largely confined to west central Africa, and in addition to Cameroon, makes a major contribution to the epidemics of Guinea-Bissau, Senegal, Gambia, Nigeria, Ghana, Cote d'Ivoire and Mali (Esbjornsson *et al*., 2011; Hamel *et al*., 2007; Sankalé *et al*., 2007; Delgado *et al*., 2008; Toni *et al*., 2007; Imamichi *et al*., 2009). In the most recent overview of global subtype distribution, CRF02_AG made up 8% of all HIV-1 infections, representing the fourth most prevalent HIV-1 variant worldwide, after subtypes C, A and B (Hemelaar *et al*., 2011). Interestingly, this was an increase of over 60% from the three year period prior to that.

Given the high degree of HIV diversity in Cameroon, several studies have questioned whether CRF02_AG has a replicative advantage over other variants circulating there. There is some evidence of CRF02_AG variants having both greater replicative fitness than subtype A and G variants *in vitro* (Njai *et al*., 2006; Konings *et al*., 2006), and yielding viral loads which are five-fold higher than those found in infections with other HIV-1 group M subtypes and recombinants (Fischetti *et al*., 2004).

Constantly improving phylogenetics-based analytical techniques and rapidly expanding HIV sequence datasets allow for better characterisation of diverse sequences, and promise to yield important insights into the origin, evolution and spread of HIV-1.

Given the potential impact of HIV-1 diversity on both vaccine development and the sustainability of antiretroviral therapies, it is particularly important that molecular epidemiological surveillance is continued in HIV diversity hotspots such as Cameroon.

The study described in this chapter characterised the diversity of *gag* and *nef* genes of Cameroonian HIV-1 isolates. These genes are particularly relevant because they encode highly immunogenic proteins that are frequently included in candidate vaccines (Masemola *et al*., 2004; McMichael and Haynes, 2012; Stephenson *et al*., 2012). To obtain a phylogenetic view of Cameroonian HIV diversity that explicitly accounted for the confounding effects of recombination, extensive recombination-aware phylogenetic analyses were performed with these new sequences along with publically available homologous HIV-1M *gag* and *nef* sequences from the Congo basin and a representative selection of the major known HIV lineages from the rest of the world. This study also characterised nine new HIV-1 near full length sequences from Cameroon.

2.2 MATERIALS AND METHODS

2.2.1 Study participants

Anonymously-donated HIV-infected blood units (n=59) were collected between December 2006 and August 2007 from Yaoundé Central Hospital, Cameroon, in a study approved by the National Ethics Committee of the Cameroonian Ministry of Health and the University of Cape Town Research Ethics Committee. Although no data on risk factors for HIV was available for the blood donors, they are believed to represent the general adult population of Yaoundé. All donors were antiretroviral therapy naïve and only age and gender information were obtained. CD4 counts were performed by flow cytometric analysis (Becton Dickenson) and viral load was determined from plasma by a second-generation real-time reverse transcription RT polymerase chain reaction (PCR; Abbott).

2.2.2 PCR amplification

2.2.2.1 Gag and Nef sequences

RNA was extracted from plasma samples using a Magna-Pure compact Nucleic Acid extractor (Roche). HIV *gag* and *nef* complementary DNA (cDNA) were generated using the Invitrogen Thermoscript^{TM} RT-PCR system (Invitrogen). cDNA from the RT step was PCR amplified as described previously (Bredell *et al*., 2007) using non-specific HIV-1 primers for HIV-1 full length *gag* (Bredell *et al*., 2007) and *nef* (Artenstein *et al*., 1996) genes. The *gag* and *nef* genome fragments were amplified, producing 1.5 kb fragment spanning the whole *gag* region and 700 kb sequences spanning the entire *nef* region. PCR was performed using the Expand High Fidelity kit (Roche-Mannheim). The first and second round reactions were performed using 1ul dNTP (10mM), 5ml 10X buffer plus $MgCl_2$ (15mM), 1ul of each primer (10mM), 0.75ul Expand High Fidelity Enzyme (5U/ul), water and template, in 50ul total reaction volume with no hot-start method. For *gag*, first round cycling conditions were: 94^0C, 2 min, and then 10 cycles of 94^0C 15 s, 52^0C 30s, 72^0C 1 min, followed by 20 cycles of 940C 15s, 55^0C 2 min, 72^0C 1.5 min and then 72^0C 7 min. The second round cycling conditions consisted of 94^0C 2 min, followed of by 35 cycles of 94^0C 15s, 55^0C 30s, 72^0C 1.5 min and then 72^0C 7 min. The *gag* first round primers were: Gag D forward (HXB2: 626-644; 5'–TCTCTAGCAGTGGCGCCCG) and Gag D reverse (HXB2: 2402-2382; 5'–AATTCCTCCTATCATTTTTGG). The second round reaction was with Gag A forward (HXB2: 683-704; 5'-CTCTCGACGCAGGACTCGGCTT) and Gag C reverse (HXB2: 2356-2334; 5'–TCTTCTAATACTGTATCATCTGC); in some cases, Gag D forward was replaced by Gag Polm1 reverse (HXB2: 2632-2610; 5'-TCTGTCAATGGCCATTGTTTAAC).

For *nef*, the cycling conditions were the same for both first and second round, consisting of 94^0C 2 min followed by 35 cycles of 94^0C 15s, 55^0C 15s, 72^0C 50s and then 72^0C 7 min. The *nef* first round primers were: Nef outer5-Ie (HXB2: 8513-8533; 5'–GTGCCTCTTCAGCTACCACCG) and Nef outer3-3e (HXB2: 9808-9488; 5'–AGCATCTGAGGGTTAGCCACT). The nef second round primers were Nef inner5-Ie (HXB2: 8698-8717; 5'-TGGACAGAYAGGGTTATAGAA) and Nef inner 3-7e (HXB2: 9467-9448; 5'-CACCTCCCCTGGAAAGTCCCC). All sequences had at least two clear readings in each direction for completion.

2.2.2.2 Near full length sequences

RNA from plasma samples selected based on phylogenetic analyses of the *gag* and *nef* sequences were manually extracted using the QIAamp Viral RNA Mini Kit (QIAGEN, Roche). Virtually full-length HIV cDNA was generated using the Thermoscript^{TM} RT-PCR system (Invitrogen) and was amplified. Limiting template dilution into the first round was performed to obtain single genome amplificons (SGA) (Salazar-Gonzalez *et al*., 2009) with 1 ul, 1:3, 1:9, and 1:27 dilutions of cDNA for initial screening of the cDNA of the first round, and with each dilution consisting of eight replicate reactions. If only non-specific bands were visible after gel electrophoresis of second round products, further dilution (at 1:50, 1:100, 1:200 *etc*) of cDNA was performed for subsequent reactions. Additionally, if there was no amplification following initial screening, 2ul to 5ul of undiluted cDNA was added to further reactions in order to obtain a full-length (~9kb) product. The desired number of positive full-length products equated to: the number of positive full-length amplicons divided by the number of reactions and should be >20%. This percentage results in the likely production of SGAs, which will, when sequenced, have no "double-peaks" (Salazar-Gonzalez *et al*., 2009). The virtually full-length genomes were amplified using the first primers (Treurnicht *et al*., 2010), 1.U5C (HXB2: 538-571; 5'-GGGTGAGTGCTCTAAGTAGTGTGTGCCCGTCTGT) 1.U5Cb (HXB2: 538-571; 5'-GGGTGAGTGCTCTAAGTAGTGTGTGCCCATCTGT), 1.3.3PIC (HXB2: 9625-9665; 5'-GGGACTTAGAGCACTCAAGGCAAGCTTTATTG), followed by amplification with the second round primers, 2.U5C (HXB2: 540-580; 5'-GGCCGCGGATCCAGTAGTGTGTGCCCGTCTGTTGTGTGACT) and 2.3.3plC (HXB2: 9650-9604; 5'-GGCCGCGCGGCCGCTAGAGCACTCAAGGCAAGCTATTGAGGCTTA). PCRs was performed as described (Treurnicht *et al*., 2010) using the Expand Long Template kit (Boehringer-Mannheim) and a hot-start method. The first and second round reactions were performed using 0.9ul dNTP (20mM), 5ml 10X buffer plus MgCl2 (15mM), 0.3ul of each primer (50pmoles/ul), 0.75ul Expand High Fidelity enzyme (5U/ul), water and template in a 50ul total reaction volume. Cycling conditions were: 94^0C for 2 min, then ten cycles of 94^0C for 10s, 60^0C for 30s and 68^0C for 8 min. This was followed by 20 cycles where the annealing temperature was 68^0C. The final extension step was 68^0C for 20 min. In some cases, multiple second round PCR amplifications were combined to provide sufficient template for sequencing.

2.2.3 DNA sequencing

Sequencing was performed at the University of Stellenbosch core facility (*www.us.ac.za*) using four primers for Gag, two for Nef and 32 for HIV full length. Fifty full length HIV-1 *gag* sequences and 55 *nef* genes from 59 HIV-infected blood donors in Cameroon were obtained. In addition, nine near full length HIV genomes were obtained.

2.2.4 Analysis

2.2.4.1 Gag and Nef sequences

Sequenced fragments were assembled using ChromasPro. Full length *gag* and *nef* sequences were generated, together with a representative selection of 270 *gag* and 279 *nef* HIV sequences from the rest of the world and all other published *gag* (266) and *nef* (278) sequences from Cameroon and other west central African countries available in the Los Alamos National Library (LANL; *http://hiv-web.lanl.gov/content/hiv-db*) and Genbank databases (May 2012). These were aligned using MUSCLE (Edgar, 2004) with default settings and manual editing of alignments in MEGA5. The representative sequences chosen from the rest of the world were specifically selected to include the broadest diversity of sequences previously identified as belonging to known HIV-1 group M subtypes and CRFs. This was achieved by constructing maximum likelihood trees from all available *gag* and *nef* sequences for each subtype and CRF, and selecting one sequence from each of the up to ten most basal lineages from the root of these subtypes and CRFs. Maximum likelihood phylogenetic trees were constructed from these sequences with 100 full maximum likelihood bootstrap replicates implemented in PHYML (Guindon *et al*., 2005), following either complete removal of recombinant sequence fragments or the division of recombinant sequences into their constituent fragments by a blinded fully exploratory screen for recombination using RDP4 (Martin *et al*., 2011). The *gag* tree was rooted using HIV-1 group N, O and P and SIVcpz isolates, while the *nef* tree was rooted with HIV-1 group N, O and P isolates. The recombination screen was fully exploratory in that every sequence was analysed for evidence of both intra- and inter-subtype recombination. Either full *nef* and *gag* sequences or the sub-fragments of these sequences identified as having recombinant origins were classified as belonging to particular HIV subtypes if they clustered with reference sequences of these subtypes.

2.2.4.2 Near full length sequences

Sequenced fragments were assembled using ChromasPro. Near full length sequences were generated and aligned using MUSCLE with manual editing in MEGA5, together with a representative selection of 395 near full length sequences from the rest of the world and all 144 published full length sequences from Cameroon and other west central African countries that were available in the LANL (*http://hiv-web.lanl.gov/content/hiv-db*) and Genbank (Jjune 2013) databases. Computations were performed using facilities provided by the University of Cape Town's ICTS High Performance Computing Facility (*http://hpc.uct.ac.za*). As for *gag* and *nef* sequences, the same method was used to select a representative selection of the major known HIV lineages from the rest of the world. A maximum likelihood phylogenetic tree was constructed from these sequences with 100 full maximum likelihood boostrap replicates using Fastree. Only HIV-1 group M sequences were included in this analysis and the phylogenetic tree was midpoint rooted.

Divergent sequences were defined as those residing on isolated branches outside of subtrees containing previously defined HIV-1 subtype or CRF lineages. Outlier sequences, on the other hand, were defined as those residing on basal branches of subtrees containing previously defined HIV-1 subtype or CRF lineages.

2.3 RESULTS

2.3.1 Characteristics of study individuals

Clinical and demographic data of the 59 HIV-infected Cameroonian study participants are summarised in Table 2.1. Individuals were between 19 and 54 years of age, with a median age of 31 years, and consisted of 46 males and 13 females. The median CD4 cell count was 432 cells/mm^3 (interquartile range or IQR 301-665). All individuals were antiretroviral therapy-naïve, and there was a wide range of viral levels, with a median HIV-1 RNA viral load of 4.93 Log RNA copies/ml (IQR 3.29-6.32). The HIV seroconversion dates for these individuals were not known.

Table 2.1 Characteristics of study individuals (n=59)

Age[a] (years)	31 (21-54)
Sex (Male:Female)	46:13
CD4 count[a] (cells/mm^3)	432 (301-665)
Viral load[a] (Log RNA copies/ml)	4.93 (4.4-5.3)

[a]Median and IQR

2.3.2 HIV-1 distribution in Cameroon based on *gag* and *nef* sequences

Fifty full length HIV-1 gag and 55 nef genes were sequenced from 59 HIV-infected blood donors in Cameroon. All sequences were derived from HIV-1 group M viruses (Table 2.2). The sequences clustered with different subtypes and CRFs and were distributed throughout the HIV-1 group M *gag* and *nef* phylogenetic trees (Figure 2.1), an observation that is consistent with the breadth of HIV-1 diversity previously described in Cameroon.

CRF02_AG-like viruses dominated the subtype distribution, infecting 50% of the 46 participants for which both genes were sequenced (Figure 2.2 and Table 2.2). Participants infected with viruses having both *nef* and *gag* sequences that clustered within known HIV-1group M subtypes included those belonging to subtypes G, D, A, and F. Subtype G sequences accounted for 11% of infections, subtype D for 4% and sub-subtypes A1 and F2 for 2% each. In addition to CRF02_AG, other CRFs identified were CRF37_cpx (4%), and CRF01_AE and CRF36_cpx (2% each). In two samples for which only *gag* or *nef* sequences was typed, these were classified as belonging to CRF11_cpx. Notably, despite subtypes B and C collectively accounting for approximately 60% infections worldwide (Hemelaar *et al.*, 2011), none of these new sequences were classified as belonging to either of these subtypes.

Table 2.2 Subtype distribution of HIV-1 in Cameroon

Sample ID	*gag* gene	*nef* gene	Genotype
BS01	CRF02_AG	CRF02_AG	**CRF02_AG**
BS02	A-like[b]	CRF02_AG	**URF**
BS03	G	G	**G**
BS04	G	ND[c]	**G[a]**
BS05	CRF02_AG	ND[c]	**CRF02_AG[a]**
BS06	CRF02_AG	CRF02_AG	**CRF02_AG**
BS09	CRF02_AG	A1	**URF**
BS10	A1	A1	**A1**
BS11	CRF02_AG	F	**URF**
BS12	G	G	**G**
BS13	CRF02_AG	A1	**URF**
BS14	CRF02_AG	CRF02_AG	**CRF02_AG**
BS16	CRF02_AG	CRF02_AG	**CRF02_AG**
BS18	ND[c]	CRF02_AG	**CRF02_AG[a]**
BS19	CRF02_AG	CRF02_AG	**CRF02_AG**
BS20	ND[c]	CRF02_AG	**CRF02_AG[a]**
BS21	CRF02_AG	CRF02_AG	**CRF02_AG**
BS22	CRF02_AG	CRF02_AG	**CRF02_AG**
BS23	CRF02_AG	CRF02_AG	**CRF02_AG**
BS24	CRF37_cpx	CRF37_cpx	**CRF37_cpx**
BS25	CRF02_AG	F	**URF**
BS26	CRF01_AE[b]	CRF01_AE	**CRF01_AE**
BS27	CRF37_cpx[b]	CRF37_cpx	**CRF37_cpx**
BS29	CRF02_AG	U[b]	**URF**
BS30	D	D	**D**
BS31	ND[c]	CRF02_AG	**CRF02_AG[a]**
BS32	CRF02_AG	CRF02_AG	**CRF02_AG**
BS35	ND[c]	CRF11_cpx	**CRF11_cpx[a]**
BS38	CRF02_AG	CRF02_AG	**CRF02_AG**
BS39	CRF02_AG	CRF02_AG	**CRF02_AG**
BS40	CRF36_cpx	CRF36_cpx	**CRF36_cpx**
BS42	URF	CRF01_AE[b]	**URF**
BS43	CRF02_AG	CRF02_AG	**CRF02_AG**
BS44	ND[c]	CRF02_AG	**CRF02_AG[a]**
BS45	CRF02_AG	CRF02_AG	**CRF02_AG**
BS46	G	G	**G**
BS47	CRF02_AG	CRF02_AG	**CRF02_AG**
BS48	G	G	**G**
BS49	F2	F	**F2**
BS50	CRF02_AG	CRF02_AG	**CRF02_AG**
BS51	G	G	**G**
BS53	CRF02_AG	CRF02_AG	**CRF02_AG**
BS54	D	D	**D**
BS55	CRF02_AG	F	**URF**
BS56	CRF02_AG	CRF02_AG	**CRF02_AG**
BS57	CRF11_cpx[b]	ND[c]	**CRF11_cpx[a]**
BS64	CRF02_AG	CRF02_AG	**CRF02_AG**
BS65	CRF22_01A1	CRF01_AE	**URF**
BS66	CRF02_AG	CRF02_AG	**CRF02_AG**
BS71	CRF02_AG	CRF02_AG	**CRF02_AG**
BS72	CRF36_cpx[b]/F2[b]	CRF01/F	**URF**
BS73	CRF02_AG	CRF02_AG	**CRF02_AG**
BS74	ND[c]	A-like[b]	**A-like[a]**
BS75	CRF02_AG	CRF02_AG	**CRF02_AG**
BS77	CRF02_AG	CRF02_AG	**CRF02_AG**
BS78	ND[c]	CRF01_AE	**CRF01_AE[a]**
BS79	ND[c]	CRF02_AG	**CRF02_AG[a]**
BS81	CRF02_AG	ND[c]	**CRF02_AG[a]**
BS82	ND[c]	CRF02_AG	**CRF02_AG[a]**

[a]Genotype was uncertain in samples for which only a single gene was determined ; [b]Outlier; [c]ND: Not done

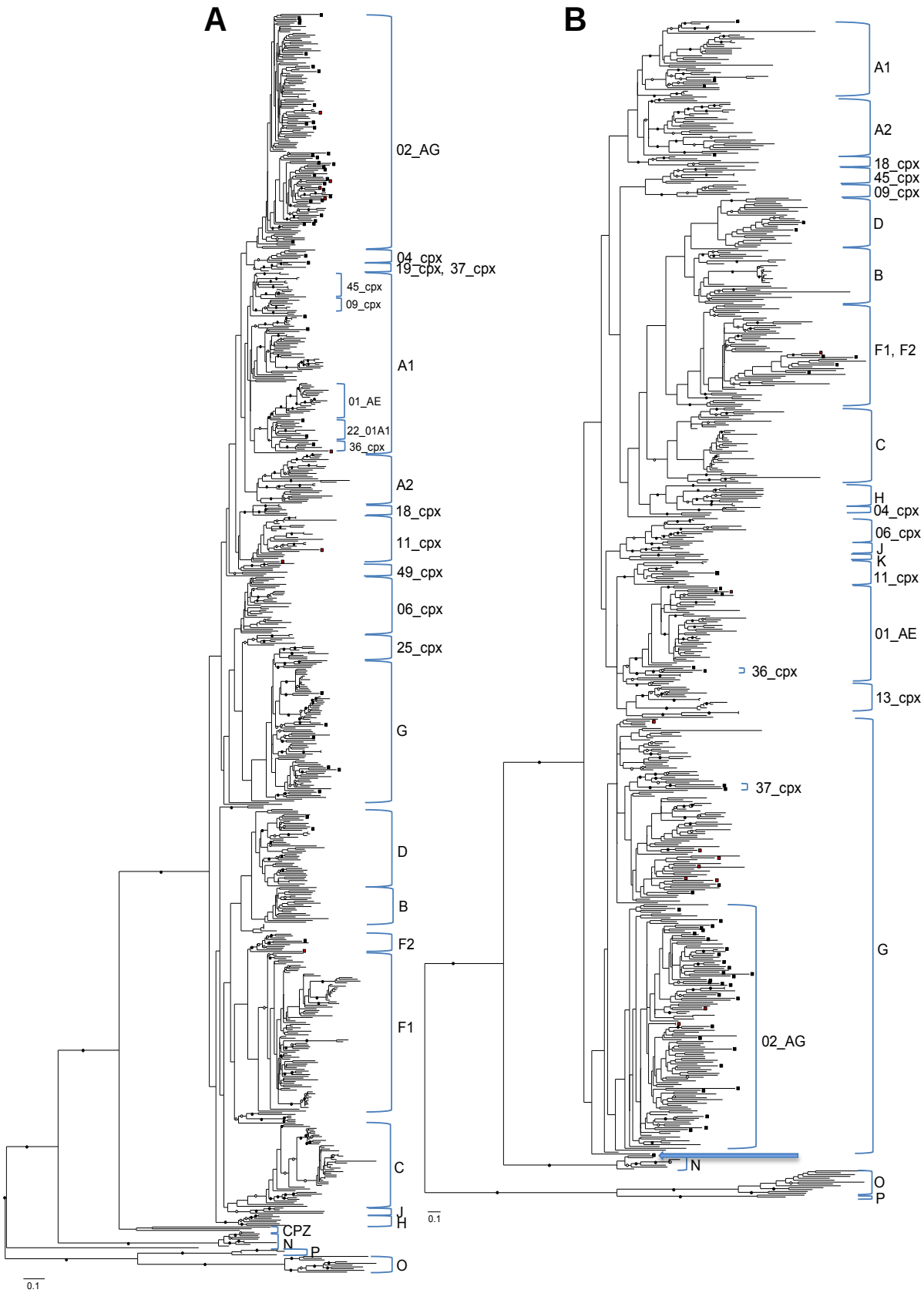
A
02_AG
04_cpx
19_cpx, 37_cpx
45_cpx
09_cpx
A1
01_AE
22_01A1
36_cpx
A2
18_cpx
11_cpx
49_cpx
06_cpx
25_cpx
G
D
B
F2
F1
C
J
H
CPZ
N
P
O
0.1
B
A1
A2
18_cpx
45_cpx
09_cpx
D
B
F1, F2
C
H
04_cpx
06_cpx
J
K
11_cpx
01_AE
36_cpx
13_cpx
37_cpx
G
02_AG
N
O
P
0.1

Figure 2.1 (previous page) Maximum likelihood trees indicating the phylogenetic relationships between (A) 727 gag and (B) 628 nef sequences of HIV-1. The trees were constructed from these sequences with 100 boostrap replicates following removal of recombinant sequence fragments by a blinded fully exploratory screen for recombination using RDP3. Black squares at the end of the branches represent the gag and nef sequences sampled from Cameroon in this study, while red squares represent intragene recombinant fragments in our the samples. The *gag* tree was rooted using HIV-1 group N, O, P and SIVcpz isolates, while the *nef* tree was rooted with HIV-1 group N, O and P isolates. Solid and open circles indicate branches with greater than 70% and 50% bootstrap support, respectively. The arrow in the *nef* tree indicates an outlier of both subtypes G and CRF02_AG.

In 10/46 samples for which both *nef* and *gag* sequences were analysed, the genes were classified as belonging to different subtypes from one another. One of the two gene sequences from 8/10 of these patients was classified as CRF02_AG (Figure 2.2 and Table 2.2).

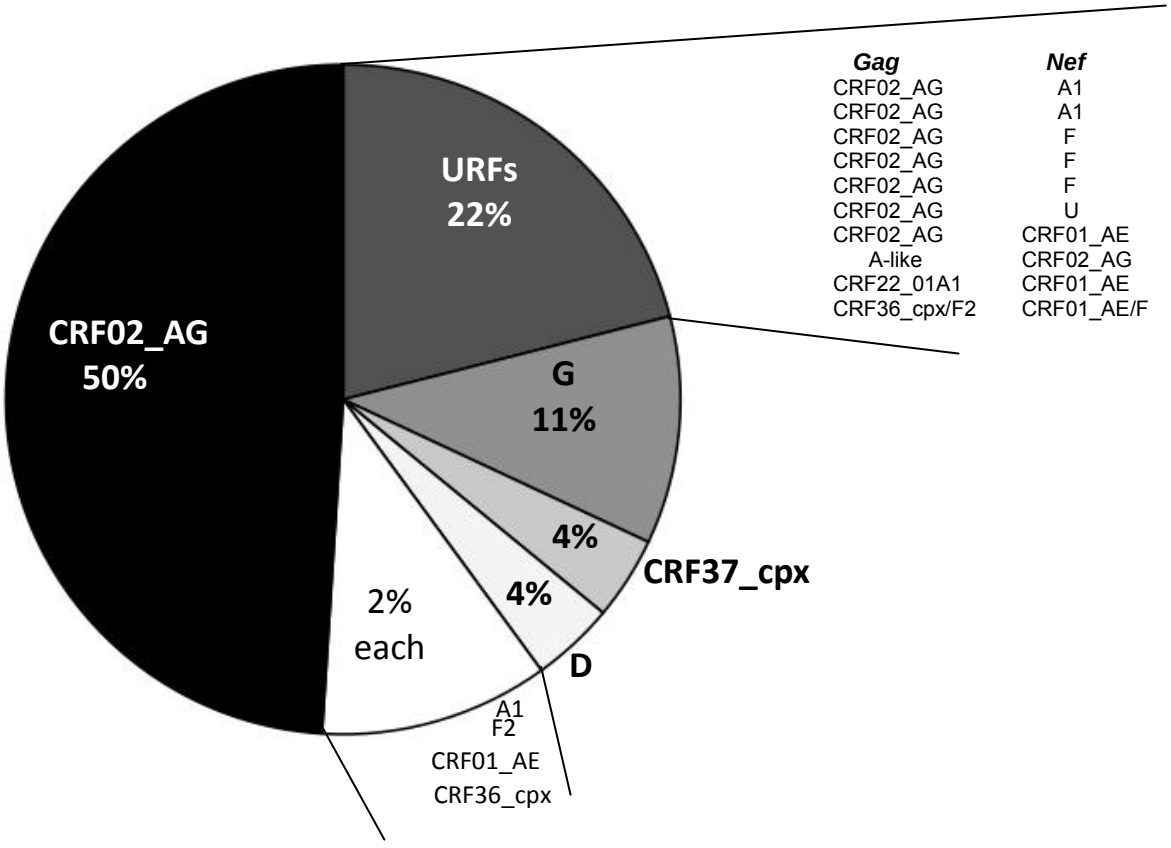

Figure 2.2 Pie chart summarising the distribution of HIV-1 group M subtypes and recombinant forms full length *gag* and *nef* gene sequencing (n=46). Intergene recombinants are detailed in the right panel. The 13 samples that were typed in only one of the genes were excluded from this analysis.

2.3.3 Identification of intra- and inter-subtype *gag* and *nef* recombinants

Numerous recombination breakpoints were also detected within the genome regions analysed. Among the newly sequenced *gag* genes, three (6%) were identified as containing recombination breakpoints between gene segments that phylogenetically clustered within two distinct groups of the same subtype, indicating that they were likely intra-subtype recombinants. Whereas two of these (BS05 and BS55) were CRF02_AG/02_AG recombinants, one (BS57) was a CRF11_cpx/11_cpx recombinant (Table 2.3 and Figure 2.3). Four of the newly sequenced *nef* genes (7%) also showed evidence of intra-subtype recombination, including three (BS12, BS48 and BS51) which were intra-subtype G recombinants and one (BS39) which was an intra-CRF02_AG recombinant (Table 2.3 and Figure 2.3).

Whereas one of the newly sequenced *gag* genes (BS72) was apparently derived through inter-subtype recombination between F2 and CRF36_cpx parental viruses, one of the *nef* genes was apparently derived through recombination between F and CRF22_01A1 parental viruses (Table 2.3).

Table 2.3 Inter- and intra-subtype recombinants

Sample ID	*gag* gene	*nef* gene
BS02	A-like[a]	CRF02_AG
BS09	CRF02_AG	A1
BS11	CRF02_AG	F
BS13	CRF02_AG	A1
BS25	CRF02_AG	F
BS29	CRF02_AG	U[a]
BS42	CRF02_AG	CRF01_AE[a]
BS39	CRF02_AG	CRF02_AG/CRF02_AG
BS55	CRF02_AG/CRF02_AG	F
BS05	CRF02_AG/CRF02_AG	ND[b]
BS65	CRF22_01A1	CRF01_AE
BS72	CRF36_cpx[a]/F2[a]	CRF01_AE/F
BS57	CRF11_cpx[a]/CRF11_cpx	ND[b]
BS12	G	G/G
BS48	G	G/G
BS51	G	G/G

[a]Outlier; [b]ND: Not done

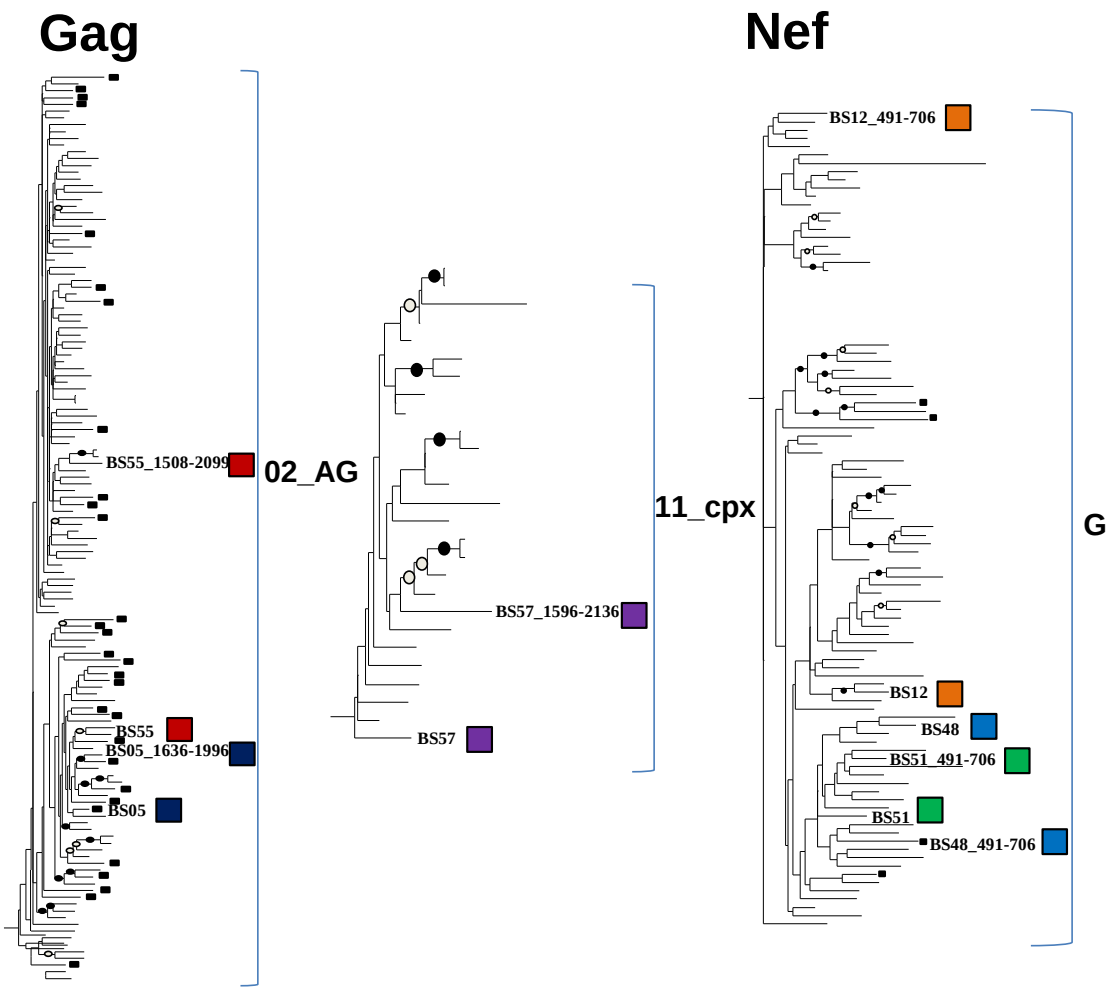

Figure 2.3 Maximum likehood subtrees indicating examples of the intragene recombinants in gag and nef. The trees were constructed following removal of recombinant sequence fragments by a blinded fully exploratory screen for recombination using RDP4. Each colour represents fragments of the same sequence.

2.3.4 Viruses with highly divergent *gag* and *nef* sequences

The phylogenetic analysis of *gag* sequences derived from the Cameroonian samples further revealed five other Cameroonian *gag* sequences determined here that branched very near the base of the subtypes that they grouped with. These were: BS02 from the base of CRF09_cpx/CRF45_cpx, BS27 from the base of CRF37_cpx, BS26 from the base of CRF01_AE, BS57 from the base of the CRF11_cpx and BS72 from the base of the F2 and CRF36_cpx clades (Figure 2.4). Similarly, in *nef*, three such sequences also branched from near the base of the subtypes that they were most closely clustered with: BS74 near the base

of the A subtype, BS42 near the base of the CRF01_AE subtype and BS29 near the base of the CRF02_AG/G subtypes (Figure 2.4).

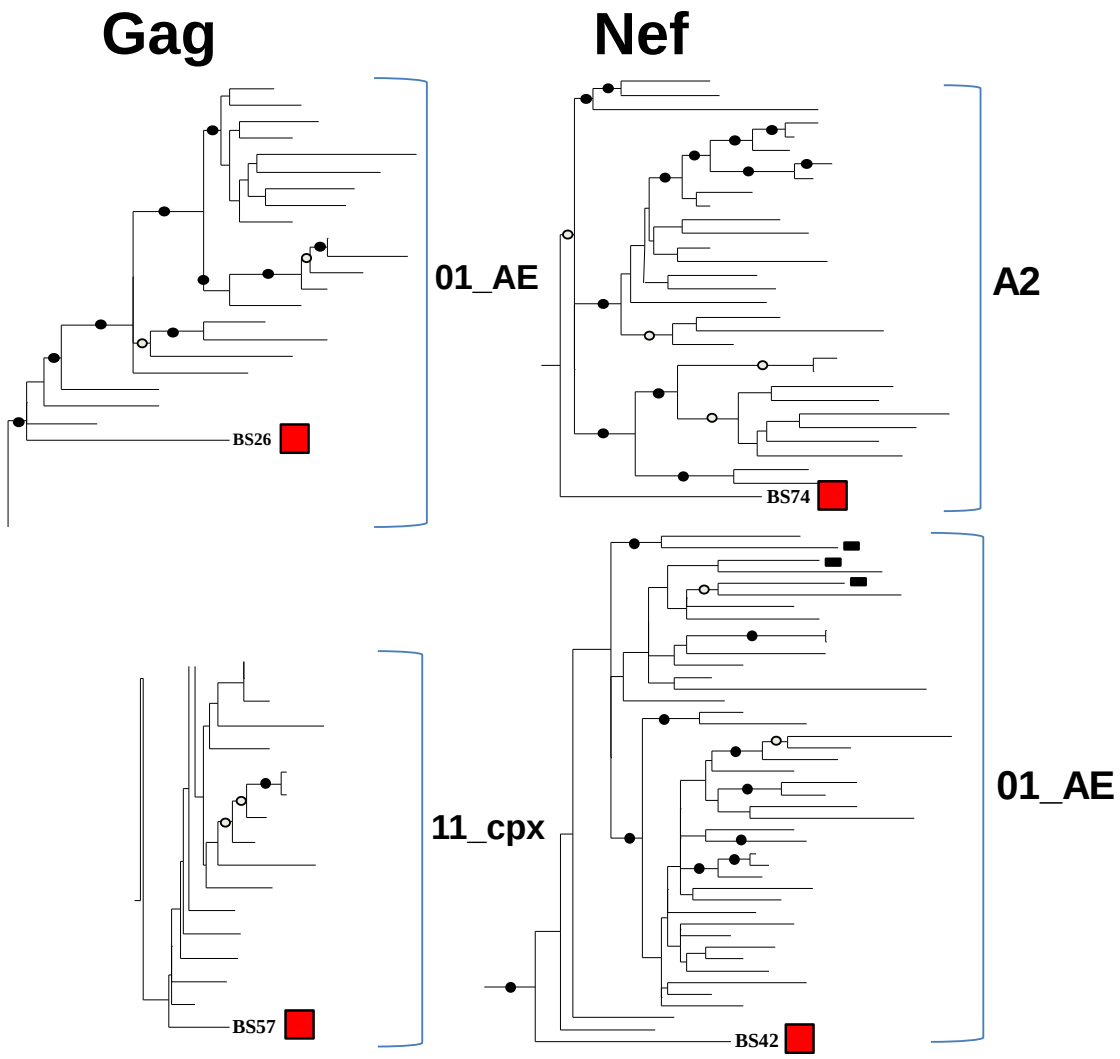

Figure 2.4 Maximum likehood subtrees indicating examples of the divergent outlier sequences in *gag* and *nef*. The phylogenetic analysis of *gag* and *nef* sequences derived from the Cameroonian samples revealed sequences (represented here with a red square) that were situated at the base of the subtypes that they grouped with.

Finally, the blind recombination screen also revealed that some divergent Cameroonian viruses previously identified as being complex recombinants, such as CRF11_cpx, is a highly divergent but not obviously recombinant lineage forming an isolated branch in both phylogenetic trees (at least in gag and nef; red portion in Figure 2.5)

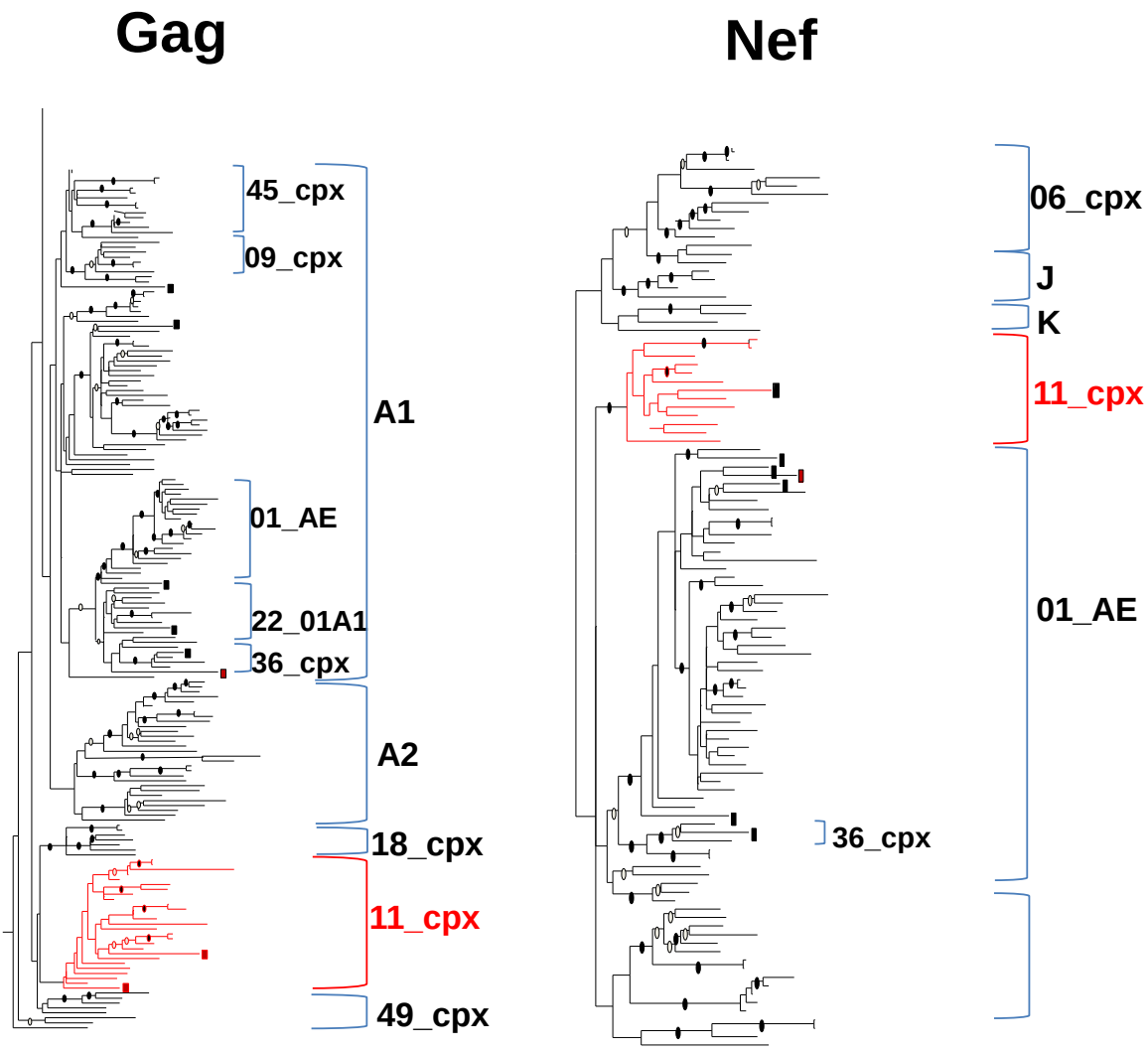

Figure 2.5 Maximum likehood subtrees showing the portions of subtype CRF11_cpx both in *gag* and *nef*. CRF11_cpx may represent at least in *gag* and *nef* a highly divergent not obviously recombinant lineage forming and isolated branch in both phylogenetic trees (red portion in both trees).

2.3.5 Near full length HIV-1 sequences from Cameroon

Nine viruses were selected for further full genome sequencing based on the results of the *gag* and *nef* phylogenetic analyses. These samples were chosen either because they were divergent sequences residing on isolated branches of subtrees containing previously defined HIV-1 subtype or CRF lineages, or because they were outlier sequences residing on basal branches of subtrees containing previously defined HIV-1 subtype or CRF lineages.

Based on the maximum likelihood phylogenetic trees generated using these and other representative sequences both from the global HIV-1group M epidemic and from central west-Africa (Figures 2.6 and 2.7), several observations were made for the nine full genome sequences determined here.

BS29 had a divergent *nef* sequence that was an outlier of the subtypes G and CRF02_AG groups, but had a full genome sequence that clustered within the subtype CRF02_AG subtree. For BS57 although the *gag* sequence was an outlier of all know CRF11_cpx lineages, the full BS57 sequence clustered within the CRF11_cpx subtree. For BS46 and BS48, both had divergent *gag* sequences branching near the base of the subtype G subtree, but have full genome sequences that clustered within the subtype G subtree (albeit with a divergent subtype G subtype). For BS40, as was the case for its *gag* sequence, was an outlier of the only two CRF36_cpx lineages that are available in the LANL. For BS13, it was a divergent sequence residing on isolated branch of CRF02_AG subtree in *gag*; the full genome sequence was an outlier of all the CRF02_AG sequences sampled to date. This newly acquired sequence may help trace the temporal and spatial origins of this important CRF as well as resolve the controversy surrounding the potentially recombinant origins of subtype G (Zhang *et al*., 2010; Carr *et al*., 1998; Abecasis *et al*., 2007).

Two full length sequences, BS11 and BS55, were both too divergent to be placed within any existing subtype or CRF grouping and therefore remained unclassified. They may in fact belong to a novel CRF or subtype, since they clustered with three other previously characterised Cameroonian sequences. The full length viral genome of BS72 was also too divergent to be placed within any existing subtype or CRF grouping.

Finally, the phylogenetic tree revealed that some of the Cameroonian sequences previously identified as URFs are divergent outliers of some of the major HIV-1M subtypes. Sequences 02CM.1807LE is an outlier of a group of subtypes including B, D, F, C and K; sequence 02CM.1710LE is an outlier of subtype CRF11_cpx; sequence 02CM.1895LE is an outlier of subtype CRF01_AE; sequence 97CA-MP645M/O is outlier of another group of major HIV-1M lineages including CRFs 09_cpx, 36_cpx, 01_AE, 22_01A1 and 02_AG (red arrows in Figures 2.6 and 2.7). These results therefore provide further evidence that Cameroon is a major hotspot of HIV diversity.

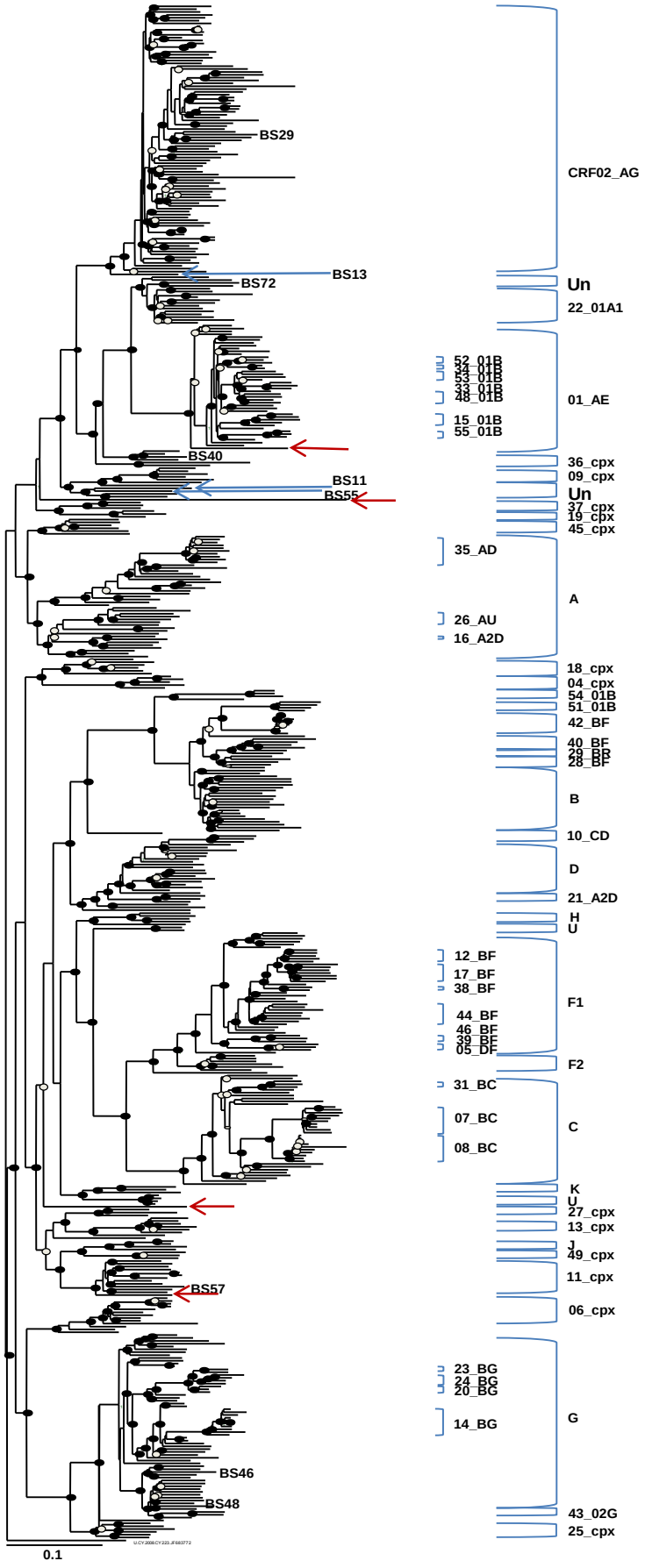
BS29
BS13
BS72
BS40
BS11
BS55
BS57
BS46
BS48
0.1
CRF02_AG
Un
22_01A1
52_01B
44_01B
53_01B
33_01B
48_01B
01_AE
15_01B
55_01B
36_cpx
09_cpx
Un
37_cpx
19_cpx
45_cpx
35_AD
A
26_AU
16_A2D
18_cpx
04_cpx
54_01B
51_01B
42_BF
40_BF
29_BF
28_BF
B
10_CD
D
21_A2D
H
U
12_BF
17_BF
38_BF
F1
44_BF
46_BF
39_BF
05_DF
F2
31_BC
07_BC
C
08_BC
K
U
27_cpx
13_cpx
J
49_cpx
11_cpx
06_cpx
23_BG
24_BG
20_BG
G
14_BG
43_02G
25_cpx

Figure 2.6 (previous page) Maximum likehood tree indicating the phylogenetic relationships between 539 full length sequences of HIV-1, indicating the nine new full length sequences generated from Cameroon. The tree was constructed from these sequences with 100 boostrap replicates. Red arrows in the tree indicate previously identified URF sequences from Cameroon that are divergent outliers. The tree was rooted using an HIV-1 sequence from Cyprus (U.CY.2008.CY223.JF683772). Solid and open circles indicate branches with greater than 0.95 and 0.8 midpoint support, respectively.

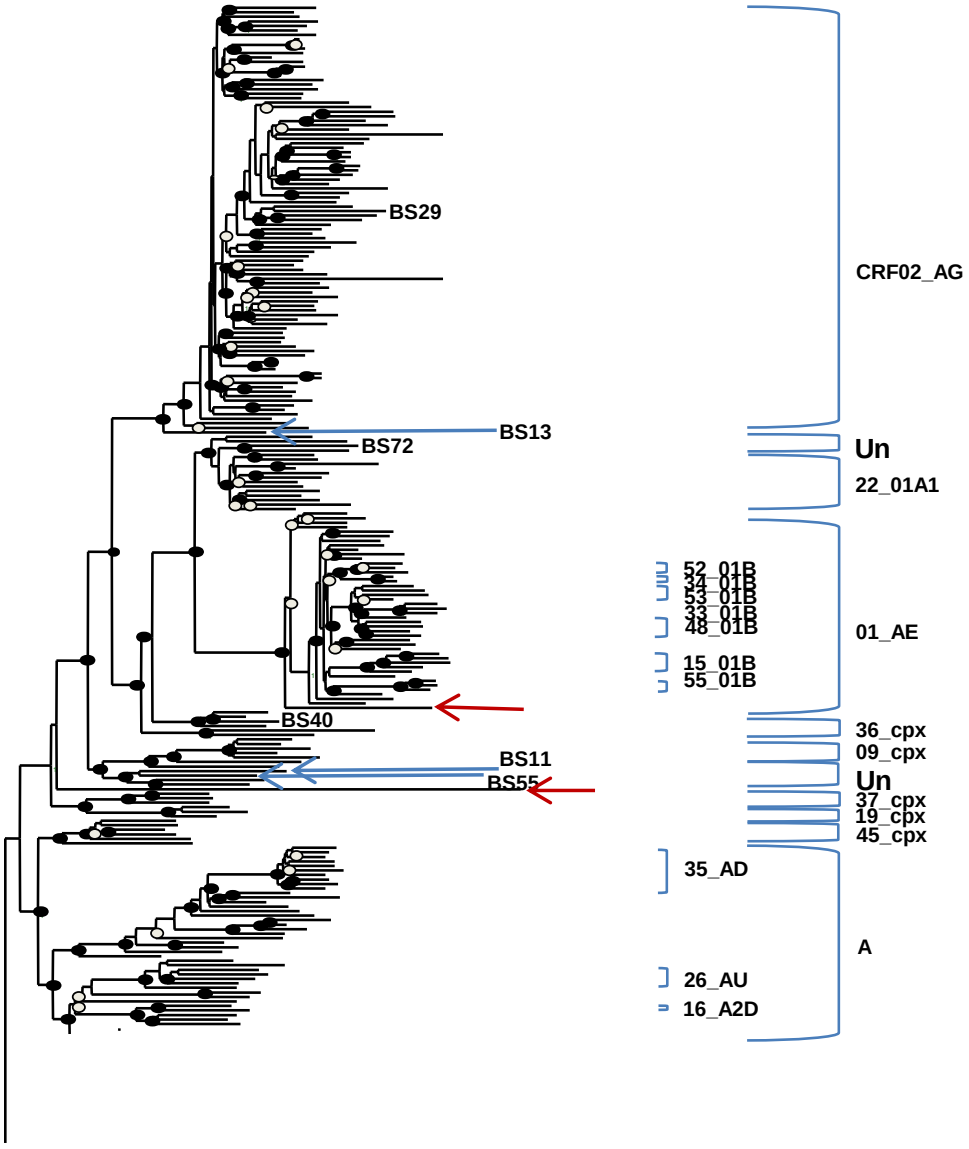

Figure 2.7 Maximum likehood subtree showing the unclassified (Un) viruses sequenced in this study (BS72, BS11 and BS55) and the outlier sequence (BS13) of all the 02_AG viruses sequences to date. Red arrows in the tree indicate previously identified URFs sequences from Cameroon that are divergent outliers. Solid and open circles indicate branches with greater than 0.95 and 0.8 midpoint support, respectively.

2.4 DISCUSSION

Several studies have characterised HIV-1M sequences from Cameroon (Brennan *et al*., 2008; Ragupathy *et al*., 2011; Machuca *et al*., 2007; Ndembi *et al*., 2008; Carr *et al*., 2010). Analysis done here of all available full-length *gag* and *nef* gene sequences from west central Africa clearly reinforces the findings of these previous studies regarding the high degree of HIV-1group M genetic diversity in the country. Also, unsurprising in the light of previous Cameroonian diversity studies, was the finding that most of the newly sampled sequences were CRF02_AG (accounting for 50% of HIV-1M infections), with the other "pure" subtypes (G, D, A, and F) and CRFs (CRF11_cpx, 36_cpx, 37_cpx, and CRF01_AE) accounting for the remainder of infections.

The blind recombination screen also revealed that some divergent Cameroonian viruses previously identified as being CRF_cpx like CRF11_cpx may either be, or at least contain *nef* and *gag* sequences derived from, relatively "pure" but under-sampled subtype-level lineages.

Phylogenetic and recombination analyses yielded results partially consistent with previous interpretations of the origins of various CRFs. For example, the *gag* gene of CRF01_AE isolates grouped with subtype A, whereas the *nef* sequences of these viruses represented a divergent lineage. Similarly, whereas the *nef* gene of CRF37_cpx grouped within the subtype G, CRF37_cpx *gag* sequences formed a branch distinct from all other HIV lineages. Other examples include (1) the *gag* sequences of CRF36_cpx viruses grouping with subtype A while the *nef* sequences of these viruses groups with CRF01_AE; (2) the *gag* sequence of CRF13_cpx grouping with subtype G while the *nef* sequences formed an isolated branch; (3) the *nef* gene of CRF02_AG and subtype G grouping together whereas they respectively form isolated clusters of sequences in Gag.

Both the *gag* and the *nef* sequences of several additional apparent CRFs such as CRF06_cpx, CRF09_cpx, CRF11_cpx and CRF45_cpx formed isolated branches in the phylogenetic analysis, suggesting that, even if these sequences are indeed recombinant, they contain genome fragments derived from highly divergent HIV-1group M parental viruses.

CRF02_AG and subtype G viruses are broadly distributed across west central Africa and have apparently been circulating stably there for many years (Brennan *et al*., 2008; Hamel

et al., 2007; Faria *et al*., 2012). This notion is consistent with the presence of fragments of these viruses having been identified in a large number of CRFs and URFs that have been sampled from this region. Analysis performed in this chapter, demonstrated that these two subtypes are highly diverse, and in most instances where *gag* and *nef* sequences from an individual patient had discordant subtype/CRF classifications, the sequence of one of the genes clustered within the CRF02_AG subtype. This further reinforces the notion that this viral subtype is a major contributor of genetic material to new recombinants (Zhang *et al*., 2010). An alternative explanation, however, could be that the *gag* and *nef* genes were amplified from different viruses co-infecting the same patients. Ongoing molecular and clinical surveillance will reveal whether new recombinants will begin to circulate stably, will harbor biological properties that favor their transmission, or will impact clinical outcomes.

Carr *et al*. (2010) recently identified sequences that were outliers of various HIV-1group M subtypes, and presented analyses that many of these viruses were likely URFs, which might explain the phylogenetic placement of these sequences on the outskirts of known subtypes (Carr *et al*., 2010). Although the majority of the outlier viruses found in this study were also URFs, they remained outliers after the removal of recombinant segments. It thus appears that these sequences represent viruses that are genuinely highly divergent and contain sequence fragments that have been acquired from previously unknown early divergent HIV-1group M lineages. Such sequences could help tremendously with efforts to piece together the early evolutionary history of HIV-1 group M.

The characterisation of nine new full genome sequences for some of the most divergent viruses identified during the *gag* and *nef* sequence analyses confirmed the great extent of the HIV diversity in Cameroon. Some of the newly sequenced viruses, such as BS11, BS55, BS72 and other Cameroonians viruses appeared to form separate, highly divergent clusters within the analysed phylogenetic trees. This suggests that some divergent Cameroonians viruses previously identified as being difficult to classify URFs might, rather than simply being phylogenetically misplaced inter-subtype recombinants, be the extant descendants of early diverging HIV-1M lineages. Regardless of whether or not these so-called complex URFs are recombinant, there is unquestionably a far more diverse pool of HIV sequences circulating amongst humans than presently accounted for in the current HIV-1 group M classification. Some of the divergent lineages, such as that represented here by

BS13, which is an outlier of the important CRF02_AG cluster, might help resolve controversies surrounding the early origins of subtypes G and CRF02_AG (Zhang *et al*., 2010; Carr *et al*., 1998; Abecasis *et al*., 2007), and provide invaluable data for the computational inference (and even perhaps the chemical synthesis and biological characterisation) of early HIV-1M ancestral sequences.

In summary, this work demonstrates the predominance in an urban Cameroonian setting of HIV-1 CRF02_AG viruses alongside viruses belonging to a multiple known and unknown HIV-1 group M subtypes lineages. Further characterisation of the divergent sequences identified here may certainly provide new insights into the evolution and emergence of HIV-1group M over a century ago in west central Africa.

CHAPTER 3: INVESTIGATING T CELL IMMUNITY IN A MULTICLADE HIV-1 EPIDEMIC IN CAMEROON

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3.1 INTRODUCTION

The design of immunogens capable of eliciting effective T cell immune responses against HIV is challenged by the extreme diversity of the virus, as well as the genetic heterogeneity of human populations. The protective efficacy of a vaccine is likely to vary in different populations infected with different HIV clades, unless effective approaches to target the variability of diverse viral strains are developed.

Artificial sequences such as consensus group M constitute an immunogen designed to cope with the high degree of HIV-1 diversity (review by Gao, 2004). They consist of a sequence where the most common amino acid found at each position is selected from a set of available sequences (Korber *et al*., 2001; Novitsky *et al*., 2002). They can reduce the genetic distance between a vaccine strain and circulating viruses, and may be particularly useful in regions where multiple HIV-1 clades and recombinant forms co-circulate. Other centralised sequences included (i) centre-of-tree or COT (the point in a phylogenetic tree that minimises the evolutionary distances to all sampled viruses); or (ii) most recent common ancestor or MRCA (the derived progenitor to a set of sequences (reviewed by Ellenberg *et al*., 2002, Mullins *et al*., 2004; Nickle *et al*., 2002). Preclinical studies testing consensus M-based immunogens both in murine (Gao *et al*., 2005; Weaver *et al*., 2006, 2010) and non-human primate models (Santra *et al*., 2008) have demonstrated stronger HIV-specific T cell responses compared to immunogens based on a single clade. To date, there have been no vaccine trials in humans that have tested and compared the immunogenicity of consensus M immunogens in different regions. Therefore, their potential for eliciting broad immune responses and for providing protection against multiple subtypes from group M is unknown.

Numerous studies have however characterised the immunological recognition of HIV-1 consensus M peptides in HIV-infected individuals, and the results suggest a broad recognition amongst individuals infected with different subtypes (Rutebemberwa *et al*., 2005; Bansal *et al*., 2006; Malhotra *et al*., 2007; Frahm *et al*., 2008; Serwanga *et al*., 2012; Côrtes *et al*., 2013). One such a study evaluated HIV-1 specific T cell responses to seven different Gag peptide reagents, including peptides based on subtype B consensus, subtype B isolate HXB2, ancestral group M, ancestral subtype B and consensus A, C and group M in 43 subtype B-infected individuals from the US (Bansal *et al*., 2006). Out of the 42 reactive

peptides, 29 (69%) demonstrated cross-reactivity among all the seven Gag peptide reagents tested in at least one of the study individuals investigated. Cross-reactivity was highest for p24 Gag (71%), following by 15 (67%) and lastly p17 (63%). This study also evaluated responses to these peptide reagents in a limited number of subtype C-infected participants from Zambia (n=13) of which reactive peptides were only confirmed in six individuals. Overall, when subtype B and C infected populations were assessed, the subtype consensus peptides that matched the infecting virus subtype detected responses of similar magnitude and breadth to consensus M and ancestral M reagents, and these were higher than responses to subtype consensus that were not matched to the infecting virus (Bansal *et al*., 2006). This study and others illustrate the value of investigating different types of centralised reagents to assess the effect of genetic diversity on HIV-specific T cell responses in infected individuals. However, many of these studies have used peptide pools to test the reactivity to consensus M reagents, and few data are available regarding differential targeting at the epitope level, and therefore questions remain regarding the specificity and frequency of consensus M peptides that are targeted within a population or between populations. It may be particularly important to generate data from different epidemics in order to understand to what extent the diversity of HIV impacts the immunological recognition of consensus M reagent sets, especially where rare clades may be under-represented in the sequence databases.

One country at the extreme end of the spectrum of viral diversity is Cameroon in west central Africa, where several groups have characterised the HIV-1 epidemic (Machuca *et al*., 2007; Brennan *et al*., 2008; Ndembi *et al*., 2008; Ragupathy *et al*., 2011; Tongo *et al*., 2013). Along with almost every known subtypes, there is a predominance of recombinant forms of HIV-1 in Cameroon, particularly CRF02_AG, which is also the main source of the epidemic also in other parts of the region (Mamadou *et al*., 2002; Pandrea *et al*., 2002; Djoko *et al*., 2010; Tebit and Arts, 2010; Ajoge *et al*., 2011). In addition to the viral heterogeneity, the Cameroonian population is a mosaic of more than 250 ethnic groups with extensive HLA diversity (Ellis *et al*., 2000; Torimiro *et al*., 2006). Despite the viral epidemic being so well characterised, there is a paucity of immunological studies on HIV infection from Cameroon, with only a single published study to date on T cell responses in HIV-infected individuals from Cameroon (Gupta *et al*., 2006). This contrasts with the urgent need to test candidate vaccines in a region such as this, where multiple HIV-1 clades and recombinant forms circulate.

The study described in this chapter investigated the T cell reactivity and immunodominance patterns towards HIV-1 consensus group M Gag and Nef in HIV-infected Cameroonians. These two HIV proteins were focused on as they are frequently targeted by HIV (Masemola *et al*., 2004; Mendiratta *et al*., 2008), and included in most candidate vaccines (McMichael e*t al*., 2012; Stephenson *et al.*, 2012). Consensus M peptide reagents were used to detect T cell responses, since they were hypothesised to provide greater coverage of more conserved regions of the HIV genome. These analyses were performed in a highly diverse epidemic, namely Cameroon, where individuals infected with the predominant CRF02_AG subtype were compared with those infected with diverse non-CRF02_AG subtypes. Furthermore, the results from this multiclade epidemic from Cameroon were compared to T cell responses measured in individuals from South Africa, a monoclade C epidemic, using an identical protocol and reagents (data provided by Lycias Zembe), as part of a multi-country initiative to map immune reactivity to HIV in different regions of Africa.

3.2 MATERIALS AND METHODS

3.2.1 Study individuals and infecting viruses

Fifty-seven HIV-infected study participants from Cameroon were involved in this part of the study. They were described in Chapter 2. The infecting HIV-1 subtype was determined and also described in the previous chapter, in 53 of the study participants. This chapter also includes data from 44 HIV-infected participants from South Africa. These data were provided by Lycias Zembe (PhD dissertation, University of Cape Town, graduation June 2012). Donors had a CD4 count above 200 cells/mm^3 and were recruited from a clinic in Cape Town, in a study approved by the Research Ethics Committee of the University of Cape Town. Each participant provided written informed consent. CD4 count was determined using FlowCARE PLG (Beckman Coulter, CA, USA) and plasma viral load determined using NucliSENS EasyQ® HIV-1 (Version 2.0, BioMerieux SA). The median viral load of these participants was 3.9 Log RNA copies/ml (IQR 2.7-4.7) and the median CD4 was 441 cells/mm^3 (334-580). All study participants from both sites were ART-naïve.

3.2.2 Processing of peripheral blood mononuclear cells

Blood collected from each participant was processed within 8 hours of collection. Peripheral blood mononuclear cells (PBMC) were obtained using standard Ficoll-Hypaque (Sigma, St. Louis) density gradient separation and cryopreserved in liquid nitrogen. For ELISPOT assays, frozen samples were thawed, and the cells were rested in RPMI-HEPES 1640 containing 20% foetal calf serum, 50 U/ml penicillin and 50 µg/ml streptomycin (all Invitrogen) at 37°C and 5% CO_2 overnight.

3.2.3 Synthetic HIV-1 peptides

A set of 182 peptides based on the HIV-1 group M consensus sequence from 2001 (14 to 16 mers overlapping by 11 amino acids) spanning full-length Gag and Nef were obtained from the NIH AIDS Research and Reference Reagent Program. They were used at a final concentration of 1.5 µg/ml in ELISPOT assays. For screening, peptides were arranged in a pool-matrix (Table A1 in Appendix A) format as previously described (Masemola *et al*., 2004) to predict single reactive peptides. Pools and single peptides were tested in duplicate.

3.2.4 IFN-γ ELISPOT assays

For analysis of T cell responses, PBMC were tested using the IFN-γ ELISPOT assay (ELISPOT screening plate is shown in Table A2, Appendix A), as described previously (Zembe *et al*., 2011). Briefly, microtiter polyvinyledene plates (Millipore were coated with anti-IFN-γ monoclonal antibody 1-D1K (Mabtech), and 100 000 cells were incubated in each well with peptide pools. Control wells included media only, cells and media and Phytohaemagglutinin (PHA, BD Biosciences). Following overnight incubation, plates were washed and incubated biotinylated anti-IFN-γ 7-B6-1 (Mabtech), washed again and incubated with streptavidin-HRP conjugate (BD Pharmingen, Canada), and then with NovaRed substrate (Vector Laboratories). Spots were counted using an automated ELISPOT plate reader (CTL Analyzer, CTL Technologies, Cleveland, OH) and expressed as spot forming units per million (SFU/10^6) PBMC. ELISPOT test acceptance criteria were as follows: (1) >300 spots in the positive wells; (2) <100 spots in the negative control wells; and (3) <5 spots in the wells containing media only. Overall, an HIV-specific response was regarded as positive when it was ≥100 SFU/10^6 PBMC after background subtraction, and at least three

times the mean of the background response (media and cells only). Individual reactive peptides predicted from the pools were confirmed in a second assay using single peptides.

The total response magnitude of response was determined by summing responses to all the protein-specific pools for each sample. To enumerate the breadth of the responses, recognition of the stronger of two adjacent peptides were considered as a response to one peptide/epitope to avoid overestimation. In the case of three adjacent peptides, the weakest of all three peptides was excluded, and the responses were counted as recognition of two peptides. In the case of four adjacent peptides, they were counted as two.

3.2.5 Statistical analysis

Statistical analyses were performed using Prism 5 (GraphPad Software, San Diego, CA). All data were analysed using non-parametric statistics, and expressed as medians with interquartile ranges or ranges. Differences were considered statistically significant when p was <0.05.

3.3 RESULTS

3.3.1 Characteristics of the study population and infecting viruses from Cameroon

Clinical and demographic data of 53 HIV-1 infected Cameroonian blood donors in whom the infecting viral subtype was characterised are shown in Table 3.1. The median age of the cohort was 31 years (IQR 21-54), and the majority of the individuals were male (79%). The median CD4 count was 457 cells/mm^3 (IQR 301-665), and plasma HIV-1 RNA viral load was 4.94 $\log_{10}$ copies/ml (IQR 4.4-5.3). All individuals were ART-naïve, and HIV infection dates were not known. The genetic diversity of HIV-1 for these 53 study participants was reported in the previous chapter. Briefly, 49% (26/53) of individuals were infected with CRF02_AG, 38% (20/53) with a variety of circulating recombinant forms or pure subtypes, found at frequencies of between 2-11% each, and the remaining 15% (7/53) with viruses that showed discordant clade distributions in *gag* and *nef* (Table 3.1). There were no significant

differences in clinical or demographic characteristics of CRF02_AG and non-CRF02_AG-infected individuals, although there was a trend towards lower CD4 counts in the latter group.

Table 3.1 Clinical characteristics and infecting subtype of study participants from Cameroon (n=53)

CRF02_AG					Non-CRF02_AG					
Sample	Age	Sex	CD4 count	Log Viral load	Sample	Age	Sex	CD4 count	Log Viral load	Infecting Clade
BS01	23	M	976	5.46	BS02[d]	ND[b]	M	838	4.29	A-like
BS02[a]	ND[b]	M	838	4.29	BS03	ND[b]	M	ND[b]	5.54	G
BS05[c,*]	42	M	552	4.89	BS04	ND[b]	M	480	4.82	G
BS06	32	M	765	4.94	BS09[e]	38	F	574	4.70	A1
BS09[c]	38	F	574	4.70	BS10	34	M	1083	4.83	A1
BS11[c]	23	M	666	5.53	BS11[e]	23	M	666	5.53	F
BS13[c]	31	M	489	5.12	BS12	36	M	334	4.84	G
BS14	49	M	620	4.85	BS13[e]	31	M	489	5.12	A1
BS16	32	M	574	4.35	BS24	44	M	504	5.20	CRF37_cpx
BS18[a,*]	41	M	521	4.39	BS25[e]	37	M	42	4.64	F
BS19	34	M	630	5.54	BS26	30	M	430	5.13	CRF01_AE
BS20[a,*]	24	M	782	4.27	BS27	29	M	278	5.45	CRF37_cpx
BS21	31	M	862	4.62	BS29[e]	22	M	388	5.19	U
BS22	27	F	1972	4.41	BS30	28	M	242	5.37	D
BS23	32	M	390	4.86	BS35	54	M	434	4.22	CRF11_cpx
BS25[c]	37	M	42	4.64	BS40	26	M	567	5.08	CRF36_cpx
BS29[c]	22	M	388	5.19	BS46	46	F	230	4.90	G
BS31[a,*]	31	F	131	3.51	BS48	29	M	878	5.12	G
BS32	27	M	663	6.18	BS49	42	M	400	5.22	F2
BS38	44	F	681	5.22	BS51	31	M	278	5.41	G
BS39	27	M	373	5.09	BS54	22	F	254	4.88	D
BS43	38	M	774	4.49	BS55[e]	29	M	337	5.70	F
BS45	29	M	312	4.93	BS57	31	M	359	4.48	CRF11_cpx
BS50	35	M	599	4.25	BS78	52	M	278	5.21	CRF01_AE
BS53	31	M	515	5.32	BS65	49	M	282	4.82	URF
BS55[c]	29	M	337	5.70	BS72	21	M	276	5.53	URF
BS56	28	M	242	5.37	BS74	26	F	427	3.91	A-like
BS64	28	M	732	5.27						
BS73	27	F	141	6.32						
BS75	29	F	152	5.43						
BS77	31	F	321	5.00						
BS79[a,*]	30	F	289	4.05						
BS82[a,*]	42	M	622	3.29						
Median	31	25:8[f]	574	4.93	Median	31	23:4[f]	394	5.12	

[a]CRF02_AG in *nef* only; [c]CRF02_AG in *gag* only (genotype differed between *gag* and *nef* genes); [b]ND: Not determined; [d]Non-CRF02_AG in *gag* only; [e]Non-CRF02_AG in *nef* only (genotype differed between *gag* and *nef);* [f]Number Male to Female; *Only genotyped in one gene; U= Unknown, URF= Unique Recombinant Form.

3.3.2 Relationship between viral sequences and peptide reagents for CRF02_AG and non_AG subtypes

In order to determine the relationship between infecting viruses and group M consensus peptides that were to be used as immunological reagents in the ELISPOT assay, amino acid distances were calculated. The median amino acid distance between viral sequences and consensus M peptide reagents was significantly lower for Gag (median 11%, range 9–13%) than for Nef (median 15%, range 10–22%). The CRF02_AG and non-CRF02_AG sequences did not differ in distance to the consensus M sequence in Gag (Figure 3.1A). For Nef, however, non-CRF02_AG clades exhibited a wider range of distances from consensus M (10 to 22% compared to 11 to 19% for CRF02_AG viruses), and the median amino acid distance to consensus M for non-CRF02_AG subtypes was significantly higher than CRF02_AG sequences (17% compared to 14%, p=0.0008; Figure 3.1A).

To complement the genetic distance results, the putative epitope coverage of consensus M for Gag and Nef proteins were calculated using Epicover (www.hiv.lanl.gov), by determining the proportion of exactly-matched 9 mers, or 9 mer coverage with one or two amino acid mismatches. There were no significant differences in epitope coverage between CRF02_AG and non-CRF02_AG viruses for either Gag or Nef (Figures 3.1B and C). Overall, the consensus peptides had the same pattern of coverage even when epitopes with 1 or 2 amino acid mismatches were taken into consideration in CRF02_AG and non-CRF02_AG subtypes, reflecting the central nature of Group M consensus peptides.

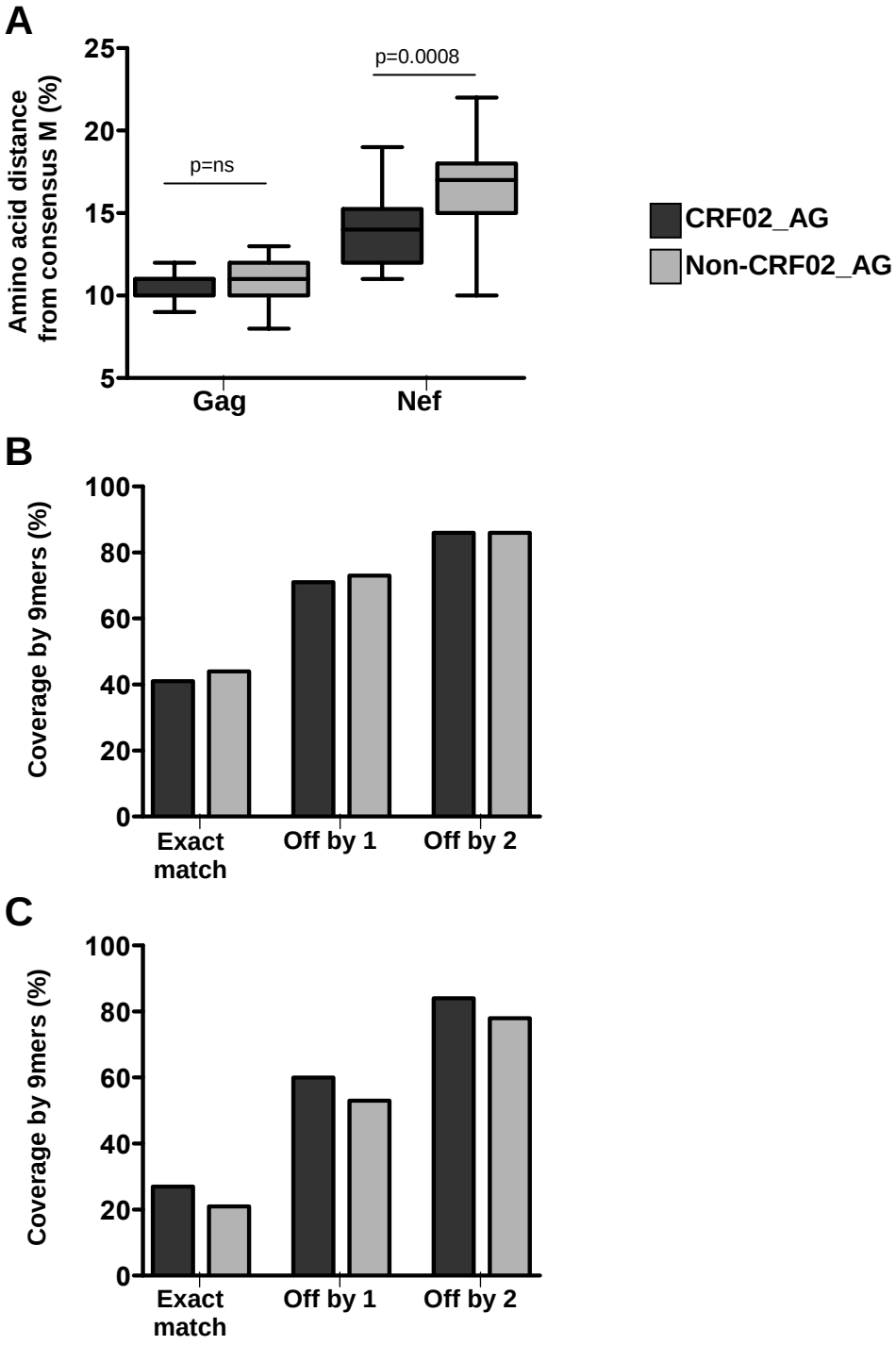

Figure 3.1 The relationship between infecting viral sequences and peptide amino acid sequences. (A) Amino acid distances between infecting viral sequences and consensus M peptide sequences for Gag and Nef, shown for CRF02_AG (dark grey) and non-CRF02_AG viruses (light grey). Differences were tested for significance by the Mann-Whitney U test. (B) Predicted 9 mer epitope coverage of consensus M Gag, and (C) Nef peptide sequences and CRF02_AG and non-CRF02_AG sequences. Coverage of 9 mers exactly matched to the viral sequences, 9 mers with one mismatch and 9 mers with two mismatches, are shown for Gag and Nef, determined using Epicover software (*www.hiv.lanl.gov*)

3.3.3 T cell responses in CRF02_AG and non-CRF02_AG-infected individuals from Cameroon

Specific T cell responses to Gag and Nef were investigated using the IFN-γ ELISPOT assay, using peptides spanning HIV-1 Group M consensus Gag and Nef. Ninety-five percent (54/57) of individuals recognised at least one of the peptide pools tested. Of these 54 responders, nine participants showed no detectable Gag and seven no detectable Nef-specific responses. Strikingly, peptides from 16 of the 17 tested pools covering Gag and Nef were targeted (Figure 3.2), suggesting a broad cross-reactivity to the group M reagents. Among the Gag responding individuals, Pool 7 (P7; Gag p24) was the most targeted pool recognised by 23/57 (42%) study participants. It was followed by P1 (Gag p17; 39%), P5 (Gag p24; 26% participants), P4 (Gag p24; 25%), P8 (Gag p24; 21%), P6 (Gag p24; 19%), P10 (Gag p24; 9%), P11, P12 (Gag p15) and P2 (Gag p17), targeted by 7% of participants each, and P9 (Gag p24; 4%). In Nef, P15 (Nef central) was the most targeted pool and was recognised by 35% participants, followed by P14 (Nef central; 29%) and finally P13, P17 and P16 (all from Nef outer), targeted respectively by 12%, 5% and 4% of participants (Figure 3.2).

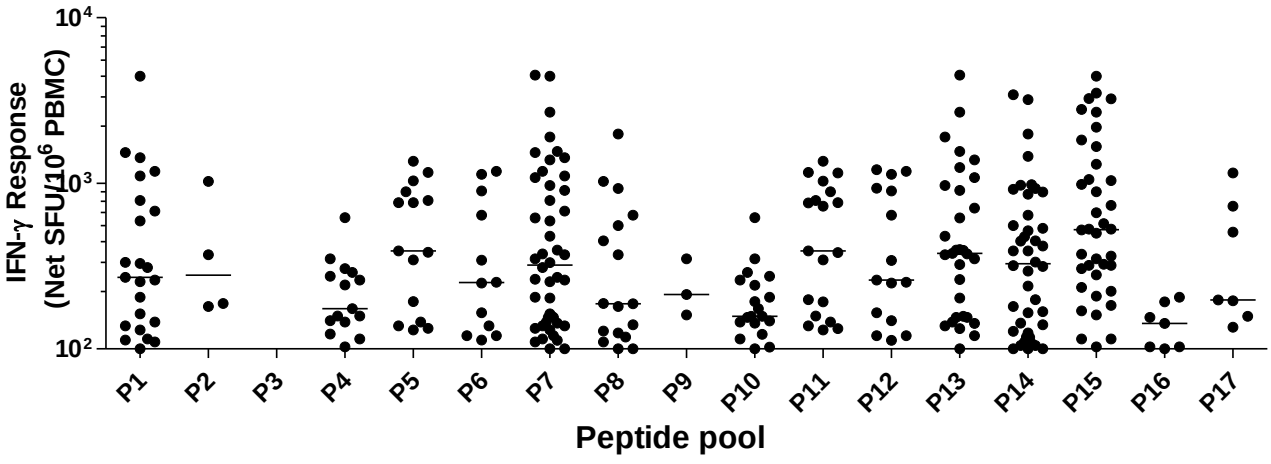

Figure 3.2 T cell responses to Gag and Nef pools. HIV-specific T cells were detected in 16 out of 17 pool used. Each dot represents a study participant. Responses are expressed as net SFU/106 PBMC.

The frequency of responders in the CRF02_AG and non-CRF02_AG-infected groups was compared, to determine whether the more diverse group of viruses demonstrated a different immunogenicity profile. Forty-five samples were typed in the *gag* gene, 27 of which were classified as CRF02_AG and 18 as non-CRF02_AG. For *nef*, 26 of 51 samples typed were CRF02_AG, and 25 genotyped as non-CRF02_AG. There was no difference in the frequency of responders in each group, with 85% and 78% for Gag for the CRF02_AG and non-CRF02_AG-infected groups, respectively, and 88% for both groups for Nef reactivity (Figure 3.3A).

The total magnitude of responses differed substantially among responders (Figure 3.3B), a likely reflection of a range of factors, including immunocompetence, different levels of viral replication and time since infection. The total magnitude of responses against the entire Gag and Nef protein ranged from 107 to 7865 SFU/10^6 PBMC (median, 1038). At the protein level, the median response to Gag was 1045 SFU/10^6 PBMC (range, 107-4508); whilst the median response to Nef was 884 SFU/10^6 PBMC (range, 115-7865). In the Gag region, there was a trend towards a higher total magnitude of responses ($p=0.0624$) in the CRF02_AG infected individuals compared to the non-CRF02_AG infected population (Figure 3.3B). In the Nef region, despite a difference in the genetic distance to consensus M peptides between the CRF02_AG and the non-CRF02_AG viruses, the magnitude of ELISPOT responses was similar between the two groups (Figure 3.3B). In terms of the breadth of the response, the median number of Gag peptides targeted in the CRF02_AG-infected individuals was double that of the non-CRF02_AG-infected population, although this difference was not significant (Figure 3.3C). The breadth of responses per person was equivalent for Nef, with a median of two targeted peptides for responders (Figure 3.3C).

Taken together, these data suggest that despite the high diversity of the HIV-1 epidemic in Cameroon, the magnitude and breadth of the immunological recognition of group M consensus peptides does not differ between CRF02_AG and non-CRF02_AG-infected individuals.

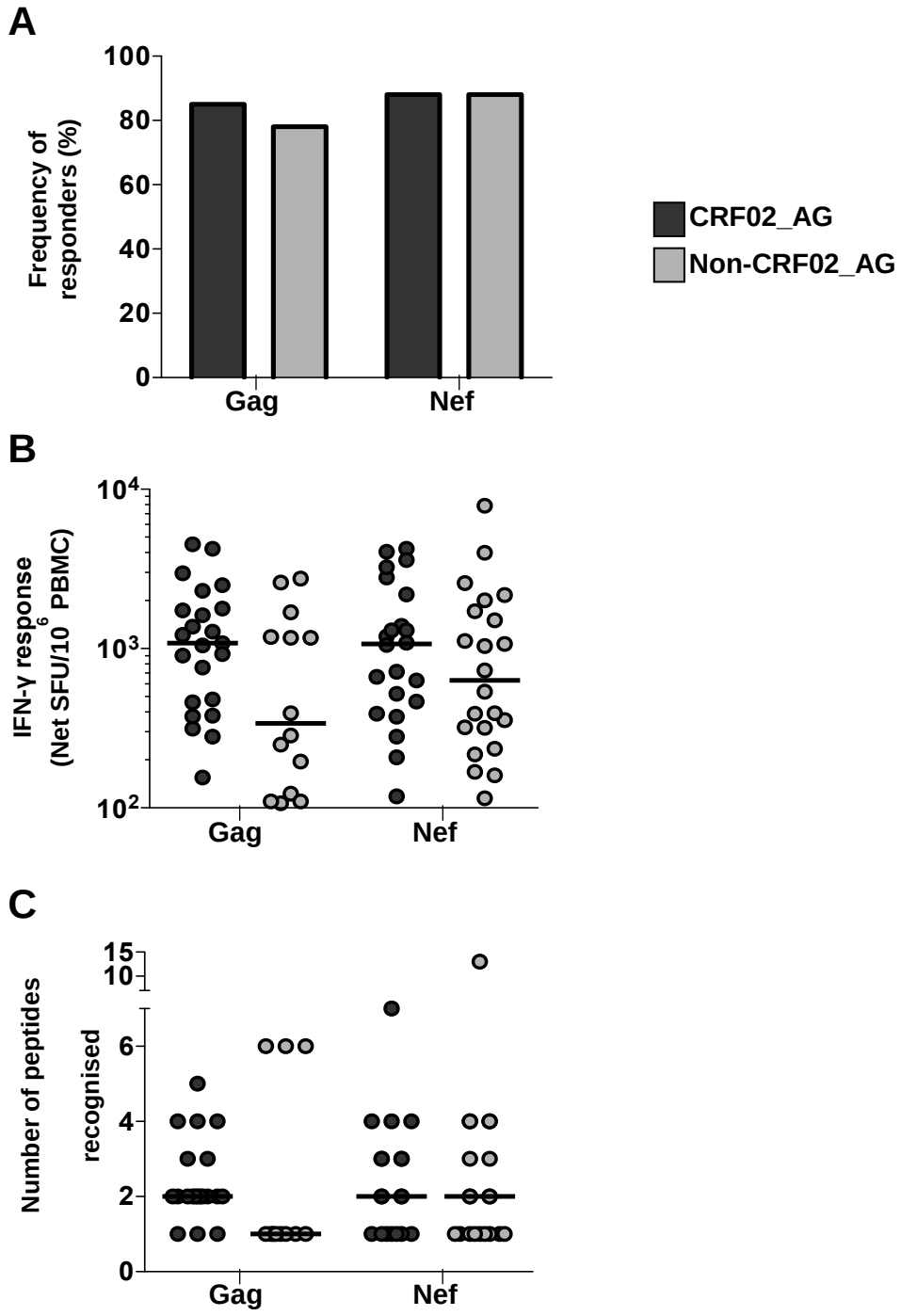

Figure 3.3 T cell responses to Gag and Nef consensus M reagents. HIV-specific T cells were detected in 23 CRF02_AG (dark grey) and 22 non-CRF02_AG-infected individuals (light grey) using the IFN-γ ELISPOT assay. (A) The frequency of responders, (B) the total magnitude of responses, and (C) the breadth of responses to Consensus M Gag and Nef peptides in CRF02_AG- and non-CRF02_AG-infected individuals. The number of responding peptides represents the minimum response, after taking into consideration overlapping peptides, as described in the Methods.

3.3.4 Immunological targeting and immunodominance in CRF02_AG and non-CRF02_AG-infected individuals from Cameroon

Having demonstrated that T cell responses were similar in frequency, magnitude and breadth between CRF02_AG and non-CRF02_AG-infected individuals from Cameroon, it was next determined whether there were differences in the specificity of peptide recognition across Gag and Nef. For Gag, CRF02_AG and non-CRF02_AG-infected individuals targeted a similar total number of peptides (27 and 22, respectively, Figure 3.4A). The majority of the peptides were recognised by only one individual (Figure 3.4B), and approximately one third of peptides (11/38) were commonly recognised by both groups (Figure 3.4C). Only two peptides were recognised by five or more (>25%) of individuals in the CRF02_AG group, and only one peptide (Gag5) was commonly recognised by at least 15% of the study participants (three individuals in the CRF02_AG-group and four in the non-CRF02_AG group), indicating a profound lack of immunodominance (peptides that are commonly targeted within a given population) of targeted peptides (Figure 3.4A). For Nef, peptides targeted by only one participant each also represented the majority of those recognised (Figures 3.4D and E).

In contrast to Gag, however, a greater proportion of peptides were targeted by two or more individuals (Figure 3.4D and E), and four peptides were commonly targeted by at least three individuals. The pattern of shared recognition also differed in Nef compared to Gag, with three times more peptides exclusively targeted in the diverse non-CRF02_AG group compared to the CRF02_AG group (10 vs 3), and more than half of all targeted Nef peptides (14/27) were recognised by both groups (Figure 3.4F).

It is interesting to note that some peptides were preferentially targeted in one group compared to another; for example, in Gag, $Gag77_{290\text{-}304}$ was targeted by 7 (35%) participants in the CRF02_AG while only 1 participant (9%) targeted the same peptide in the non-CRF02_AG group; $Gag78_{294\text{-}308}$ was targeted by 3 (15%) participants infected with CRF02_AG and no participants infected with non-CRF02_AG viruses; $Gag93_{349\text{-}363}$ was targeted by 0 and 3 (27%) participants, respectively, in the CRF02_AG and non-CRF02_AG group. In Nef, $Nef21_{79\text{-}92}$, $Nef24_{90\text{-}104}$ and $Nef28_{104\text{-}118}$ were targeted by 3 (15%) and 0, 1 (5%) and 4 (21%), and 6 (30%) and 1 (5%) participants, respectively, in the CRF02_AG and non-CRF02_AG groups (Figure 3.4A and D).

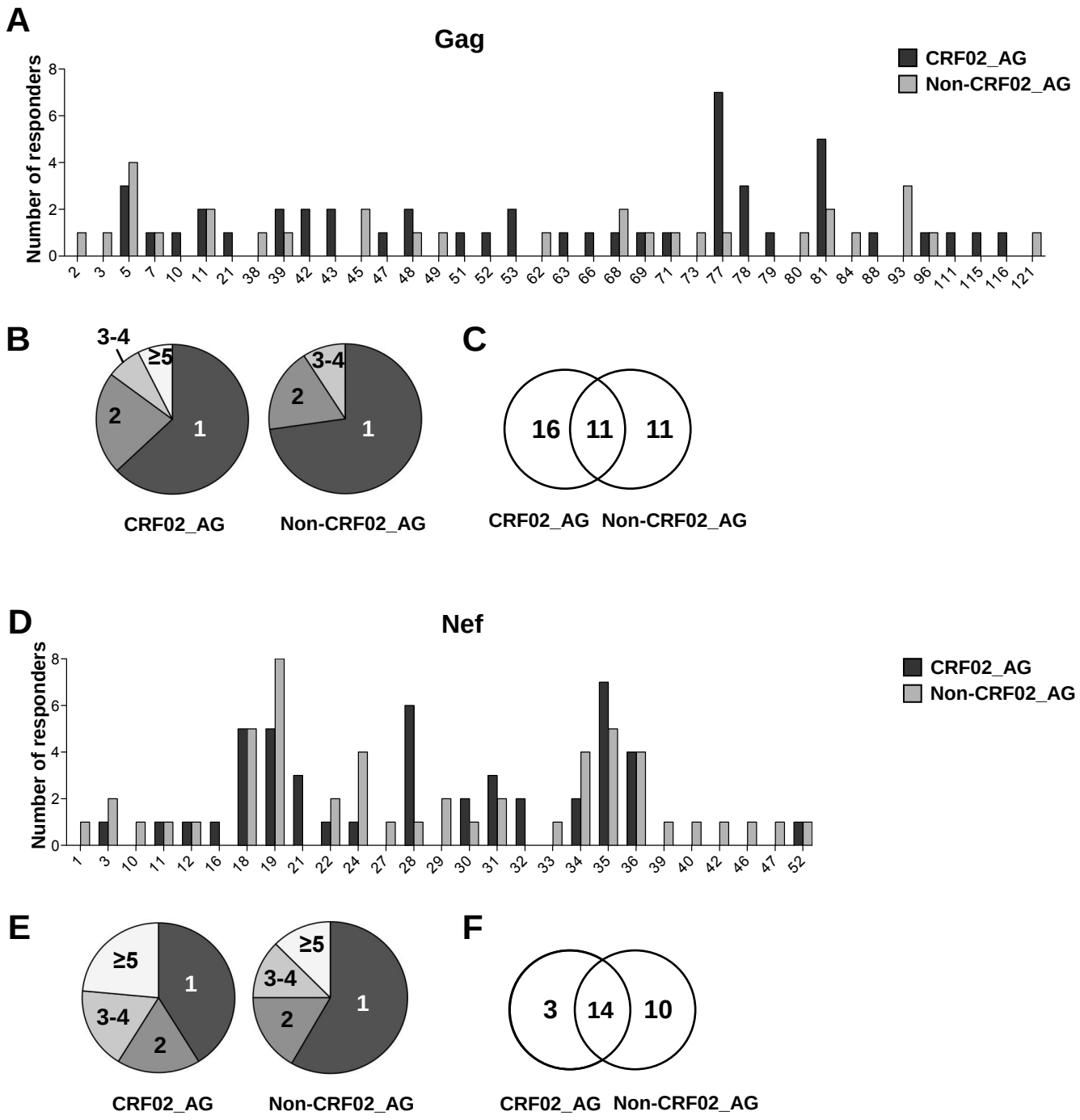

Figure 3.4 Pattern of recognition of gag and Nef consensus M peptides in CRF02_AG and non-CRF02_AG infected Cameroonians. The number of individuals specifically recognizing consensus M peptides in (A) Gag (38 peptides), in (D) Nef (27 peptides), shown for CRF02_AG (dark bars) and non-CRF02 virus infection (light bars). Pie charts represent the proportion of individuals recognizing 1, 2, 3-4 and or more than 5 peptides in Gag (B) and Nef (E) in donors infected with CRF02_AG and non-CRF02_AG HIV-1 clades. The Venn diagrams show the total numbers of commonly and exclusively recognised peptides in Gag (C) and Nef (F) in CRF02_AG and non-CRF02_AG-infected participants. ELISPOT confirmation at the peptide level was performed for Gag in 20 and 11 individuals, respectively, in the CRF02_AG and non-CRF02_AG group; and in Nef, on 20 and 19, respectively, in the CRF02_AG and non-CRF02_AG group.

3.3.5 Similarities between a multiclade (Cameroon) and a monoclade epidemic (South Africa)

Having observed differences and similarities between a homogeneous CRF02_AG and a diverse non-CRF02_AG group in Gag and Nef, the diverse epidemic in Cameroon as a whole was next compared to a more homogenous monoclade epidemic in South Africa. Table 3.2 shows the characteristics of the Cameroonian cohort together with 44 HIV-infected individuals from South Africa, who were tested in an identical manner for ELISPOT responses to HIV-1 group M consensus Gag and Nef peptides (data provided by Lycias Zembe). CD4 counts were similar between the two groups, however viral loads were one log higher in the Cameroonian group ($p<0.0001$). Full-length *gag* and *nef* sequences were generated from 23 and 19 South African samples, respectively, and 100% were pure HIV-1 clade C viruses (data provided by Lycias Zembe, PhD thesis from the University of Cape Town, June 2012), consistent with previous studies demonstrating the predominant monoclade C of the epidemic in South Africa (Zembe *et al*., 2011; Iweriebor *et al*., 2011).

Amino acids distances between infecting viral sequences and consensus group M peptides were similar between the South African monoclade and Cameroonian multiclade epidemic in both Gag (11%) and Nef (16% and 15%, respectively; Table 3.2 and Figure A3 in Appendix A3). As for the within Cameroon CRF02_AG and non-CRF02_AG comparison, there was a trend towards a higher magnitude of responses in the monoclade compared to the multiclade epidemic in Gag ($p=0.073$; Table 3.2 and Figure A4 B in Appendix A4), and the median number of peptides targeted was similar. In Nef, the magnitude of ELISPOT responses was similar between the two cohorts, and the median number of targeted peptides was double in Cameroon (Table 3.2 and Figure A4 C in Appendix A4).

When targeting of HIV-1 group M consensus Gag and Nef T cell responses in the mono- versus multiclade epidemic was examined, the pattern appeared very similar to the within-Cameroon homogenous versus diverse group comparison. Although a large number of peptides across Gag were targeted (74/129), the majority of peptides were targeted by only one individual in both groups (Figures 3.5A and B). There were few immunodominant responses in either group, and only two shared immunodominant responses that were recognised by four or more individuals from both groups (Figure 3.5A).

Table 3.2 Clinical Characteristics and Immunological Responses of Study Participants from South Africa and Cameroon

		South Africa	Cameroon	p-value
Number of participants		44	57	
CD4 count (cells/mm^3)[a]		441 (334-580)	494 (301-665)	0.5215
Viral load (Log RNA copies/ml)[a]		3.9 (2.7-4.7)	4.9 (4.4-5.3)	0.003
Amino acid distance to Con M[a] **(%)**	**Gag**[c]	11 (11-12)	11 (10-12)	0.1790
	Nef[c]	16 (14-16)	15 (13-17)	0.1730
Frequency of responders (%)	**Gag**	75	79	
	Nef	66	82	
Magnitude of response[a] **(SFU/10^6 PBMC)**	**Gag**	1455 (566-3448)	1064 (330-1769)	0.0730
	Nef	770 (340-1615)	828 (361-1786)	0.8163
Breadth of response[b] **(number of peptides)**	**Gag**	2.5 (1-10)	2 (1-9)	0.8721
	Nef	1 (1-6)	2 (1-13)	0.2260

[a]Median and interquartile range; [b]Median and range; [c]Twenty-three samples in Gag and 19 in Nef were sequenced for the South Africa cohort.

A similar number of Gag peptides were recognised in the two groups, namely 50 peptides for Cameroon and 53 for South Africa; one third of these were commonly recognised by both groups (Figure 3.5C). In Nef, although peptides targeted by only one individual still made up the majority of those recognised, a greater sharing of targeted peptides was apparent (Figures 3.5D and E), as was observed for the within-Cameroon comparison of diverse and homogenous groups. More peptides were targeted in the highly diverse viral epidemic from Cameroon compared to South Africa (27 vs 16), and approximately half of all Nef peptides were recognised by both groups (Figure 3.5F). As for the within-Cameroon analysis, Nef exhibited a greater immunodominance compared to Gag, with five peptides being commonly recognised across the two populations, despite the fact that an equal frequency of peptides was recognised in each protein (31/53 [58%] Nef peptides and 57% in Gag). Taken together, these results demonstrate very similar findings for homogeneous CRF02_AG-infected individuals compared to diverse non-AG-infected individuals within Cameroon, and a

homogeneous monoclade epidemic in South Africa compared to a diverse multiclade epidemic in Cameroon.

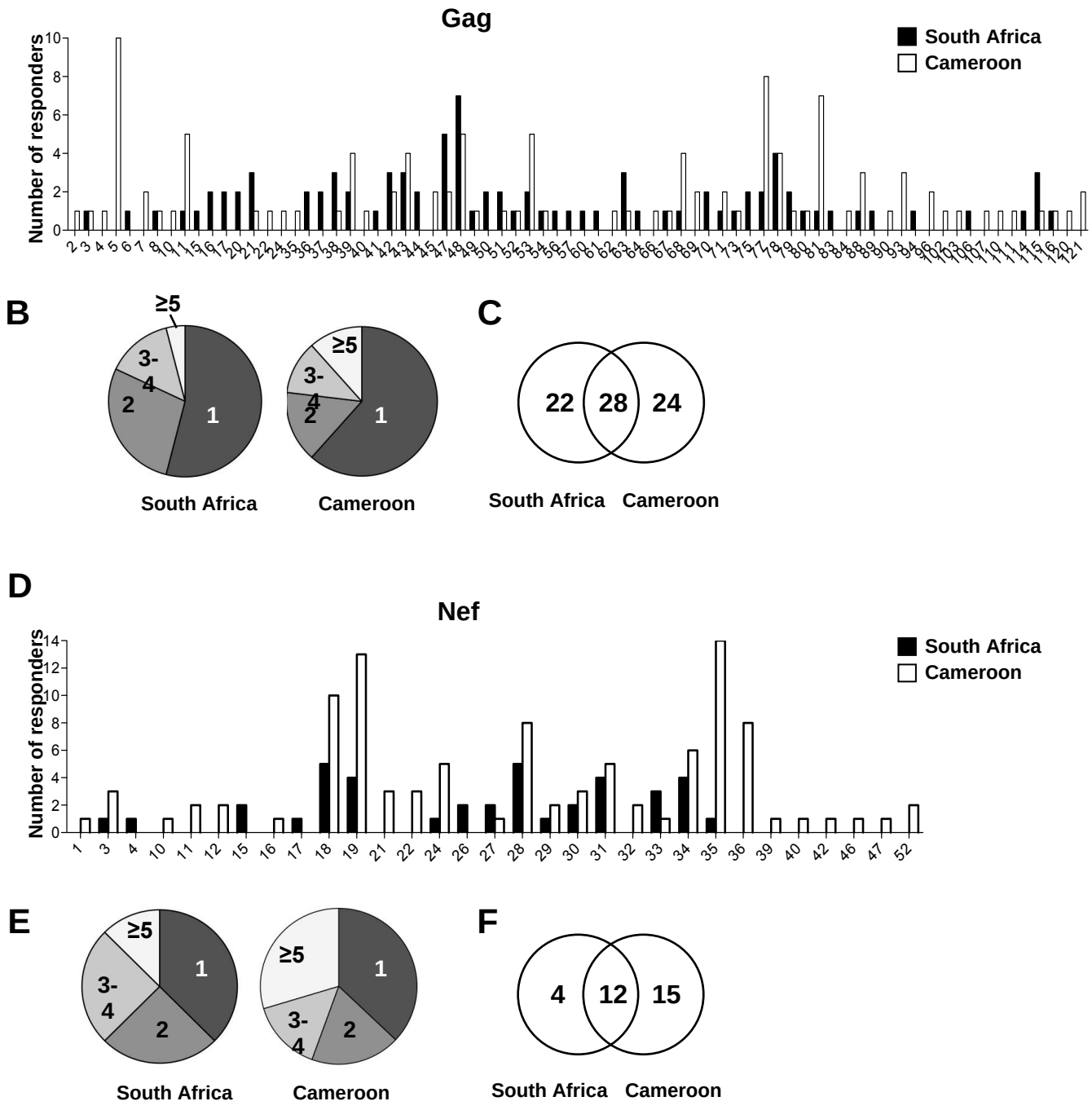

Figure 3.5 Pattern of recognition of Gag and Nef consensus M peptides in HIV-infected individuals from Cameroon and South Africa. The number of individuals specifically recognizing consensus M peptides in (A) Gag (74 peptides), in (D) Nef (31 peptides), shown for Cameroon (white bars) and South Africa (black bars). Pie charts represent the proportion of individuals recognizing 1, 2, 3-4 and or more than 5 peptides in Gag (B) and Nef (E) in HIV- infected individuals from Cameroon and South Africa. The Venn diagrams show the total numbers of commonly and exclusively recognised peptides in Gag (C) and Nef (F) in Cameroon and South Africa. ELISPOT confirmation at

the peptide level was performed on 26 and 41 participants for Gag, and 17 and 41 participants for Nef, for South Africa and Cameroon, respectively.

The identification of regions of HIV commonly targeted in different populations has importance for the development of a universal HIV vaccine. The peptides that were commonly recognised between Cameroon and South Africa were further investigated. A total of 40 commonly targeted peptides were identified, 28 in Gag and 12 in Nef protein (Table 3.2). Of these, 24/40 (60%) have previously been associated with a beneficial effect on viral load (Mothe *et al*., 2011), with 20 in Gag and four in Nef. Further characterisation of these peptides demonstrated that 21/28 (75%) in Gag and 9/12 (75%) in Nef were located in the conserved regions of these proteins, namely p24 for Gag and the central region of Nef, and all contained previously identified epitopes, confirming that consensus M reagents identify responses targeting conserved regions of HIV

In addition to the low frequency of immunodominant commonly recognised peptides between the two geographically-distinct epidemics, there was a clear signal of preferential targeting in the different populations, as had been observed in the within Cameroon comparison. For example, peptide $Gag5_{17\text{-}31}$ was targeted by 10 (24%) participants in Cameroon and not targeted at all in South Africa; $Gag47_{176\text{-}190}$ was targeted by 2 (5%) participants in Cameroon and by 5 (19%) participants in South Africa; $Gag77_{290\text{-}304}$ was targeted by 8 (20%) participants in Cameroon and only 2 (8%) participants in South Africa; $Gag81_{305\text{-}318}$ by 7 (17%) participants in Cameroon and only 1 (4%) participant in South Africa. In the Nef protein, $Nef33_{124\text{-}138}$ was targeted by 1 (2%) participant in Cameroon and by 3 (18%) participants in South Africa; $Nef35_{131\text{-}145}$ was targeted by 14 (34%) participants in Cameroon, and only a single participant (6%) in South Africa (Table 3.3).

These differences in recognition are likely driven by viral and host genetic factors within the two HIV-infected populations.

Table 3.3 Commonly recognised peptides between monoclade (South Africa) and multiclade (Cameroon) epidemics

Peptide No	Peptide sequence	Location	Frequency of responders (%)		Beneficial[a]
			South Africa	Cameroon	
Gag					
3	SGGKLDAWEKIRLRP	p17	4	2	No
8	YRLKHLVWASRELER	p17	4	2	No
11	LERFALNPGLLETS	p17	4	12	Yes
21	LYNTVATLYCVHQRI	p17	12	2	Yes
38	GQMVHQAISPRTLNA	p24	12	2	No
39	HQAISPRTLNAWVKV	p24	8	10	No
42	VKVIEEKAFSPEVIP	p24	12	5	Yes
43	EEKAFSPEVIPMFSA	p24	12	10	Yes
47	SEGATPQDLNTMLNT	p24	19	5	Yes
48	TPQDLNTMLNTVGGH	p24	27	12	Yes
49	LNTMLNTGVGHQAAM	P24	4	2	Yes
51	GGHQAAMQMLKDTIN	p24	8	2	Yes
52	AAMQMLKDTINEEAA	p24	4	2	Yes
53	MLKDTINEEAAEWDR	p24	8	12	Yes
54	TINEEAAEWDRLHPV	p24	4	2	No
63	GTTSTLQEQIAWMTS	P24	12	2	Yes
67	NPPIPVGEIYKRWII	p24	4	2	No
68	PVGEIYKRWIILGLN	p24	4	10	No
71	GLNKIVRMYSPVSIL	p24	4	5	Yes
73	YSPVSILDIRQGPK	p24	4	2	Yes
77	KEPFRDYVDRFFKTL	p24	8	20	Yes
78	RDYVDRFFKTLRAEQ	p24	15	10	Yes
79	DRFFKTLRAEQATQ	p24	8	2	Yes
80	FKTLRAEQATQDVKN	p24	4	2	Yes
81	RAEQATQDVKNWMT	p24	4	17	Yes
88	CKTILKALGPGATL	P24	4	7	No
115	RQANFLGKIWPSNKG	p2p7p1p6	12	2	Yes
116	FLGKIWPSNKGRPGN	p2p7p1p6	4	2	Yes
Nef					
3	SIVGWPAVRERIRRT	outer region	6	7	No
18	GFPVRPQVPLRPMTY	outer region	29	24	No
19	RPQVPLRPMTYKAAL	outer region	24	32	No
24	FLKEKGGLEGLIYSK	central region	6	12	No
27	LIYSKKRQEILDLWV	central region	12	2	No
28	KKRQEILDLWVYHTQ	central region	29	20	No
29	EILDLWVYHTQGYFP	central region	6	5	No
30	LWVYHTQGYFPDWQN	central region	12	7	No
31	HTQGYFPDWQNYTPG	central region	24	12	No
33	WQNYTPGPGIRYPLT	central region	18	2	No
34	TPGPGIRYPLTFGW	central region	24	15	No
35	PGIRYPLTFGWCFKL	central region	6	34	No

[a]"Yes" indicates that peptides have been classified as beneficial for lower viral load by Mothe *et al*., 2011.

3.4 DISCUSSION

The substantial genetic diversity of HIV is a major challenge for vaccine development. Whilst there is baseline knowledge of HIV diversity in most countries, there is limited information on the relationship between genetic diversity and cross-clade immune reactivity. Immunological responses to HIV-1 consensus M-based peptides were characterised in two distinct African HIV-1 epidemics, namely Cameroon in west central Africa, the origin of the HIV-1 pandemic (Tongo *et al*., 2013; Carr *et al*., 2010), a country with virtually every known clade of HIV-1 circulating (Machuca *et al*., 2007; Brennan *et al*., 2008; Ndembi *et al*., 2008; Ragupathy *et al*., 2011; Tongo *et al*., 2013), which has an evolving epidemic in which HIV-1 recombinants predominate (Carr *et al*., 2010), within an ethnically diverse population; and South Africa, located at the southern-most tip of Africa, with one of the highest adult prevalences globally at 18% (UNAIDS, 2012), but with a homogeneous clade C epidemic (Zembe *et al*., 2011; Iweriebor *et al*., 2011). The primary analysis in Cameroon sought to describe the differences in immunological responses to the predominant CRF02_AG subtype with other rarer circulating subtypes and recombinants, and determine whether the use of consensus clade M reagents could broadly detect T cell responses, in light of the high levels of viral diversity. This has implications for the broader context of performing vaccine trials in the region (Ellenberger *et al*., 2002; Stephenson and Barouch, 2013). There was no difference in the frequency, cumulative magnitude or breadth of IFN-γ responses to Gag or Nef between the two groups. This was despite significantly higher genetic distances between the non-CRF02_AG viruses and consensus M sequences for Nef. Differences between the two groups emerged at the peptide level, particularly in the Nef protein, where there were a higher number of targeted peptides in the non-AG subtypes compared to CRF02_AG, although a lack of immunodominance was evident, with most peptides targeted by only a single participant. The same observation was seen when the diverse epidemic of the Cameroon group as a whole was compared with a monoclade C epidemic in South Africa. These data suggest that higher HIV-1 diversity leads to the detection of broader HIV-1 specific responses at the cohort level, but not at the individual level. Thus, the use of consensus M reagents did indeed result in broad recognition in HIV-infected individuals, consistent with studies in other populations (Rutebemberwa *et al*., 2005; Bansal *et al*., 2006; Malhotra *et al*., 2007; Frahm *et al*., 2008; Serwanga *et al*., 2012; Côrtes *et al*., 2013).

This work formed part of a multi-country study examining the immune reactivity of Group M consensus Gag and Nef using identical methodology. The findings generated here are similar to those of Serwanga and colleagues who examined immune responses in Uganda, a predominant clade A and D epidemic (Serwanga *et al*., 2012). Interestingly, of the seven most targeted peptides that they identified, five were also commonly targeted peptides in our study. One limitation of our study was that we did not performed HLA typing in our cohort; this would have allowed us to better see the impact of HLA on targeting of peptide. Despite this, our finding plus Serwanga *et al*. clearly demonstrates that consensus M reagents, despite being central to all the group M viruses, identify a narrow immunodominance spectrum in different HIV clade epidemics and populations. This could be because consensus M sequences do not take into account the true extent of HIV-1 diversity (Walker and Korber, 2001), as well as the HLA composition in different populations having an effect on immunodominance (reviewed in Goulder and Watkins, 2008). In addition, the observation that there were immunodominance differences between cohorts favours the use of full-protein (not epitope-based) immunogens in the design of an HIV vaccine; this is mainly because different viruses and human drive immune response to specific epitopes. Therefore, assessing reactivity to newer vaccine immunogens that incorporate both human and HIV diversity, such as the conserved elements or mosaic immunogens (Korber *et al*., 2009), may increase recognition and give a truer picture of reactivity.

A major question that still remains in vaccine development is the importance of breadth of the T cell response compared to specificity of the response. Mothe *et al.* (2011) performed a large study to identify peptides that were beneficial for slower disease progression. These were frequently located in HIV Gag and Pol, but rarely in the more variable proteins such as Env and Nef. Despite the low frequency of immunodominant commonly-recognised peptides between the two geographically distinct epidemics in this study, 50% of the commonly recognised peptides could be considered beneficial according to Mothe *et al*., all of these located in Gag. Although very few peptides were identified as beneficial by Mothe *et al*., in Nef in that study, it exhibited a greater immunodominance in the presentis study compared to Gag, with three peptides being commonly recognised by at least 20% of the study participants in both epidemics. These peptides might still induce strong and effective immune responses, as Mudd *et al*. (2012), have shown that few some epitopes in Nef were associated with viral control in macaques that expressed Mamu-B*08 in a vaccine study.

(Mudd *et al*., 2012). Thus, the current view on what may constitute a protective cellular immune response to HIV is likely biased towards immunodominant responses and those restricted by frequent HLA class I alleles and HLA alleles associated with superior disease outcome.

In conclusion, these data show that the central nature of HIV-1 consensus M sequences resulted in their broad recognition by T cells, both in a highly diverse HIV epidemic from Cameroon, and a homogeneous epidemic from South Africa. As expected, different patterns of immunodominance prevailed at each site, likely governed by both viral and host HLA differences. Here, the most striking finding was that the consensus M reagents failed to identify highly immunodominant peptides that were common within or between homogeneous and diverse HIV epidemics. The implications for the development of a global HIV vaccine are that immunogens should take into consideration not only the overall diversity of the virus but also should address the diversity at the population level. Further refinement of immunogens based on consensus sequences, taking into account human diversity, is required for the development of a globally relevant vaccine.

CHAPTER 4: CHARACTERISATION OF HIV-SPECIFIC T CELL RESPONSES TO POTENTIAL T CELL EPITOPES (PTEs) IN A HIGHLY DIVERSE EPIDEMIC FROM CAMEROON

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4.1 INTRODUCTION

The development of a safe, globally effective and affordable vaccine offers the best hope for the future control of the HIV pandemic. One of the major challenges in developing such a vaccine is the high degree of genetic diversity the virus exhibits. This enormous variation in HIV-1 poses a barrier to both the design of a protective vaccine and the evaluation of such a vaccine, since a globally effective vaccine will most likely need to elicit cross-protective immunity against all circulating strains that an individual may encounter (McMichael and Hanke, 2003; Mullins and Jensen, 2006; reviewed by Nabel, 2001). To date, all vaccine candidates tested in human clinical efficacy trials have used single or multiple representatives of HIV from different subtypes, for example the RV144 trial tested subtypes B and CRF01_AE (Pitisuttithum *et al*., 2006), the STEP and Phambili trials used subtype B (Buchbinder *et al*., 2008; Gray *et al*., 2011) and the HVTN505 trial tested subtype B for *gag*, *pol* and *nef* genes and the *env* gene from HIV subtypes A, B and C (Catanzaro *et al*., 2006; 2007). In the absence of any efficacy from all but one of these trials, and no T cell correlates of efficacy in a vaccine trial, we still do not know what kind of viral sequence or group of sequences is required to induce T cells that can cross-react and cross-protect against a broad spectrum of circulating viruses.

Traditionally, the main approaches at attempting to improve immunogenic coverage have focused on the use of consensus sequences, MRCA and COT (Ellenberger *et al*., 2002; Gaschen *et al*., 2002; Korber *et al*., 2001; Mullins *et al*., 2004; Roland *et al*., 2007). These approaches for generating an artificial sequence minimise the genetic distance from circulating strains of the virus. A more recent and novel approach, mosaic sequences, involves the use of algorithms to improve 9mer epitope coverage that can accurately reflect immune responses to virus encountered by the host (Nickle *et al*., 2007; Fischer *et al*., 2007). For example, across a specific HIV protein sequence alignment, multiple 9mer epitopes at a given position which can vary in one or more amino could exist, reflecting the diversity within that set of sequences. Thus, when these multiple epitopes variants are included in an immunogen, they could be presented to CD8+ T lymphocytes and could then induce an immune response against multiple different variants of the virus. These 9mer epitopes embedded in multiple overlapping mosaic sequences are known as Potential T cell Epitopes or PTEs (Li *et al*., 2006). In a mosaic approach, software algorithms combine sequences which allow the

processing and expression of a maximum of variants of the same epitope. Therefore, sequences based on the mosaic approach which are similar to naturally circulating HIV-1, would demonstrate a greater depth or coverage of potential T-cell epitopes for different HIV proteins (Fischer *et al*., 2007; Thurmond *et al*., 2008; Corey and McElrath, 2010). The optimisation process can be performed for a single HIV subtype or for all the group M subtypes. This is a great advantage over consensus sequences, which are also artificially derived but consist of a unique sequence that at each site contains the amino acid that was most common amongst the aligned sequences that it was generated to represent.

Preclinical vaccine trials comparing these new mosaic immunogens containing multiple PTE variants, to consensus and natural sequences in rhesus macaques, showed an improvement in the T cell immune response generated. In one study (Barouch *et al*., 2010), bivalent mosaic Gag, Pol and Env antigens expressed by replication incompetent Ad26 were administrated to rhesus macaques, and compared to group M consensus, bivalent subtypes B and C and optimal natural subtype C immunogens also expressed by the same vector. Breadth and magnitude of vaccine-elicited HIV-specific T cell responses were assessed by IFN-γ ELISPOT assays using PTE peptides based on the mosaic sequences. The total number of Gag-, Pol- and Env-specific cellular responses to PTE peptides elicited by the mosaic antigens was 3.8-fold higher than the number of responses induced by the consensus or natural sequence antigens. More importantly, the mosaic vaccine induced T cell responses that recognised more variants within an epitope (depth) than consensus or natural sequence antigens. A second similar preclinical study compared responses in animals receiving either mosaic or consensus group M Gag and Nef immunogens delivered by a DNA prime–recombinant vaccinia virus boost regimen (Santra *et al*., 2010). T cell immune responses were also assessed by IFN-γ ELISPOT assays using sets of peptides from subtypes A, B, C and G. Here, a greater magnitude, breadth and depth of responses to Gag mosaic immunogens were observed compared to consensus, but no difference was seen for Nef. These experimental results indicated that in addition to the vector involved, reagents used to map T cell responses influenced the outcome of experiments, with differences between the mosaic and consensus and natural sequences being less apparent when evaluated with subtype-specific peptides compared to PTE peptides (Barouch *et al*., 2010; Santra *et al*., 2010).

In the absence of clinical trials evaluating these new vaccine immunogens in humans, it is important to understand the potentially relevant responses that T cell-based vaccines to these mosaic sequences could elicit or detect. One of the ways to achieve this is to use the peptides based on the mosaic immunogens (PTE peptide sets) to stimulate PBMC from HIV-infected individuals and evaluate their recognition. This may provide some information on whether mosaic vaccines, if tested in a particular population, would generate responses that were cross-reactive to the circulating HIV strains present in that population. To date, only a single study has investigated the ability of a subtype B PTE peptide set based on Nef to detect T cells from HIV subtype B-infected individuals (Malhotra et *al*., 2007). T cells responses were evaluated with subtype B consensus and PTE Nef peptides in 23 individuals using the IFN-γ ELISPOT assay. Although the specificity of responses was comparable when the two sets of peptides were used, both the breadth and the magnitude of responses detected were significantly higher when PBMC were stimulated with PTE peptides compared to consensus peptides (Malhotra et *al*., 2007).

This chapter describes the ability of T cells from HIV-infected blood donors from Cameroon, with a highly diverse HIV epidemic, to recognise multiple group M peptide variants using PTE peptide sets for Gag and Nef. These results were compared with responses detected using consensus M peptides, generated in the previous chapter. The hypothesis was that the use of PTE peptides would improve the immunological detection of responses compared to the use of consensus peptides.

4.2 MATERIALS AND METHODS

4.2.1 Study individuals

Thirty-two individuals from the cohort of HIV-infected blood donors from Cameroon that had previously been screened for consensus M responses by ELISPOT (reported in Chapter 3) were selected for this study. Selection was based on individuals mounting an HIV-specific response to consensus group M peptides greater or equal to 100 SFU/10^6 PBMC, and sufficient PBMC sample being available for further study.

4.2.2 Synthetic HIV-1 peptides

A set of 320 Gag and 127 Nef peptides based on HIV-1 group M PTE sequences spanning the entire Gag and Nef proteins (Los Alamos National HIV sequence database, February 2005) were obtained from the NIH AIDS Research and Reference Reagent Program. The peptides are 15 amino acids in length and contain naturally occurring 9mer epitopes from HIV. For screening, peptides were arranged in pools using the Deconvolution program (Mario Roederer, Vaccine Research Center, NIH; Tables B1 and 2 in Appendix B) to allow for single peptide mapping using IFN-γ ELISPOT. There were 63 pools in total, 40 in Gag and 23 in Nef. In Gag, each pool consisted of 32 peptides, and in Nef, there were 7-20 peptides in each pool. The pattern of responses to the pools indicated specific candidate reactive peptides for confirmation. Group M PTE peptides contain 9mer epitopes with a frequency equal to or greater than 15% in any one of the subtypes A, B, C and non-A,B,C. Single peptides from all the variants were available as 1mg lyophilised preparations. These were reconstituted to 30µl aliquots of 10mg/ml as stocks and then further diluted to 40µg/ml as peptide pools or individual peptides and stored at -80^0C. They were used at a final concentration of 1.5µg/ml in IFN-γ ELISpot assays.

4.2.3 IFN-γ ELISPOT assay

HIV-specific T cell responses were assessed by the IFN-γ ELISpot assay (ELISPOT screening plate layout is shown in Table B3, Appendix B), as previously described in Chapter 3 of this thesis. The same protocol was used as previously described, with the same criteria for positivity. Experiments comparing the two sets of reagents (Consensus and PTE peptides) were not carried out simultaneously. Therefore, to ensure that a valid comparison could be made between the same PBMC tested several years apart, various analyses were performed. There was a correlation between the consensus and PTE ELISPOT responses (Figure B1, Appendix B) when PBMC were stimulated with the CEF control peptide pool ($p<0.0001$), as well as when exactly matched peptides, or peptides shifted by one amino acid, between the two sets were compared PBMC ($p<0.0001$). These results indicate that the quality of PBMC sample and the reagents used in the ELISPOT gave equivalent responses and results from the two studies on the same samples could be compared.

4.2.4 Analysis of peptide responses

The breadth of responses or number of epitopic regions only take into account the number of distinct peptides with no inclusion of variants. As it was in chapter 3, recognition of the stronger of two adjacent peptides was considered as a response to one peptide/epitopic region to avoid overestimation. In the case of three adjacent peptides, the weakest of all three peptides was excluded, and the responses were counted as recognition of two peptides. The total magnitude of response was determined by summing responses to all peptides or epitopic regions with the highest response among variants for each sample. To delineate the number of variants, PTE peptides with aa overlap of $\leq$10 were counted as one peptide. The depth of the T cell response was determined by taking all the reactive peptides plus their reactive variants.

4.2.5 Statistical analysis

Statistical analyses were performed by using GraphPad Prism version 5.0 (GraphPad Software). Total magnitude and breadth of responses to PTE and consensus peptide sets for matching donors were compared using Wilcoxon nonparametric two-tailed paired tests. Mann-Whitney nonparametric analysis for unpaired measures was used to test for significant differences between Gag and Nef PTE peptide responses. Differences were considered statistically significant when p was <0.05.

4.3 RESULTS

4.3.1 Participants and viruses

Clinical and demographic data of the 32 HIV-infected Cameroonian participants included in this chapter are summarised in Table 4.1. These individuals were selected from the full cohort described in the previous chapters based on their total magnitude of response to group M consensus Gag or Nef peptides reactivity (of at least 100 SFU/10^6 PBMC), and with sufficient PBMC vials available for further testing. Twenty-two individuals were screened in Gag and they were 27 in Nef. Individuals had a median age of 31 years (IQR 21-54) and the majority were male, with a ratio of 23:9. The median CD4 count was 494 cells/mm^3 (IQR 312-663) and a median viral load of 4.9 Log RNA copies/ml (IQR 4.4-5.3).

Table 4.1 Characteristics of study individuals (n=32)

Sample ID	Age	Sex	CD4 count	Log Viral load	Infecting virus	
					Gag	Nef
BS 09	38	F	574	4.7	CRF02_AG	A1
BS 11	23	M	666	5.5	CRF02_AG	F
BS 14	49	M	620	4.8	CRF02_AG	CRF02_AG
BS 16	32	M	574	4.3	CRF02_AG	CRF02_AG
BS 18	41	M	521	4.4	ND[a]	CRF02_AG
BS 20	24	M	782	4.3	ND	CRF02_AG
BS 22	27	F	1972	4.4	CRF02_AG	CRF02_AG
BS 23	32	M	390	4.9	CRF02_AG	CRF02_AG
BS 25	37	M	42	4.6	CRF02_AG	F
BS 27	29	M	278	5.5	CRF37_cpx	CRF37_cpx
BS 29	22	M	388	5.2	CRF02_AG	U
BS 30	28	M	242	5.4	D	D
BS 35	54	M	434	4.2	ND	CRF11_cpx
BS 38	44	F	681	5.2	CRF02_AG	CRF02_AG
BS 39	27	M	373	5.1	CRF02_AG	CRF02_AG
BS 40	26	M	567	5.1	CRF36_cpx	CRF36_cpx
BS 46	46	F	230	4.9	G	G
BS 48	29	M	878	5.1	G	G
BS 49	42	M	400	5.2	F2	F
BS 50	35	M	599	4.3	CRF02_AG	CRF02_AG
BS 51	31	M	278	5.4	G	G
BS 53	31	M	515	5.3	CRF02_AG	CRF02_AG
BS 54	22	F	254	4.9	D	D
BS 55	29	M	337	5.7	CRF02_AG	F
BS 64	28	M	732	5.3	CRF02_AG	CRF02_AG
BS 65	49	M	282	4.8	CRF22_01A1	CRF01_AE
BS 72	21	M	276	5.5	CRF36_cpx/F2	CRF01_AE/F
BS 73	27	F	141	6.3	CRF02_AG	CRF02_AG
BS 74	26	F	427	3.9	ND	A
BS 77	31	F	321	5.0	CRF02_AG	CRF02_AG
BS 78	52	M	278	5.2	ND	CRF01_AE
BS 79	30	F	289	4.0	ND	CRF02_AG
Median (IQR)	31 (21-54)	23:09[b]	494 (312-663)	4.9 (4.4-5.3)		

[a]Not Done, [b]Number Male to Female

In this subset of selected individuals, based on the HIV *gag* and *nef* sequences generated in Chapter 2, CRF02_AG predominated, accounting for 44% (14/32) of the studied infections, followed by subtype G (9%, 3/32), subtype D (6%, 2/32), CRF37_cpx, CRF11-_cpx, CRF36_cpx, F2, CRF01_AE (3% each with 1/32). In addition, 22% (7/32) of the studied viruses had *nef* and *gag* genes from viruses belonging to different subtypes. In Gag, 16 out of 22 individuals (73%) were CRF02_AG. Nef was more diverse than Gag, with 11/27 (41%) infected with CRF02_AG and 16/27 (59%) other subtypes.

Both clinical characteristics and immune responses to consensus M (magnitude and breadth) were similar between this subset of the cohort and the full cohort described in Chapter 3.

4.3.2 Frequency, magnitude and breadth of responses to HIV-1 Group M PTE peptides

HIV-specific responses to PTE Gag and Nef proteins were investigated using the IFN-γ ELISPOT assay in HIV-infected individuals from Cameroon. Twenty two individuals were screened in Gag and 27 in Nef. Ninety-one percent (20/22) in Gag and 85% (23/27) in Nef of all individuals recognised at least one of the PTE peptides. Two donors in Gag and four in Nef showed no detectable response to the PTE peptides even though they targeted group M consensus peptides.

The level of immune reactivity to the PTE peptides within each individual tested was examined next. Confirmation of reactive peptides was performed in 17 individuals that had responded to both Gag and Nef PTE peptides. The range of HIV-specific T cell responses differed substantially among different individuals, with the total magnitude of responses against the entire Gag and Nef protein ranging from 230 to 5790 SFU/10^6 PBMC. At the protein level, the median response to Gag was 1510 SFU/10^6 PBMC (range 370-5790), whilst the median response to Nef was 1320 SFU/10^6 PBMC (range 230-4190). There was no significant difference between the overall magnitude of the response to Gag versus Nef ($p=0.3262$; Figure 4.1 A).

Subsequently, analyses of the HIV-specific T cell responses from study individuals at the single peptide level were performed. A total number of 83 out of 320 (26%) peptides

(including variants) were recognised in Gag. In Nef, substantially more peptides were targeted, namely 81 out of 127 (64%) of peptides. To determine the breadth of response, a strategy outlined in the Methods section was used. As observed with the magnitude of responses, the breadth of responses also varied substantially among study participants. The median number of peptides recognised per individual was 2, both in Gag (range, 1 to 6) and Nef (range, 1 to 7; Figure 4.1B).

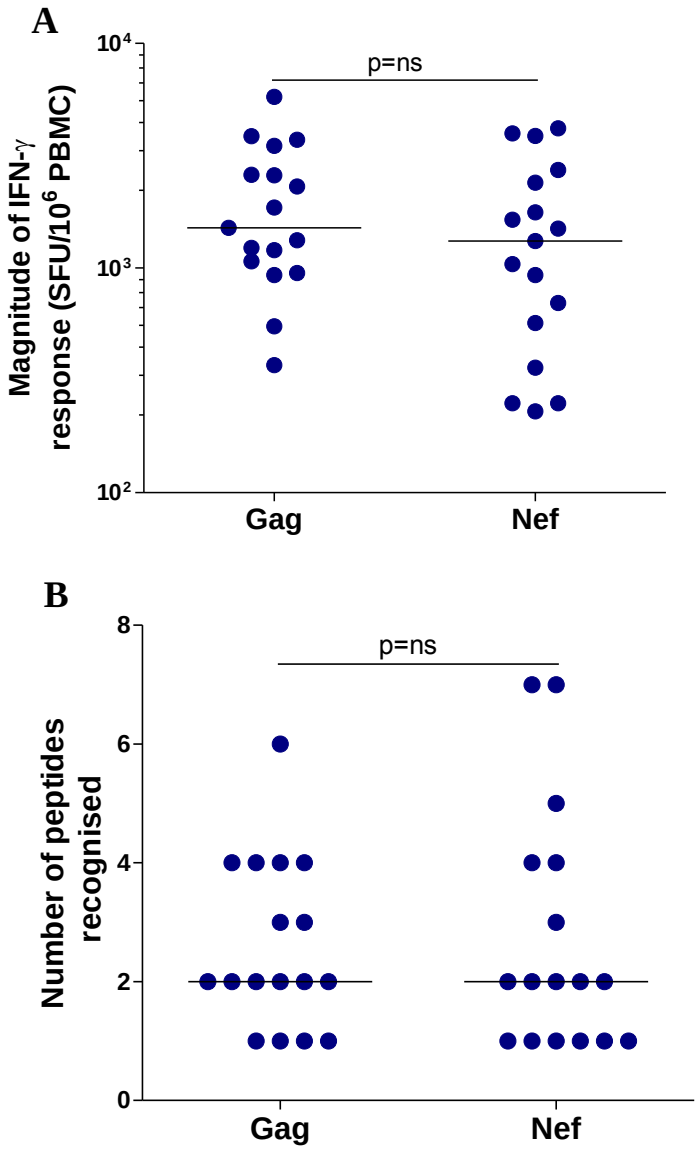

Figure 4.1 Total HIV-specific IFN-γ producing T cells against group M PTE Gag and Nef peptides. (A), total magnitude of responses to Gag and Nef individual peptides recognised in 17 study subjects. (B) Total number of peptides recognised in Gag and Nef proteins. The number of responding peptides represents the minimum response, after taking into consideration overlapping peptides and excluding variants, as described in the Methods.

4.3.3 Comparison of the reactivity of PTE peptides and consensus M peptides

4.3.3.1 Magnitude and breadth of responses

The magnitude and breadth of Gag and Nef reactivity for PTE and consensus group M peptides were next compared in the 17 responding study individuals. In Gag, the magnitude of responses per individual was significantly higher when PTE peptides were used compared to when consensus peptides were used (Figure 4.2A; left panel). The median response magnitude was 1510 and 1013 SFU/10^6 PBMC (p=0.0045) for PTE versus consensus peptides (range, 370-5790 and 128-4850, respectively; Figure 4.2A; left panel). In Nef, in contrast to what was observed for Gag, the magnitude of IFN-γ responses was similar between the two sets of peptides (Figure 4.2A; right panel). The median magnitude of response was 1320 and 1342 SFU/10^6 PBMC (p=0.2977) for PTE versus consensus peptides (range, 230-4190 and 260-3266, respectively; Figure 4.2A; right panel). The magnitude of responses followed a similar pattern for both proteins when only corresponding or matched peptides in the two panels were compared (Figure 4.3). Although the total magnitude of response was similar for Nef when the two sets of peptides were used, eight individuals representing 47% of the cohort had an increased magnitude of responses when PTE peptides set were used, compared to only five (29%) individuals who showed an increased magnitude of responses with consensus peptides (Figure 4.4A).

Surprisingly, the overall breadth of IFN-γ ELISPOT responses to PTE peptides was similar to what was observed with consensus M peptides. A median of 2 Gag peptides were targeted when PTE and consensus peptides panel were used (range 1-6 with both set of reagents; Figure 4.2B; left panel). In Nef, a median of 2 peptides were also targeted when PTE peptides were used compared to consensus (range 1-7 for PTE and 1-5 for consensus; Figure 4.2B; right panel). There was also a similarity in the total number of epitopic regions targeted within the two proteins when the two sets of reagents were used. In Gag, study participants responded to a total of 46 peptides from consensus M, whilst they targeted 44 PTE epitopic regions. In Nef, there was a total number of 38 and 46 peptides targeted to consensus M and PTE peptides, respectively. It appears from these data that PTE peptides were not able to dramatically increase the breadth of responses detected.

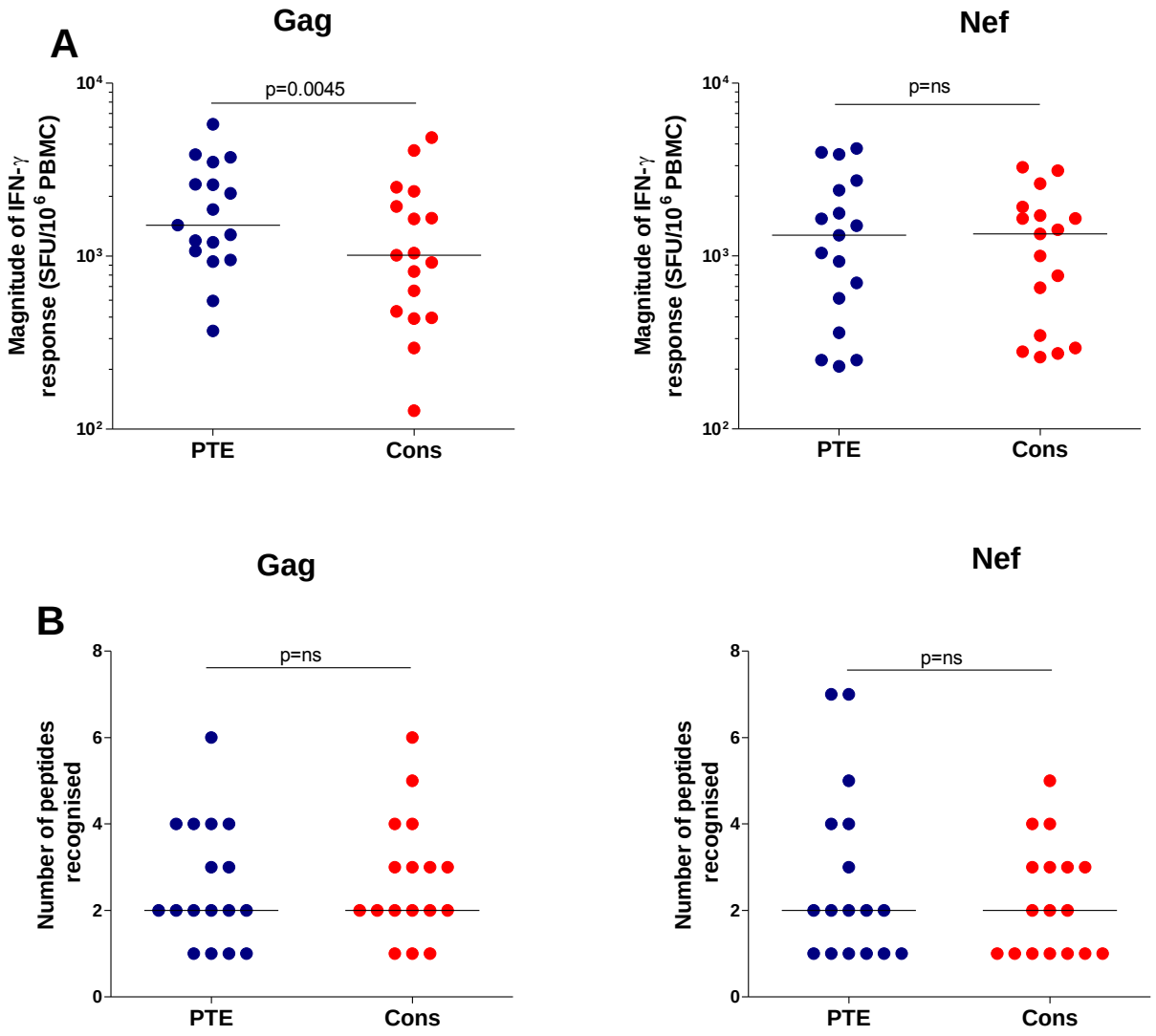

Figure 4.2 T cell responses to Gag and Nef consensus and PTE M reagents. HIV-specific T cells were detected in 17 infected individuals using PTE peptides (blue) and consensus peptides (red) and ELISPOT assay. (A) The total magnitude of responses (Gag, left panel; Nef, right panel), and (B) the breadth of responses (Gag, left panel; Nef, right panel). The number of responding peptides or epitopic regions represents the minimum response, after taking into consideration overlapping peptides and variants, as described in the Methods. Tcell responses between consensus and PTE peptide sets were compared using Wilcoxon paired tests.

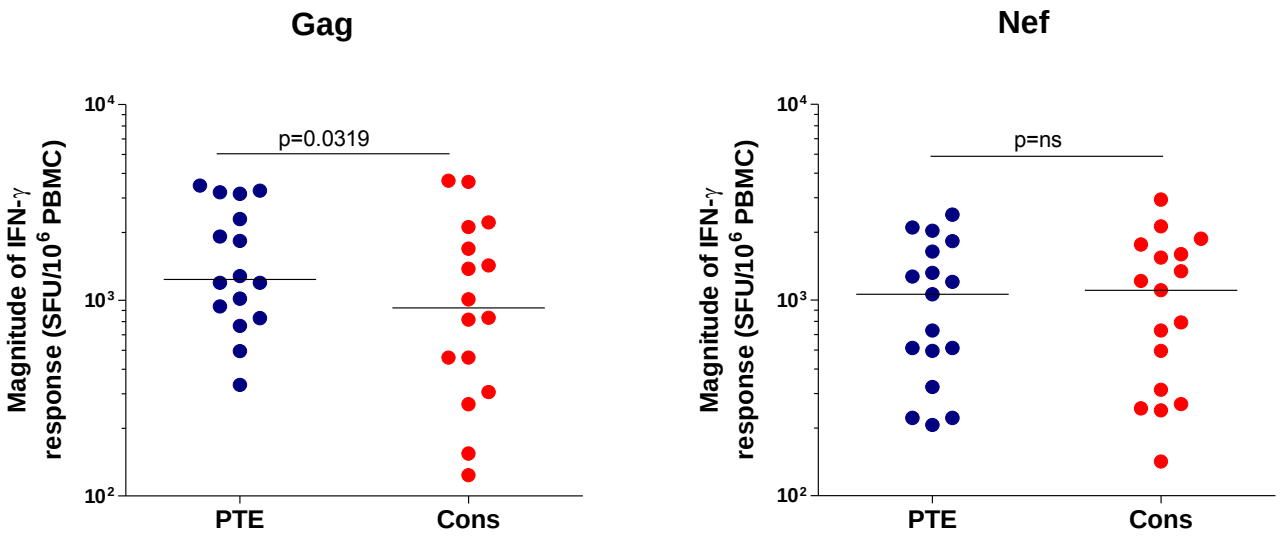

Figure 4.3 The total magnitude of responses from matched individuals for PTE and consensus group M peptides in Gag and Nef. Each dot represents a study participant and T cell responses between consensus and PTE peptide sets were compared by the use of Wilcoxon paired test.

However, when the data were looked at in more detail, some interesting observations emerged. Six individuals, representing 35% of the cohort, had a higher number of peptides or epitopic regions detected when PTE peptides set were used compared to three (18%) of individuals with consensus peptides in Nef (Figure 4.4B). When more PTE peptides were recognised, four out the six (67%) of individuals were infected with CRF02_AG, and when there were a higher number of consensus peptides recognised, all three individuals were infected with non-CRF_02AG subtypes. In Gag, an equal number of individuals (five representing 29% of the cohort) had a higher number of peptides detected when PTE peptides or consensus reagent were used (Figure 4.4C).

There was also a clear signal that the same peptide was targeted (overlap by $\geq$ 10 amino acid) when both sets of reagents were used. For example in Gag, the majority of targeted consensus peptides (30/46, 65%) were also recognised when PTE peptides were used (Table 4.2). The majority of targeted consensus peptides was also targeted when using the PTE peptide set in Nef (28/38, 74%; Table 4.2).

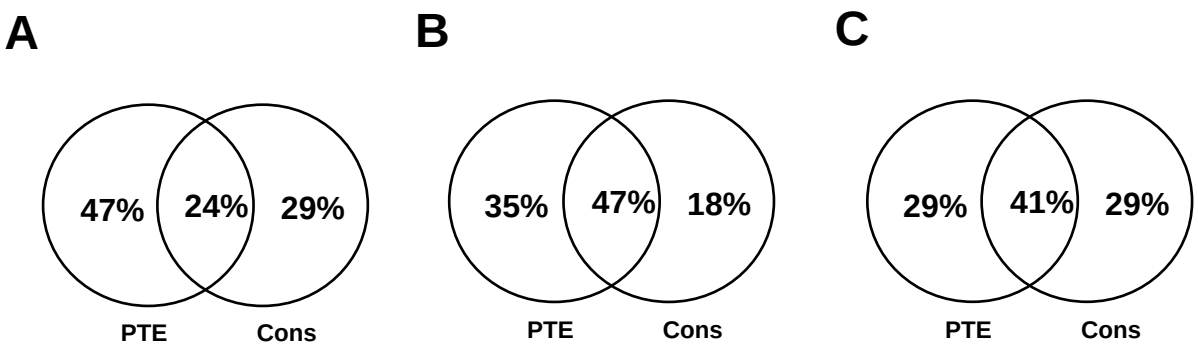

Figure 4.4 Comparison of PTE and consensus peptide set reactivity. (A) Magnitude of responses in Nef, (B) breadth of responses in Nef, and (C) breadth of responses in Gag.

Thus, the magnitude of T cell responses in the Gag protein was significantly higher when PTE peptides were used in this highly diverse HIV-infected population compared to consensus peptides, although this was not the case for Nef. In addition, whilst the overall median breadth of the response in the cohort did not change, a proportion of individuals in Nef had an increased number of responses.

To determine whether the diversity within the cohort affected the immune recognition when the two sets of peptide were used, the cohort was divided into CRF02_AG-infected individuals and non-CRF02_AG. This could be performed only with Nef responses, with nine and eight individuals in each group, respectively. The comparison could not be performed with Gag responses, since the majority of individuals were infected with CRF02_AG. In the CRF02_AG-infected group, the magnitude of Nef responses was similar when the two sets of peptide were used. The median magnitude of response was 1770 and 1420 SFU/10^6 PBMC, respectively, with PTE and consensus peptides (Figure 4.5A; left panel). The breadth of responses was also similar, with a median of two peptides recognised in the two sets of peptides (Figure 4.5B; left panel). In the non-CRF02_AG infected individuals, the same was observed. The magnitude of responses was similar when consensus peptides were used compared to PTE peptides. The median magnitude of response was 1171 and 635 SFU/10^6 PBMC ($p=0.9$), respectively with consensus and PTE peptides (Figure 4.5A; right panel). The median breadth of responses was also similar when the two sets of reagents were used, with 2

vs 1.5 targeted peptides (p=0.2) with consensus and PTE respectively (Figure 4.5B; right panel). In addition, a total number of 49 peptides (including variants) were targeted in the CRF02_AG group, while 31 were targeted in the non-CRF02_AG group.

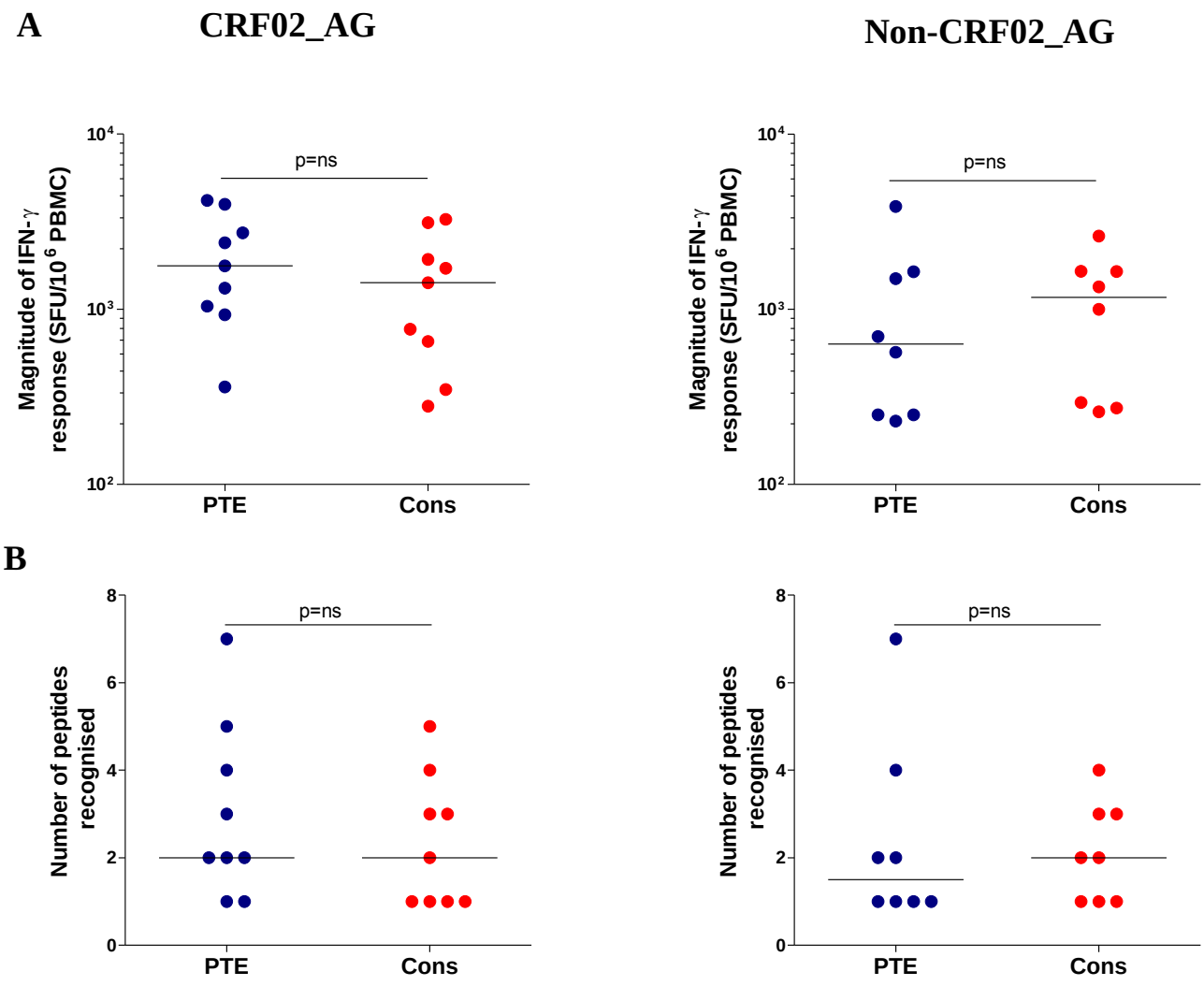

Figure 4.5 Comparison of Nef responses in CRF02_AG and non-AG-infected individuals. HIV-specific T cells were detected in nine CRF02_AG (right panel) and eight non-CRF02_AG-infected individuals (left panel) using the PTE (blue) and consensus (red) peptides. (A) the total magnitude of responses, and (B) the breadth of responses to Nef peptides detected in CRF02_AG and non-CRF02_AG-infected individuals.

4.3.3.2 Distribution of reactive PTE and Consensus peptides in Gag and Nef

It was next determined how targeted peptides from the two sets of peptides were distributed across the Gag and Nef proteins. Figure 4.6 shows the position of all the targeted consensus and PTE peptides across Gag and Nef. In Gag, the highest density of targeted peptides was observed for Gag p24 (amino acids 150-336). In both sets of reagents, reactive domains detected with both the consensus and PTE peptide sets were similar, spanning the entire protein; meanwhile, a minor acluster between amino acids 71-83 was exclusively detected using PTE peptides, and by only one study participant. Peptides that were targeted by at least three study participants with the two sets of reagents spanned amino acids 162-186 and 296-311 (Figure 4.6).

In Nef, the pattern of T cell responses between the two set of reagents was similar, with the central region, spanning amino acids 74-150 being the most frequently targeted (Figure 4.6). Reactivity was thus distributed along the most conserved regions of Gag and Nef for both PTE and consensus peptides.

4.3.3.3 Depth of responses

Next, the depth of the response, which is the cross-reactivity to different peptide variants, was determined for the PTE peptide set. For several of these peptides, recognition of multiple variants was detected with the PTE peptide set, with up to five variants being recognised for particular peptides (Table 4.2 and 4.3). An example of this can be seen with the most frequently targeted peptide when the two sets of reagents were used. Consensus Gag81 was targeted by five (29%) study participants while its corresponding PTE peptide was targeted by six (35%) participants; among these six participants, all except one also recognised at least one variant of this peptide, with some individuals reacting to three variants (Table 4.2). In Nef, consensus Nef35 was targeted by 8 (47%) study participants and its corresponding PTE peptide was recognised by seven (41%) participants, all of whom also recognised at least one variant of this peptide, with some individuals reacting to three variants (Table 4.3). In addition, out of a total of 83 peptides plus variants recognised in Gag, only 12 (14%) had sequence that matched the autologous viral sequence. The same trend was observed in Nef, where only 8/81 (10%) of targeted PTE peptides matched the corresponding

viral sequence. Despite this, multiple peptide variants were recognised by individuals even when sequences did not match the autologous virus sequences (Tables 4.2 and 4.3).

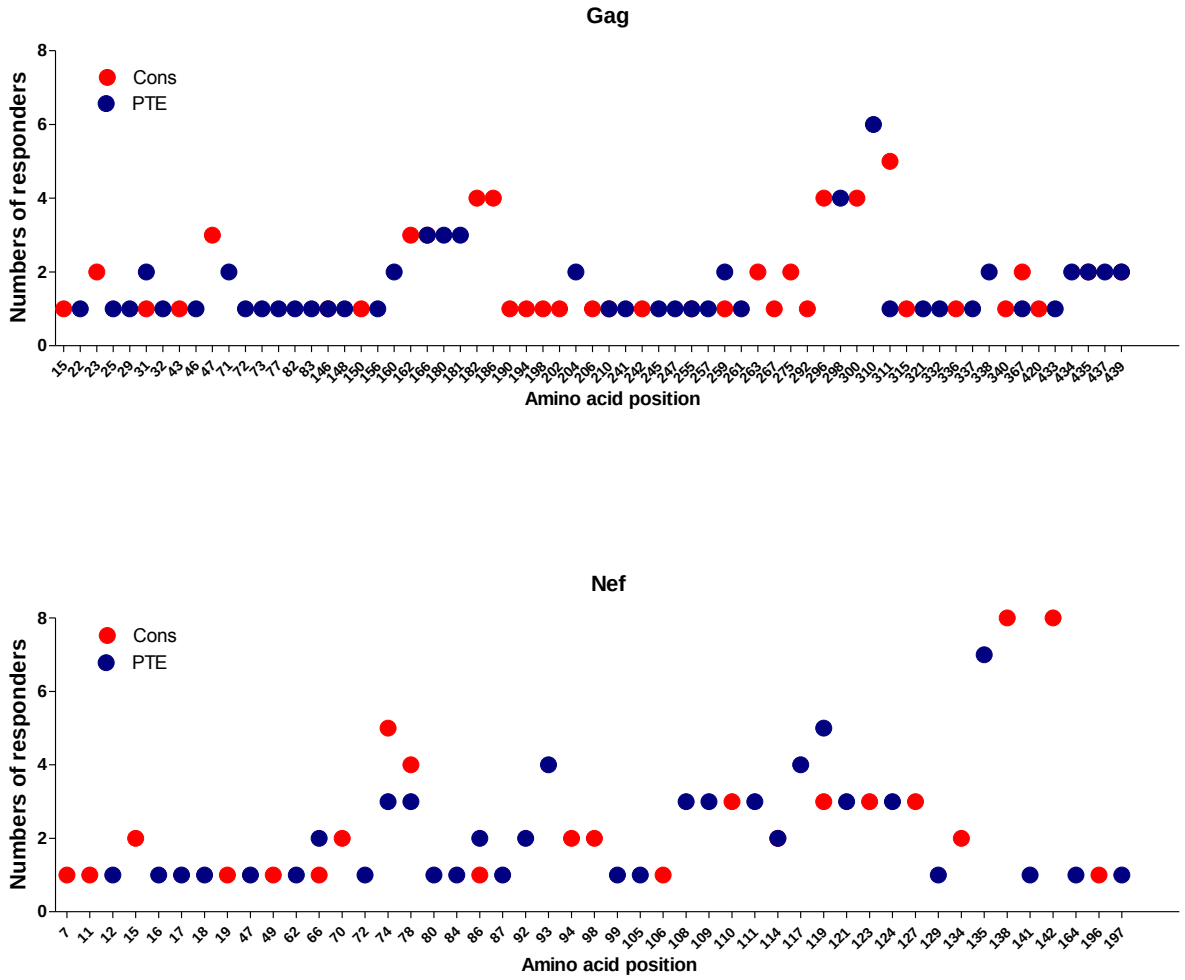

Figure 4.6 Reactive peptide distribution across Gag and Nef. Total number of targeted PTE (blue) and consensus (red) peptides are displayed and shown is the mid-point amino acid position for the peptide.

Figure 4.7 shows the proportion of recognised peptides with targeted variants in Gag and Nef. Individuals targeted a total of 44 peptides or epitopic regions in Gag. Out of these peptides, 25 (57%) had also at least one variant targeted, while 19 had no targeted variant. The opposite was observed in Nef, where a total of 46 epitopic regions were targeted. Out of these, only 18 (39%) had at least one variant that was also targeted (Figure 4.7A). Thus, the majority of targeted peptides in Gag had at least one variant targeted, but this was not the case

with Nef. Within Nef, although the majority of targeted peptides had no variant targeted in both the CRF02_AG and non-CRF02_AG infected individuals, there was a greater number of peptides and variants targeted in the CRF02_AG-infected cohort (27 vs 19; Figure 4.7 B).

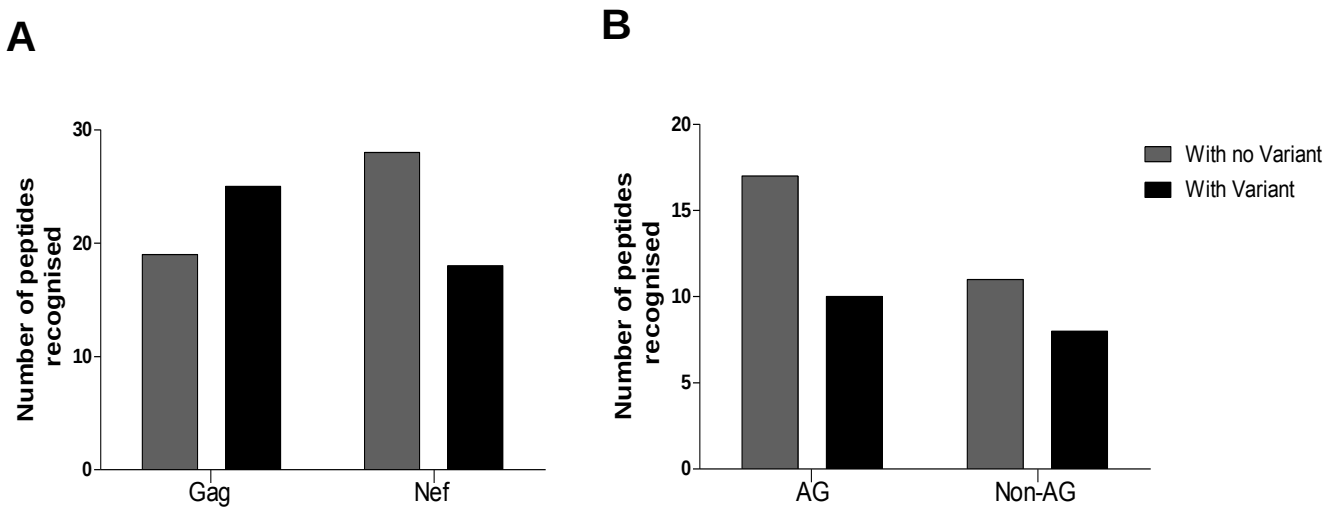

Figure 4.7 Proportion of recognised peptides with targeted variants. The number of peptides that were targeted along with variants (black bar) or with no targeted variants (grey bar) in (A) Gag and Nef and (B) in CRF02_AG and non-CRF02_AG-infected individuals for Nef.

When the magnitude of responses was compared amongst variants of the same targeted peptide, there was an overall trend of similarity. There were 25 cases in Gag where a particular peptide and at least one variant were targeted. In 19 (76%) of these cases, there was no substantial variation in the magnitude of responses (a difference was considered substantial when the magnitude was three times greater). For example, BS39 in Gag recognised four different peptides, of which three had one variant and one had three variants. In all these peptides and their variants, there was no substantial variation in the magnitude of responses and all the variants had a sequence that contained mismatches with respect to the autologous virus (Table 4.2). In Nef, there were 19 cases when a particular peptide was targeted with at least one variant. In 17 (89%) of cases, the magnitude of responses to the variant(s) was not substantially different. One example is BS27, who targeted two peptides, of which one with two variants had similar magnitudes of responses. The peptide sequences also mismatched the autologous viral sequence (Table 4.3). On the other hand, there were cases where the

magnitude of responses between a peptide and its variant(s) was drastically different. For example, BS29 in Gag targeted four peptides, including two with two variants each. In one of these peptides, the magnitude of responses was drastically reduced in one variant (from 1700 to 220 SFU/10^6 PBMC; Table 4.2). This was also observed in Nef, where for example BS64 recognised three peptides, of which two had two variants each, and there was a reduction of the magnitude in one variant of one of the peptides recognised (from 890 to 160 SFU/10^6 PBMC; Table 4.3).

Table 4.2 Summary of reactive PTE and Consensus peptides in the Gag protein in responders from Cameroon

PID	PTE				Consensus				[d]Virus Sequence
	[a]Peptide no.	HXB2 aa Position	[b]Sequence	SFC/10^6 PBMC	Peptide #	HXB2 Aa Position	[c,e]Sequence	SFC/10^6 PBMC	
BS29	PTEGag260	23-37	PGGNKKYKLKHIVWA	1720	ConsGag7	25-39	GKKKYRLKHLVWASR	485	PGG-KKQYKLKHLVW
	PTEGag175	25-39	GKKHYMLKHLVWASR	1700					GKKQYKLKHLVWASR
	PTEGag79	26-40	KKKYRLKHLVWASRE	220					KKQYKLKHLVWASRE
	PTEGag75	154-168	NAVKVVEEKAFSPEV	140	ConsGag42	156-170	VKVIEEKAFSPEVIP	175	NAWVKVIEEKAFSPEV
	PTEGag181	154-168	AWVKAIEEKAFSPEV	170					NAWVKVIEEKAFSPEV
	PTEGag14	160-174	EEKAFSPEVIPMFSA	230	ConsGag43	160-174	EEKAFSPEVIPMFSA	155	EEKAFSPEVIPMFTA
	PTEGag129	332-346	TILRALGPGATLEEM	190					TILRALGPAATLEEM
BS53	PTEGag28	292-326	PFRDYVDRFFKTLRA	3630	ConsGag77	290-304	KEPFRDYVDRFFKTL	4085	PFRDYVDRFFKTLRA
	PTEGag48	292-326	PFRDYVDRFYKTLRA	2040					PFRDYVDRFFKTLRA
	PTEGag268	65-74	LPALKTGSEELRSLY	100					LQSTLRTGSEELKSLF
BS30	PTEGag67	304-318	LRAEQATQEVKNWMT	1230	ConsGag81	305-318	RAEQATQDVKNWMT	128	LRAEQATQEVKNWMT
	PTEGag98	304-318	LRAEQASQEVKNWMT	398					LRAEQATQEVKNWMT
	PTEGag232	25-39	GRKKYRLKHIVWASR	280					GKKRYKLKHIVWASR

BS77	PTEGag8	174-188	ALSEGATPQDLNTML	380	ConsGag47	176-190	SEGATPQDLNTMLNT	193	ALSEGATPQDLNMML
					ConsGag49	184-198	LNTMLNVGGHQAAM	123	
	PTEGag11	427-441	TERQANFLGKIWPSH	120	ConsGag116	433-447	FLGKIWPSNKGRPGN	603	TERQANFLGKIWPSS
	PTEGag201	428-442	ESKANFLGKIWPSNK	400					ERQANFLGKIWPSSK
	PTEGag309	429-443	RQANFLGKIWPSYKG	120					RQANFLGKIWPSSKG
	PTEGag179	431-445	ANFLGKIWPSSKGRP	280					ANFLGKIWPSSKGRP
	PTEGag83	433-447	FLGKIWPSHKGRQEN	460					FLGKIWPSSKGRPGN
BS22	PTEGag31	253-267	NPPIPVGEIYKRWII	2080	ConsGag68	257-271	PVGEIYKRWIILGLN	1508	NPPIPVGEIYKRWII
	PTEGag53	255-269	PIPVGDIYKRWIILG	1200					PIPVGEIYKRWIILG
	PTEGag223	255-269	PVPVGEIYKRWIVLG	880					PIPVGEIYKRWIILG
	PTEGag67	304-318	LRAEQATQEVKNWMT	1420	ConsGag81	305-318	RAEQATQDVKNWMT	858	LRAEQATQEVKNWMT
	PTEGag82	304-318	LRAEQATQDVKNWMT	800					LRAEQATQEVKNWMT
	PTEGag98	304-318	LRAEQASQEVKNWMT	690					LRAEQATQEVKNWMT
BS64	PTEGag47	198-212	MEMLKDTINEEAAEW	1020	ConsGag53	200-214	MLKDTINEEAAEWDR	885	MQMLKDTINEEAAEW
	PTEGag80	198-212	MHMLKETINEEAAEW	1240					MQMLKDTINEEAAEW
	PTEGag16	204-218	TINEEAAEWDRLHPV	910	ConsGag54	204-218	TINEEAAEWDRLHPV	305	TINEEAAEWDRTHPV
	PTEGag67	304-318	LRAEQATQEVKNWMT	1410	ConsGag81	305-318	RAEQATQDVKNWMT	1315	LRAEQATQEVKNWMT
	PTEGag82	304-318	LRAEQATQDVKNWMT	960					LRAEQATQEVKNWMT

	PTEGag77	179-193	ATPQDLNMMLNIVGG	300					ATPQDLNMMLNIVGG
BS39					ConsGag11	41-54	LERFALNPGLLETS	378	LERFALNPGLLDTA
					ConsGag51	192-206	GGHQAAMQMLKDTIN	248	GGHQAAMQMLKDTIN
					ConsGag76	286-300	RQGPKEPFRDYVDRF	198	RQGPKEPFREYVDRF
	PTEGag28	292-306	PFRDYVDRFFKTLRA	2940	ConsGag78	294-308	RDYVDRFFKTLRAEQ	3118	PFREYVDRFFKTLRA
	PTEGag48	292-306	PFRDYVDRFYKTLRA	1730					PFREYVDRFFKTLRA
	PTEGag139	331-345	KTILKALGPGATLED	620	ConsGag88	330-343	CKTILKALGPGATL	908	KAILRALGPGATLEE
	PTEGag173	331-345	KSILRALGPGASLEE	640					KAILRALGPGATLEE
	PTEGag129	332-346	TILRALGPGATLEEM	910					AILRALGPGATLEEM
	PTEGag318	332-346	SILKALGTGATLEEM	800					AILRALGPGATLEEM
	PTEGag120	71-85	GSEELRSLYNTVATL	950					GSEELKSLFNTVATL
	PTEGag123	71-85	GSEELKSLYNTVATL	770					GSEELKSLFNTVATL
	PTEGag289	76-91	KSLFNTVATLYCVHA	990					KSLFNTVATLCCVHQ
	PTEGag54	77-92	SLYNTVATLYCVHQR	670					SLFNTVATLCCVHQR
BS14	PTEGag21	235-249	DIAGTTSTLQEQIGW	1760	ConsGag63	238-252	GTTSTLQEQIAWMTS	1235	DIAGTTSNLQEQIGW
	PTEGag89	239-253	TTSTLQEQIAWMTSN	1460					TTSNLQEQIGWMTSN

	PTEGag78	241-255	STLQEQIGWMTSNPP	150					SNLQEQIGWMTSNPP
	PTEGag28	292-306	PFRDYVDRFFKTLRA	100	ConsGag77	290-304	KEPFRDYVDRFFKTL	485	PFRDYVDRFFKTLRA
	PTEGag91	361-375	RVLAEAMSQVTNSAT	150	ConsGag96	361-375	RVLAEAMSQVTNAAI	115	RVLAEAMSQVQ---QAN
					ConsGag71	269-283	GLNKIVRMYSPVSIL	105	GLNKIVRMYSPVSIL
	PTEGag43	150-164	RTLNAWVKVVEEKAF	270					RTLNAWVKVIEEKAF
	PTEGag46	326-340	ANPDCKTILKALGPA	160					ANPDCKSILKALGTG
BS09	PTEGag163	40-54	ELERFALNPSLLETT	390	ConsGag10	37-51	ASRELERFALNPGLL	135	ELERFALNPSLLETA
	PTEGag42	140-154	GQMVHQAISPRTLNA	290	ConsGag39	144-158	HQAISPRTLNAWVKV	765	GQMVHQAISPRTLNA
	PTEGag127	142-156	MVHQALSPRTLNAWV	100					GQMVHQAISPRTLNA
	PTEGag28	292-306	PFRDYVDRFFKTLRA	130	ConsGag77	290-304	KEPFRDYVDRFFKTL	605	PFRDYVDRFFKALRA
					ConsGag81	305-318	RAEQATQDVKNWMT	105	RAEQATQEVKGWMT
	PTEGag268	65-79	LPALKTGSEELRSLY	260					LQPALKTGSEELKSLF
	PTEGag209	66-80	STLKTGSEELKSLYN	180					PALKTGSEELKSLFN
	PTEGag182	67-81	ALKTGTEELRSLYNT	150					ALKTGSEELKSLFNA
BS72					ConsGag11	41-54	LERFALNPGLLETS	120	LERFALNPNLLETT

	PTEGag8	174-188	ALSEGATPQDLNTML	210	ConsGag48	180-194	TPQDLNTMLNTVGGH	180	ALSEGATPQDLNMML
					ConsGag68	257-271	PVGEIYKRWIILGLN	110	PVGEIYKRWIILGLN
					ConsGag71	269-283	GLNKIVRMYSPVSIL	130	GLNKIVRMYSPVSIL
	PTEGag67	304-318	LRAEQATQEVKNWMT	1120	ConsGag81	305-318	RAEQATQDVKNWMT	330	LRAEQATQEVKNWMT
	PTEGag82	304-318	LRAEQATQDVKNWMT	260					LRAEQATQEVKNWMT
	PTEGag98	304-318	LRAEQASQEVKNWMT	790					LRAEQATQEVKNWMT
					ConsGag96	361-374	RVLAEAMSQVTNAA	170	RVLAEAMSQVQ---
BS73					ConsGag69	261-275	IYKRWIILGLNKIVR	293	IYKRWIILGLNKIVR
					ConsGag111	414-427	WKCGKEGHQMKDCT	143	WKCGKEGHQMKDCT
	PTEGag80	198-212	MHMLKETINEEAAEW	120					MQMLKDTINEEAAEW
	PTEGag67	304-318	LRAEQATQEVKNWMT	830					LRAEQATQEVKNWMT
	PTEGag98	304-318	LRAEQASQEVKNWMT	170					LRAEQATQEVKNWMT
	PTEGag302	305-319	RAEQGTQEVKNWMTE	160					LRAEQATQEVKNWMT
BS38	PTEGag14	160-174	EEKAFSPEVIPMFSA	210	ConsGag42	156-170	VKVIEEKAFSPEVIP	145	EEKAFSPEVIPMFSA
	PTEGag201	428-442	ESKANFLGKIWPSNK	150	ConsGag115	429-443	RQANFLGKIWPSNKG	150	ERQANFLGKIWPSSK
	PTEGag309	429-443	RQANFLGKIWPSYKG	140					RQANFLGKIWPSSKG
	PTEGag179	431-445	ANFLGKIWPSSKGRP	180					ANFLGKIWPSSKGRP

	PTEGag83	433-447	FLGKIWPSHKGRQEN	340					FLGKIWPSSKGRPGN
BS16					ConsGag5	17-31	EKIRLRPGGKKKYRL	315	EKIRLRPGGRKQYKL
	PTEGag65	249-263	WMTSNPPIPVGDIYK	320	ConsGag66	249-263	WMTSNPPIPVGEIYK	165	WMTSNPPIPVGEIYK
	PTEGag140	249-263	WMTSNPPVPVGEIYK	370					WMTSNPPIPVGEIYK
	PTEGag185	251-265	TSNPPVPVGDIYKRW	300					TSNPPIPVGEIYKRW
	PTEGag31	253-266	NPPIPVGEIYKRWII	120					NPPIPVGEIYKRWII
BS11					ConsGag11	41-54	LERFALNPGLLETS	210	LERFALNPGLLES
	PTEGag75	154-263	NAVKVVEEKAFSPEV	180	ConsGag42	156-170	VKVIEEKAFSPEVIP	430	NAWVKVIEEKAFSPEV
	PTEGag181	154-263	AWVKAIEEKAFSPEV	110					NAWVKVIEEKAFSPEV
	PTEGag14	160-174	EEKAFSPEVIPMFSA	840	ConsGag43	160-174	EEKAFSPEVIPMFSA	1020	EEKAFSPEVIPMFTA
	PTEGag162	306-320	AEQATQEVKGWMTDT	840					AEQATQDVKNWMTDT
	PTEGag67	304-318	LRAEQATQEVKNWMT	120					LRAEQATQDVKNWMT
BS55	PTEGag8	174-188	ALSEGATPQDLNTML	2600	ConsGag48	180-194	TPQDLNTMLNTVGGH	1013	ALSEGATPQDLNMML
	PTEGag77	179-193	ATPQDLNMMLNIVGG	2490					ATPQDLNMMLNIVGG
BS25	PTEGag8	174-188	ALSEGATPQDLNTML	930	ConsGag48	180-194	TPQDLNTMLNTVGGH	340	ALSDGATPQDLNMML
	PTEGag77	179-193	ATPQDLNMMLNIVGG	840					ATPQDLNMMLNIVGG
					ConsGag52	196-210	AAMQMLKDTINEEAA	100	AAMQMLKDTINEEAA

BS48	PTEGag26	16-30	WEKIRLRPGGKKKYR	1230	ConsGag5	17-31	EKIRLRPGGKKKYRL	510	WEKIRLRPGGKKKYK
	PTEGag156	19-33	IRLRPGGKKHYMLKH	450					IRLRPGGKKKYKMKH
					ConsGag3	9-23	SGGKLDAWEKIRLRP	120	SGGKLDAWEKIRLRP

[a] Different peptides and their variants are separated from each other by a line; [b] PTE amino acid differences from the consensus sequence are highlighted in red; [c] Peptides in red are consensus peptides with no corresponding targeted PTE peptide; [d] Autologous virus amino acid differences from the PTE sequence are highlighted in blue; [e] Differences between consensus and viral sequences are highlighted in green

Table 4.3 Summary of reactive PTE and Consensus peptides in the Nef protein in responders from Cameroon Cameroon

	PTE				Consensus				[d]Virus Sequence
PID	[a]Peptideno #	HXB2 aa Position	[b]Sequence	SFC/10^6 PBMC	Peptide #	HXB2 Aa Position	[c,e]Sequence	SFC/10^6 PBMC	
BS29					ConsNef1	1-15	MGGKWSKSSIVGWPA	565	MGGKWSKSSIVGWPQ
	PTENef6	60-74	AQEEEEVGFPVRPQV	250					AQEDDEVGFPVRPQV
	PTENef50	78-92	PMTYKGAFDLSHFLK	110					PMTYKGAFDLGFFLK
	PTENef11	87-101	LSFFLKEKGGLDGLI	320					LGFFLKEKGGLDGLI
	PTENef8	113-127	WVYHTQGYFPDWQNY	170	ConsNef30	112-126	LWVYHTQGYFPDWQN	125	WVYHTQGYFPDWQNY
	PTENef30	123-137	WQNYTPGPGTRFPLT	580					WQNYTPGPGIRYPLT
	PTENef10	129-143	GPGVRYPLTFGWCFK	1850	ConsNef35	131-145	PGIRYPLTFGWCFKL	1935	GPGIRYPLTFGWCYK
	PTENef27	129-143	GPGIRYPLTFGWCYK	1880					GPGIRYPLTFGWCYK
	PTENef31	129-143	GPGTRFPLTFGWCFK	2090					GPGIRYPLTFGWCYK
	PTENef70	158-172	GENNSLLHPMSLHGM	350					GENNSLLHPICQHGM
BS27	PTENef29	81-95	YKGAFDLSFFLKEKG	120					FKAAFDLSFFLKEKG
	PTENef10	129-143	GPGVRYPLTFGWCFK	1260	ConsNef35	131-145	PGIRYPLTFGWCFKL	1648	GPGTRYPLTFGWCFK
	PTENef27	129-143	GPGIRYPLTFGWCYK	1380					GPGTRYPLTFGWCFK
	PTENef31	129-143	GPGTRFPLTFGWCFK	1260					GPGTRYPLTFGWCFK

BS30					ConsNef3	9-23	SIVGWPAVRERIRRT	218	---GWPAVRERIRRT
	PTENef51	68-82	FPVKPQVPLRPMTFK	280	ConsNef18	67-81	GFPVRPQVPLRPMTY	508	FPVRPQVPLRPMTFK
	PTENef4	72-86	PQVPLRPMTYKGAFD	110					PQVPLRPMTFKAALD
	PTENef11	87-101	LSFFLKEKGGLDGLI	400					LSHFLKEKGGLEGLV
					ConsNef34	128-141	TPGPGIRYPLTFGW	105	TPGPGTRYPLCFGW
	PTENef24	111-125	DLWVYNTQGYFPDWQ	400				108	DLWVYHTQGFFPDWH
	PTENef8	113-127	WVYHTQGYFPDWQNY	130					WVYHTQGFFPDWHNY
	PTENef10	129-143	GPGVRYPLTFGWCFK	490	ConsNef35	131-145	PGIRYPLTFGWCFKL	508	GPGTRYPLCFGWCFE
	PTENef27	129-143	GPGIRYPLTFGWCYK	460					GPGTRYPLCFGWCFE
	PTENef31	129-143	GPGTRFPLTFGWCFK	560					GPGTRYPLCFGWCFE
BS22	PTENef66	6-20	SKSSIVGWPEVRERM	920	ConsNef3	9-23	SIVGWPAVRERIRRT	108	SKSSIVGWPQVRERM
	PTENef120	10-24	GVGWPTVRERMRRAE	750					IVGWPQVRERMRQ--
	PTENef108	11-25	VGWPAVRERMRRTEP	600					VGWPQVRERMRQ--T-P
	PTENef122	12-26	GWPEVRERMRRAPAA	750					GWPQVRERMRQ--T-P--
					ConsNef12	43-57	ITSSNTAANNPDCAW	168	ITSSNTAHTNADCAW
	PTENef62	56-70	WPAVRERMRRAEPAA	770					WPQVRERMRQ--TPSAA

	PTENef37	80-94	TYKAAVDLSHFLKEK	110					TYKGAVDLSHFLKEK
	PTENef18	99-113	GLIYSKKRQEILDLW	400	ConsNef27	100-114	LIYSKKRQEILDLWV	108	GLIYSKKRQEILDLW
	PTENef47	102-116	YSQKRQDILDLWVYH	660	ConsNef28	104-118	KKRQEILDLWVYHTQ	928	YSKKRQEILDLWVYN
	PTENef64	103-117	SRRRQEILDLWVYNT	840					SKKRQEILDLWVYNT
	PTENef7	105-119	KRQEILDLWVYHTQG	910					KRQEILDLWVYNTQG
	PTENef17	105-119	KRQDILDLWVYNTQG	580					KRQEILDLWVYNTQG
	PTENef2	118-132	GYFPDWQNYTPGPGV	120	ConsNef32	120-134	YFPDWQNYTPGPGIR	108	GFFPDWQNYTPGPGT
BS64	PTENef33	111-125	DLWVYHTQGFFPDWH	260	ConsNef30	112-126	LWVYHTQGYFPDWQN	255	DLWVYHTQGFFPDWQ
	PTENef8	113-127	WVYHTQGYFPDWQNY	550					WVYHTQGFFPDWQNY
	PTENef13	115-129	YHTQGFFPDWQNYTP	320					YHTQGFFPDWQNYTP
	PTENef2	118-132	GYFPDWQNYTPGPGV	190	ConsNef31	116-130	HTQGYFPDWQNYTPG	545	GFFPDWQNYTPGPGT
	PTENef10	129-143	GPGVRYPLTFGWCFK	1030	ConsNef35	131-145	PGIRYPLTFGWCFKL	915	GPGTRYPLTFGWCYK
	PTENef27	129-143	GPGIRYPLTFGWCYK	890					GPGTRYPLTFGWCYK
	PTENef31	129-143	GPGTRFPLTFGWCFK	140					GPGTRYPLTFGWCYK
BS39	PTENef6	60-74	AQEEEE-VGFPVRPQ	1070	ConsNef16	60-73	AQEEEEVGFPVRPQ	348	AQEEGEVGFPVRPQV
	PTENef4	72-86	PQVPLRPMTYKGAFD	1330					PQVPLRPMTYKGALD

BS72					ConsNef18	67-81	GFPVRPQVPLRPMTY	110	GFPVRPQVPLRPMTY
	PTENef5	86-100	DLSHFLKEKGGLEGL	230	ConsNef24	90-104	FLKEKGGLEGLIYSK	150	DLGFFLKEKGGLDGL
BS79	PTENef10	129-143	GPGVRYPLTFGWCFK	1320	ConsNef35	131-145	PGIRYPLTFGWCFKL	1923	GPGVRYPLCFGWCFK
	PTENef27	129-143	GPGIRYPLTFGWCYK	1300					GPGVRYPLCFGWCFK
	PTENef31	129-143	GPGTRFPLTFGWCFK	890					GPGVRYPLCFGWCFK
BS73	PTENef33	111-125	DLWVYHTQGFFPDWH	1110	ConsNef30	112-126	LWVYHTQGYFPDWQN	2803	DLWIYHTQGYFPDWQ
	PTENef8	113-127	WVYHTQGYFPDWQNY	2280					WIYHTQGYFPDWQCY
	PTENef13	115-129	YHTQGFFPDWQNYTP	1500					YHTQGYFPDWQCYTP
	PTENef36	135-149	PLTFGWCYKLVPVDP	450	ConsNef35	131-145	PGIRYPLTFGWCFKL	463	PLTFGWCYKLVPMDP
BS20	PTENef1	66-80	VGFPVRPQVPLRPMT	410	ConsNef18	67-81	GFPVRPQVPLRPMTY	1860	VGFPVRPQVPLRPMT
	PTENef51	68-82	FPVKPQVPLRPMTFK	1510					FPVRPQVPLRPMTFK
	PTENef27	129-143	GPGIRYPLTFGWCYK	120	ConsNef35	131-145	PGIRYPLTFGWCFKL	520	GPGTRFPLTFGWCFK
	PTENef31	129-143	GPGTRFPLTFGWCFK	490					GPGTRFPLTFGWCFK
	PTENef37	80-94	TYKAAVDLSHFLKEK	100					TFKAAIDLSHFLKEK
	PTENef117	191-205	RHLARELHPEYYKNC	2090					RHIAQEMHPEFYKDC
					ConsNef17	64-77	EEVGFPVRPQVPLR	330	EEVGFPVRPQVPLR

					ConsNef34	128-141	TPGPGIRYPLTFGW	410	TPGPGTRFPLTFGW
BS35					ConsNef19	71-85	RPQVPLRPMTYKAAL	240	RPQVPLRPMTYKAAF
	PTENef47	102-116	YSQKRQDILDLWVYH	200	ConsNef28	104-118	KKRQEILDLWVYHTQ	690	YSRKRQDILNLWVYH
	PTENef 87	103-117	SQRRQDILDLWIYHT	120					SRKRQDILNLWVYHT
	PTENef7	105-119	KRQEILDLWVYHTQG	450					KRQDILNLWVYHTQG
	PTENef35	108-122	DILDLWVYHTQGYFP	250	ConsNef29	108-122	EILDLWVYHTQGYFP	715	DILNLWVYHTQGFFP
BS38	PTENef47	102-116	YSQKRQDILDLWVYH	160	ConsNef28	104-118	KKRQEILDLWVYHTQ	280	YSRKRQEILDLWIYH
	PTENef64	103-117	SRRRQEILDLWVYNT	360					SRKRQEILDLWIYHT
	PTENef7	105-119	KRQEILDLWVYHTQG	180					KRQEILDLWIYHTQG
	PTENef17	105-119	KRQDILDLWVYNTQG	160					KRQEILDLWIYHTQG
BS16	PTENef45	41-55	GAITSSNTAATNADC	180					GAVTISNTAATNANC
	PTENef51	68-82	FPVKPQVPLRPMTFK	170	ConsNef18	67-81	GFPVRPQVPLRPMTY	315	FPVRPQVPLRPMTYK
					ConsNef21	79-92	MTYKAALDLSHFLK	105	MTYKGALDLSHFLK
	PTENef11	87-101	LSFFLKEKGGLDGLI	100					LSHFLKEKGGLDGLI
	PTENef28	93-107	EKGGLDGLIYSKKRQ	100					EKGGLDGLIYSKKRQ
	PTENef10	129-143	GPGVRYPLTFGWCFK	320	ConsNef35	131-145	PGIRYPLTFGWCFKL	235	GPGIRYPLTFGWCYK

	PTENef27	129-143	GPGIRYPLTFGWCYK	380					GPGIRYPLTFGWCYK
	PTENef31	129-143	GPGTRFPLTFGWCFK	250					GPGIRYPLTFGWCYK
BS18	PTENef24	111-125	DLWVYNTQGYFPDWQ	150					DLWVYNTQGFFPDWQ
	PTENef8	113-127	WVYHTQGYFPDWQNY	200					WVYNTQGFFPDWQNY
	PTENef13	115-129	YHTQGFFPDWQNYTP	470					YNTQGFFPDWQNYTP
	PTENef2	118-132	GYFPDWQNYTPGPGV	570	ConsNef32	120-134	YFPDWQNYTPGPGIR	770	GFFPDWQNYTPGPGI
BS78	PTENef35	108-122	DILDLWVYHTQGYFP	250	ConsNef29	108-122	EILDLWVYHTQGYFP	294	EILDLWVYHTQGFLP
BS55	PTENef5	86-100	DLSHFLKEKGGLEGL	470	ConsNef23	90-104	FLKEKGGLEGLIYSK	273	DLSHFLKEKGGLEGL
	PTENef11	87-101	LSFFLKEKGGLDGLI	570					LSHFLKEKGGLEGLI
BS40	PTENef4	72-86	PQVPLRPMTYKGAFD	110	ConsNef19	71-85	RPQVPLRPMTYKAAL	700	PQVPLRPMTYKAAVD
	PTENef21	74-88	VPLRPMTYKAAVDLS	250					VPLRPMTYKAAVDLS
					ConsNef52	190-204	ALRHIARELHPEYYK	300	ARRHIARELHPEYYK

[a] Different peptides and their variants are separated from each other by a line; [b] PTE amino acid differences from the consensus sequence are highlighted in red; [c] Peptides in red are consensus peptides with no corresponding targeted PTE peptide; [d] Autologous virus amino acid differences from the PTE sequence are highlighted in blue; [e] Differences between consensus and viral sequences are highlighted in green

4.4 DISCUSSION

HIV-1 diversity represents a huge obstacle not only to the design of an effective vaccine but also to the evaluation of HIV vaccines. One of the proposed strategies to cope with this diversity is the use of mosaic constructs for vaccination. Mosaic proteins are assembled to resemble natural proteins but they maximise the coverage of potential T cell epitopes (PTEs, or epitopes of nine amino acids) for a viral population. This study aimed to evaluate the reactivity to PTE group M peptides in HIV-infected individuals from Cameroon, a country with considerable HIV-1 genetic diversity, and compare this reactivity to results obtained with consensus group M peptides generated in the previous chapter.

This study revealed that T cells from HIV-infected Cameroonians are capable of recognising multiple variants of epitopes in Gag and Nef. Although the magnitude of responses was substantially lower in Nef to responses observed by Malhotra *et al*. (2007), with a median response magnitude of 1310 SFU/10^6 here (compared to 2169 SFU/10^6 in their study), the breadth of responses was however similar in the two studies, with a median breadth of 2 epitopic regions here and in their study. The magnitude difference could be explained by the fact that Malhotra and colleagues used a PTE peptide set from subtype B to detect responses in subtype B-infected individuals, and the closer match between peptides and viral sequences likely resulted in an increased magnitude of responses. This study used group M PTE and a population infected with diverse viruses. An additional consideration is that the individuals in that study were at an early stage of HIV infection, with viral loads more than three times greater than the present study. Whilst the infection date of individuals in the present study is unknown, it is likely, from CD4 count and viral load data, that the majority were in the chronic phase of infection.

Whilst we detected a significantly greater magnitude of T cell responses with the PTE peptide set than with the consensus M set in Gag, the magnitude of responses was similar between the two sets of peptides in Nef. The breadth of responses was also similar between the two set of peptides. This was surprising, as we hypothesised that the use of PTE peptides would increase immune coverage, as previously reported (Malhotra *et al*., 2007). It may be possible that HIV sequences from Cameroon are under-represented in the database, and because some of these individuals are infected with highly diverse viruses with rare epitopes,

that the PTE peptides do not increase the rate of epitope detection. Overall, these data suggest that the PTE peptides do not sufficiently take into account the diversity represented by other subtypes and CRFs that circulate in Cameroon.

Another striking point was that the magnitude of responses to different variants of the same peptide broadly followed the same pattern of response. When the response to a particular peptide was high, the response to the variant was also high. This can be explained by the fact that the PTE set, as for the consensus peptides, include the most conserved epitopes across global lineages. This was found when the peptide distribution was mapped across the Gag and Nef proteins and most of the targeted peptides in both PTE and consensus sets were located in the conserved regions. It does appear that recognition to these conserved epitopes can tolerate some amino acid mismatch. Furthermore, most PTE variants were recognised even when there were mismatches from autologous virus sequence. This could be explained by the fact that either variations were tolerated in the epitopes, mismatches were outside the epitope, or minor viral variants were present that were not reflected in the bulk autologous sequence generated. the observation that the majority of the peptides targeted with PTE peptides set were also targeted with the consensus set and that recognition of multiple variants was detected with the PTE set constitute an improvement of the use of PTEs over consensus peptides.

This study had several limitations. Since HLA typing was not performed, it was not possible to precisely predict the epitope within the 15mer peptides tested, to understand the effect of sequence mismatches on epitope reactivity. Another limitation was the autologous viral sequence, since bulk sequencing was performed to assess the infecting subtype. This represented a bias since the method detects the most common variant in the viral population and it not excludes minor viral populations might match PTEs. Finally, only responders to consensus M peptides were chosen for this study, and some differences could have emerged if non-responders were also included in this study.

In conclusion, Gag and Nef-specific T cells capable of recognising multiple variant epitopes were commonly detected in this study using PTE peptides. The immunological responses detected in HIV-infected individuals from Cameroon did not differ substantially between these peptides and consensus peptides. However, the observation that the majority of the peptides targeted with PTE peptides set were also targeted with the consensus set, that

recognition of multiple variants was detected with the PTE set and a marginally improved magnitude of response with PTE set**,** constitute an improvement of the use of PTEs over consensus peptides.

CHAPTER 5: DISCUSSION

This thesis focused on studying HIV diversity in Cameroon and characterising T cell immune responses in HIV-infected blood donors from Cameroon. Cameroon forms part of the Congo basin, where HIV originated from, and for this reason, the region has one of the oldest epidemics in the world. Very few immunological studies on HIV are available from the whole region, with only a single published study to date on T cell responses in HIV-infected individuals from Cameroon (Gupta *et al*., 2006).

A vaccine that can induce immune responses capable of preventing or containing HIV offers the greatest hope of halting the pandemic. Some major challenges need to be overcome to develop such a vaccine, including the enormous diversity the virus exhibits. Several studies have characterised HIV diversity in Cameroon, and have found the presence of every known subtype, including many recombinant forms. Given the impact that viral diversity can pose to the development of an effective HIV vaccine, it is important to continue to monitor the diversity at the epicentre of the pandemic. Data presented in the first chapter of this thesis corroborates previous findings of extensive HIV diversity in Cameroon. CRF02_AG remains the major circulating subtype, accounting for more than half of the viruses sequenced, both in *gag* and *nef*. The remainder were primarily a range of CRFs and pure subtypes, with 22% being URF viruses. This study, using a new phylogenetic analysis tool, quantified the rate of intra-gene recombination for the first time. A blind, fully exploratory screen for recombination was performed, testing all sequences (whether a previously defined non-recombinant pure subtype or not, for evidence of recombination. This analysis allowed information such as (1), the identification of recombinants as parental sequences of other recombinants, and (2), the detection of intra-subtype recombination. The range of intra-gene recombination (recombination breakpoints between gene segments that phylogenetically clustered within two distinct groups of the same subtype, indicating that they were likely intra-subtype recombinants) in Gag and Nef genes was estimated at 6% and 7%" respectively. This therefore suggests that even a pure subtype may in fact be a recombinant of different lineages from the same subtype. These findings therefore have implications for the development of a vaccine for the region, as the range of subtypes and the amount of intra-

subtype diversity within any geographical region will significantly impact HIV vaccine efficacy.

A further observation was that some previously identified CRFs, such as CRF11_cpx, are independent lineages and not obviously recombinant subtypes, at least in *gag* and *nef*. The identification and characterisation of certain CRFs and URFs have raised some questions, for example, the presence in the genome of sequences that are of indeterminate origin (Carr *et al*., 2010). Some of these CRF_cpx sequences may therefore represent examples of under-sampled subtype-level lineages. More importantly, despite the fact that the broadest HIV diversity, representative of all subtypes, was used to perform the analyses, some newly sequenced Cameroonian viruses were still too divergent to be placed within any existing subtype or CRF grouping, and they have remained unclassified. This was observed both with partial and full genome analysis. To date, the most extensive analysis of HIV in Cameroon was performed by Carr *et al*. (2010). They also found highly divergent viruses that were outliers of some existing lineages, and suggested that these divergent sequences were URFs. An advantage of the analysis tool used in Chapter 2 was that it breaks up the different recombinant fragments of a given sequence. Thus, after removal of recombinant fragments, some highly divergent viruses sequenced in this study remained outliers in *gag* and *nef*. This implies that there is potentially a less prominent, but far more diverse, pool of viruses circulating amongst humans than the currently classified subtypes and CRFs might suggest. This further complicates the design of an effective vaccine for the region.

It is now well accepted that an effective vaccine against HIV would need to elicit both potent broadly neutralising antibodies as well as effective CD8+ T cell responses against the vast majority of HIV subtypes and recombinants, either to augment protection at the site of exposure, or potentially to reduce viral load if breakthrough infection occurs. Therefore, there has been a continued focus on HIV vaccine immunogen design to elicit T cell responses that can control viral replication and be cross-reactive (and cross-protective) against the full spectrum of HIV diversity globally. One such proposed artificial immunogen is based on a consensus sequence of group M viruses. Chapter 3 of this thesis assessed HIV-specific T cell responses to peptides based on consensus M in HIV-infected individuals from mono- and multi-clade epidemics. First, when comparing the reactivity to these reagents between CRF02_AG and non-AG infected individuals from Cameroon, no differences in the

magnitude, breadth or epitope coverage between CRF02_AG and non-CRF02_AG viruses was observed for either Gag or Nef. In addition there was poor immunodominance in both Gag and Nef in both groups, and similar results were obtained when South Africa, a mono-clade C epidemic, and Cameroon, a multi-clade epidemic, were compared. Importantly, the same observations were made by Serwanga *et al*. (2011) in Uganda, which also represents a multi-clade epidemic, with few immunodominant epitopes being described. The targeting of peptides was directed towards conserved regions in Gag and Nef, and the use of consensus group M reagents likely resulted in the decreased detection of subtype-specific responses. The poor immunodominance that these central HIV consensus M sequences detected suggests that they may not be an ideal reagent for inclusion as a vaccine immunogen. They also suggest that consensus reagents are limited in their use as a standard panel to broadly assess HIV-specific T cell responses in vaccine trials in multiple sites where different epidemics prevail.

Despite the low immunodominance described in Chapter 3, there was a clear indication of preferentially targeted peptides when both CRF20_AG and non-AG-infected individuals and mono-clade and multi-clade epidemics were compared, an observation also made in the Ugandan study of consensus M peptide reactivity (Serwanga *et al*., 2011). Some peptides were preferentially targeted in the Ugandan population compared to Cameroon and South Africa. This suggests that on one hand, the infecting virus influences T cell immune recognition, as certain epitopes would be specific to certain viruses, and on the other hand, recognition is also driven by host genetic factors, as individuals from different ethnicities would express specific HLA molecules. The implication for an HIV vaccine design is that vaccine formulation needs to take into consideration both the circulating virus and the HLA of individuals targeted by this vaccine. This finding highlights the challenges of developing a global vaccine against HIV. It is unlikely that reasonably effective consensus M HIV vaccines will provide full protection against all subtypes. This favours the design of a vaccine made from a cocktail of conserved elements representing all subtypes from different epidemics, also taking into account global HLA diversity. Alternatively, a regional vaccine development approach for specific epidemics could also be considered.

Chapter 4 of this thesis investigated whether the use of PTE peptides from group M could result in enhanced T cell detection compared to consensus M in the context of HIV diversity. Mosaic vaccines represent a novel approach that extends the coverage to multiple

9mer epitopes or PTEs, which can be targeted by the host immune response (Fischer *et al*., 2007; Nickle *et al*., 2007). Therefore, potentially relevant T cell recognition to these PTE peptides could guide in the design of a mosaic vaccine. In addition, they could also be a useful standard tool for vaccine trial evaluation. It was previously reported that the magnitude and breadth of detectable responses were enhanced when subtype B PTE peptides were used, compared to consensus B peptides, during early HIV infection in subtype B-infected individuals (Malhotra *et al*., 2007). Here, the magnitude of the response detected was significantly higher in Gag when PTE peptides were used compared to consensus peptides, but this was not the case for Nef. The breadth of responses was however similar when the two sets of reagents were used, both in Gag and Nef. Further analyses in Nef revealed that the magnitude and breadth of responses were similar in CRF02_AG and non-AG-infected individuals. It appeared that since Gag was more homogenous than Nef (more patients were infected with CRF02_AG in Gag), this resulted in the increase in magnitude detected, most likely because there were less mismatches between the peptides and the virus. The result in Nef suggests that epitopes from minor circulating viruses are underrepresented in the PTE peptide set. The overall similarity of breadth between PTE and consensus peptides underscores the fact that there are fewer characterised epitopes from viruses circulating in Cameroon present in Los Alamos HIV databases. These findings therefore suggest that PTE peptides should be optimised to include more epitopes from minor subtypes. Therefore, if a mosaic approach for an HIV vaccine is pursued, or the use of PTE peptides for vaccine testing is pursued, additional mosaic sequences may need to be included for highly diverse epidemics such as that in Cameroon.

There were several limitations to this work that could be addressed in future studies. Characterisation of HIV diversity with intra- and inter-gene recombinants was performed for only two genes, namely *gag* and *nef*. Therefore, they do not represent an accurate picture of the extent of the recombination across the full genome. Further recombination analyses will be performed with the nine full length genome sequences generated here, as well as other published sequences. This may provide some insights into the early HIV-1 group M ancestral sequences. T cell characterisation was investigated using one function, namely the production of IFN-γ. It is possible that T cell responses producing other cytokines (such as MIP-1β) were missed. Furthermore, HLA typing was not performed on the study participants. This would have permitted the prediction of epitopes within reactive peptides, which would be useful to

update the HIV immunology database. In addition, HIV typing in *gag* and *nef* was performed using bulk PCR, which limited the analyses that could be performed on reactive peptide variants and autologous virus sequences. Finally, blood samples used in this study were drawn from HIV-infected blood donors, and no information was available on how long they had been infected, and whether they were in the acute or chronic phase of infection.

The work presented in this thesis offered some strengths. These included the large number of participants enrolled in the cohort (n=57), which allowed for the analysis of two sub-groupings (CRF02_AG and non-AG-infected individuals). Furthermore, all *gag* and *nef* sequences generated have been deposited in the Los Alamos HIV sequence database, and could prove to be useful in generating a consensus sequence or for designing or updating the PTE group M peptide set.

In conclusion, the study described in this thesis provides novel data on HIV diversity in Cameroon, where under-sampled divergent viruses are still circulating, and on T cell immune responses influenced by the viral diversity present in the country. Moreover, despite the central nature of consensus group M Gag and Nef peptide reagents, as well as the greater number of variants included in consensus group M PTE peptide sets, their use to evaluate HIV-specific T cell responses may be limited due to mismatches with particular HIV subtypes. These data suggest that developing a vaccine for a highly diverse epidemic is extremely challenging, and that a globally relevant vaccine may not provide sufficient coverage across different epidemics.

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APPENDICES

Appendix A ELISPOT assay with consensus group M peptides

Table A1 Peptide pool and matrix configuration for Consensus M peptides

POOLS		1	2	3	4	5	6	7	8	9	10	11	12
	Gag	1-12	13-24	25-33	34-45	46-57	58-69	70-81	82-93	94-95	96-107	108-119	120-129
	Nef	1-12	13-24	25-36	37-48	49-53							

MATRIX		Matrix 1	Matrix 2	Matrix 3	Matrix 4	Matrix 5	Matrix 6	Matrix 7	Matrix 8	Matrix 9	Matrix 10	Matrix 11	Matrix 12
	Gag Pool 1	Gag 1	Gag 2	Gag 3	Gag 4	Gag 5	Gag 6	Gag 7	Gag 8	Gag 9	Gag 10	Gag 11	Gag 12
	Gag Pool 2	Gag 13	Gag 14	Gag 15	Gag 16	Gag 17	Gag 18	Gag 19	Gag 20	Gag 21	Gag 22	Gag 23	Gag 24
	Gag Pool 3	Gag 25	Gag 26	Gag 27	Gag 28	Gag 29	Gag 30	Gag 31	Gag 32	Gag 33			
	Gag Pool 4	Gag 34	Gag 35	Gag 36	Gag 37	Gag 38	Gag 39	Gag 40	Gag 41	Gag 42	Gag 43	Gag 44	Gag 45
	Gag Pool 5	Gag 46	Gag 47	Gag 48	Gag 49	Gag 50	Gag 51	Gag 52	Gag 53	Gag 54	Gag 55	Gag 56	Gag 57
	Gag Pool 6	Gag 58	Gag 59	Gag 60	Gag 61	Gag 62	Gag 63	Gag 64	Gag 65	Gag 66	Gag 67	Gag 68	Gag 69
	Gag Pool 7	Gag 70	Gag 71	Gag 72	Gag 73	Gag 74	Gag 75	Gag 76	Gag 77	Gag 78	Gag 79	Gag 80	Gag 81
	Gag Pool 8	Gag 82	Gag 83	Gag 84	Gag 85	Gag 86	Gag 87	Gag 88	Gag 89	Gag 90	Gag 91	Gag 92	Gag 93
	Gag Pool 9	Gag 94	Gag 95										
	Gag Pool 10	Gag 96	Gag 97	Gag 98	Gag 99	Gag 100	Gag 101	Gag 102	Gag 103	Gag 104	Gag 105	Gag 106	Gag 107
	Gag Pool 11	Gag 108	Gag 109	Gag 110	Gag 111	Gag 112	Gag 113	Gag 114	Gag 115	Gag 116	Gag 117	Gag 118	Gag 119
	Gag Pool 12	Gag 120	Gag 121	Gag 122	Gag 123	Gag 124	Gag 125	Gag 126	Gag 127	Gag 128	Gag 129		
	Nef Pool 1	Nef 1	Nef 2	Nef 3	Nef 4	Nef 5	Nef 6	Nef 7	Nef 8	Nef 9	Nef 10	Nef 11	Nef 12
	Nef Pool 2	Nef 13	Nef 14	Nef 15	Nef 16	Nef 17	Nef 18	Nef 19	Nef 20	Nef 21	Nef 22	Nef 23	Nef 24
	Nef Pool 3	Nef 25	Nef 26	Nef 27	Nef 28	Nef 29	Nef 30	Nef 31	Nef 32	Nef 33	Nef 34	Nef 35	Nef 36
	Nef Pool 4	Nef 37	Nef 38	Nef 39	Nef 40	Nef 41	Nef 42	Nef 43	Nef 44	Nef 45	Nef 46	Nef 47	Nef 48
	Nef Pool 5	Nef 49	Nef 50	Nef 51	Nef 52	Nef 53							

The pooled peptides were arranged in 12 Gag pools and five Nef pools and 12 Matrix pools. Pools were tested in duplicate and matrices in single wells.

Table A2 ELISPOT plate layout for testing Consensus M peptides

	1	2	3	4	5	6	7	8	9	10	11	12
A	Gag Pool 1	Gag Pool 5	Gag Pool 9	Nef Pool 1	Nef Pool 5	Matrix 7	Cells+Media	QC Cells+Media				
B	Gag Pool 1	Gag Pool 5	Gag Pool 9	Nef Pool 1	Nef Pool 5	Matrix 8	Cells+Media	QC Cells+Media				
C	Gag Pool 2	Gag Pool 6	Gag Pool 10	Nef Pool 2	Matrix 1	Matrix 9	Cells+Media	QC Cells+Media				
D	Gag Pool 2	Gag Pool 6	Gag Pool 10	Nef Pool 2	Matrix 2	Matrix 10	Cells+Media	QC CEF				
E	Gag Pool 3	Gag Pool 7	Gag Pool 11	Nef Pool 3	Matrix 3	Matrix 11	CEF	QC CEF				
F	Gag Pool 3	Gag Pool 7	Gag Pool 11	Nef Pool 3	Matrix 4	Matrix 12	CEF	QC CEF				
G	Gag Pool 4	Gag Pool 8	Gag Pool 12	Nef Pool 3	Matrix 5	Media	PHA	QC PHA				
H	Gag Pool 4	Gag Pool 8	Gag Pool 12	Nef Pool 4	Matrix 6	Media	PHA	QC PHA				

			Reagent number	Initials		Start Time	End Time	Initials
PtID		Coating ab			Blocking	:	:	
		Secondary ab			O/N incubation	:	:	
Cells/well		Streptavidin			2nd Ab	:	:	
		NovaRed			Streptavidin	:	:	
QC ID		Blocking media			Nova Red	:	:	
		FCS			Plate Number			
		Coating date			Plate Read By		Date	

Comments _____

This table shows the ELISPOT layout utilised for those individuals screened for consensus group M Gag and Nef and HIV-specific T cell responses. Subsequent confirmatory assays were performed on single predicted reactive peptides.

Appendix A3. The relationship between infecting viral sequences and peptide amino acid sequences in a multiclade epidemic Cameroon and monoclade epidemic South Africa

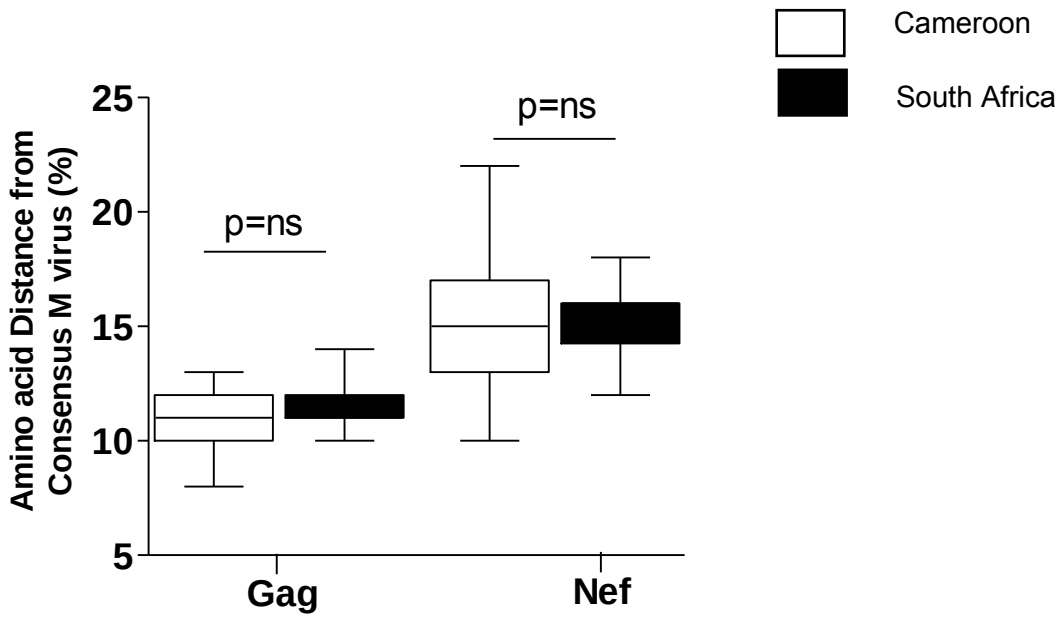

Figure A3 The relationship between infecting viral sequences and peptide amino acid sequences. Amino acid distances between infecting viral sequences and consensus M peptide sequences for Gag and Nef, shown for Cameroon (white bars) and South Africa (black bars).

Appendix A4 T cell responses to Gag and Nef consensus M reagents in a multiclade epidemic from Cameroon and monoclade epidemic from South Africa

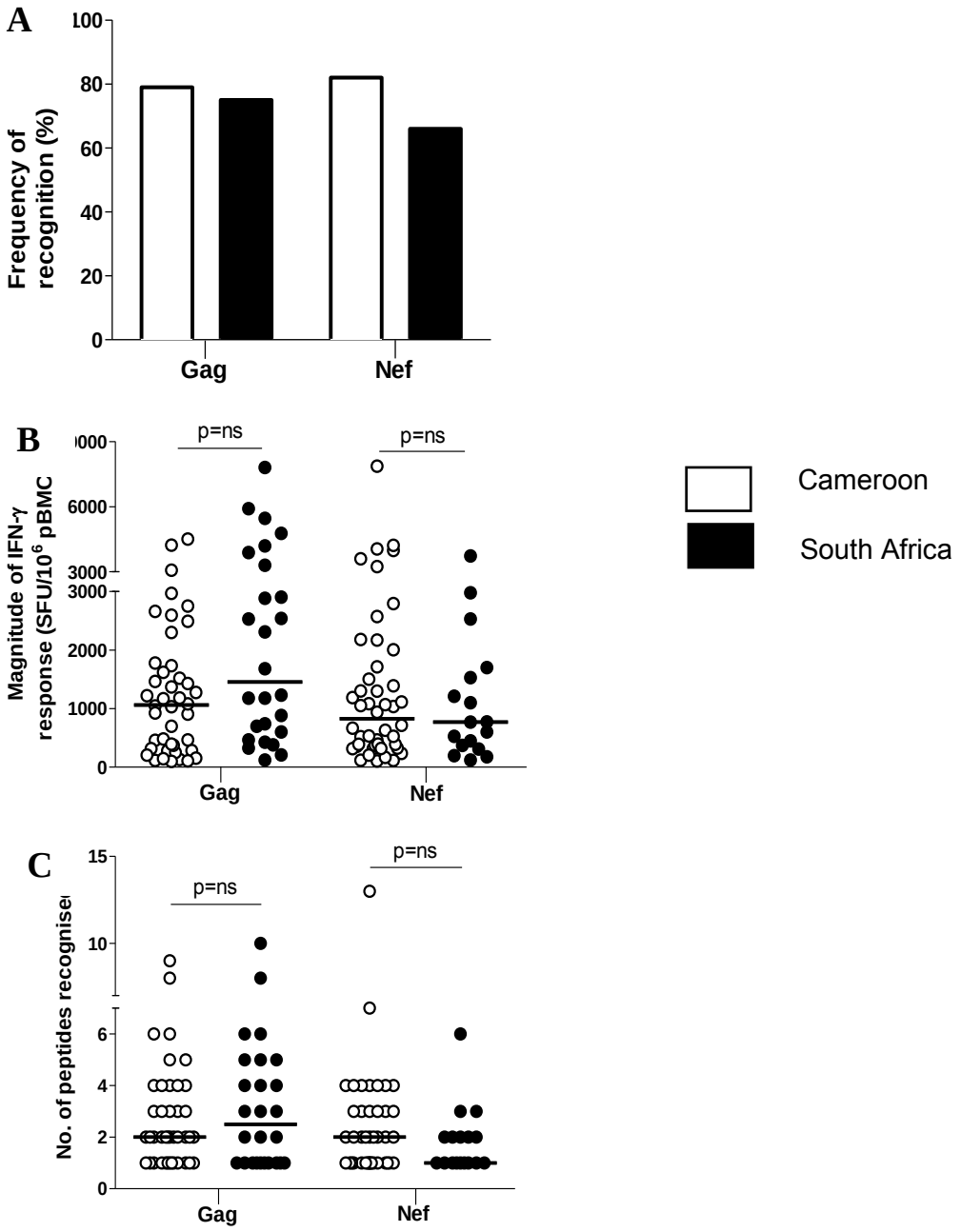

Figure A4 T cell responses to Gag and Nef consensus M reagents. HIV-specific T cells were detected in 57 individuals from Cameroon (white symbols) and 44 from South Africa (black symbols) using the IFN-γ ELISPOT assay. (A) The frequency of responders, (B) the total magnitude of responses, and (C) the breadth of responses to Consensus M Gag and Nef peptides in Cameroonian and South African infected individuals. The number of responding peptides represents the minimum response, after taking into consideration overlapping peptides, as described in the Methods.

Appendix B ELISPOT assay using PTE peptides

Table B1 Peptide pool configuration for the PTE peptide set in Gag

Pool #	Peptides in pool																															
1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
2	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64
3	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96
4	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128
5	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160
6	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192
7	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224
8	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256
9	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288
10	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320
11	1	2	3	4	33	34	35	36	65	66	67	97	98	99	129	130	131	161	162	163	193	194	195	225	226	227	257	258	259	289	290	291
12	5	6	7	8	37	38	39	68	69	70	100	101	102	132	133	134	164	165	166	196	197	198	228	229	230	260	261	262	292	293	294	295
13	9	10	11	12	40	41	42	71	72	73	103	104	105	135	136	137	167	168	169	199	200	201	231	232	233	263	264	265	296	297	298	299
14	13	14	15	16	43	44	45	74	75	76	106	107	108	138	139	140	170	171	172	202	203	204	234	235	236	266	267	268	300	301	302	303
15	17	18	19	20	46	47	48	77	78	79	109	110	111	141	142	143	173	174	175	205	206	207	237	238	239	269	270	271	304	305	306	307
16	21	22	23	24	49	50	51	80	81	82	112	113	114	144	145	146	176	177	178	208	209	210	240	241	242	272	273	274	308	309	310	311
17	25	26	27	28	52	53	54	83	84	85	115	116	117	147	148	149	179	180	181	211	212	213	243	244	245	275	276	277	312	313	314	315
18	29	30	31	32	55	56	57	86	87	88	118	119	120	150	151	152	182	183	184	214	215	216	246	247	248	278	279	280	316	317	318	319
19	58	59	60	61	89	90	91	92	121	122	123	124	153	154	155	156	185	186	187	188	217	218	219	220	249	250	251	252	281	282	283	320
20	62	63	64	93	94	95	96	125	126	127	128	157	158	159	160	189	190	191	192	221	222	223	224	253	254	255	256	284	285	286	287	288
21	1	5	9	13	33	37	62	68	71	74	93	103	106	109	138	141	144	173	176	179	208	211	214	243	246	249	278	281	284	289	292	320
22	2	6	10	14	38	58	63	65	72	75	94	104	107	110	139	142	145	174	177	180	209	212	215	244	247	250	279	282	285	290	293	296
23	3	7	11	34	39	43	64	66	73	76	100	111	112	143	146	147	178	181	182	213	216	217	248	251	253	257	283	286	297	300	304	308
24	4	8	12	15	35	40	44	46	67	69	77	101	105	108	140	148	153	175	183	189	205	210	218	240	252	254	275	280	287	309	312	316
25	16	17	29	36	41	45	55	70	78	80	89	97	102	113	132	135	149	167	170	185	206	219	221	241	245	255	258	276	288	291	294	317
26	18	21	25	42	47	59	81	83	90	95	98	114	118	125	129	133	136	164	171	184	199	202	220	234	237	256	259	260	277	295	298	318
27	19	22	26	30	48	49	56	60	84	91	96	99	115	126	130	134	137	168	172	186	203	207	222	225	228	242	261	263	266	301	313	319
28	20	23	27	31	50	52	57	61	79	85	92	119	121	127	131	150	157	161	165	190	196	200	204	226	235	238	262	264	267	299	310	314
29	24	28	32	51	53	82	86	116	120	122	128	151	154	158	162	166	169	187	193	197	201	223	227	229	231	239	265	268	269	302	305	315
30	54	87	88	117	123	124	152	155	156	159	160	163	188	191	192	194	195	198	224	230	232	233	236	270	271	272	273	274	303	306	307	311
31	1	10	23	32	38	40	61	73	74	77	87	108	110	128	146	149	159	170	184	186	207	220	221	225	229	232	257	272	277	290	292	314
32	2	7	15	25	36	37	42	69	71	76	88	105	107	111	145	150	154	172	185	190	194	196	222	237	242	245	269	273	284	315	316	320
33	3	6	31	41	43	54	56	67	68	72	90	106	114	123	139	141	147	175	187	191	195	210	223	226	239	255	260	263	267	310	313	317
34	4	11	22	39	46	52	59	75	79	80	86	101	103	117	130	155	158	161	171	177	203	206	214	228	250	256	265	276	286	289	303	318
35	5	12	21	34	47	53	60	78	82	83	93	100	104	127	140	142	152	176	180	188	193	204	216	230	231	252	264	266	288	291	293	319
36	8	9	16	28	33	44	63	81	85	91	109	115	120	121	134	135	160	174	178	183	199	215	217	227	235	253	258	262	270	295	297	311
37	13	17	24	35	45	48	57	65	92	96	102	112	116	125	136	144	151	163	165	182	202	205	224	233	243	251	259	261	282	296	300	312
38	14	19	27	49	55	58	66	84	94	97	118	124	131	132	137	153	164	169	181	192	197	209	211	236	238	246	268	281	287	298	304	309
39	18	26	29	50	62	70	95	99	113	119	122	129	138	143	156	166	167	173	189	198	201	208	213	234	240	244	275	279	283	299	301	306
40	20	30	51	64	89	98	126	133	148	157	162	168	179	200	212	218	219	241	247	248	249	254	271	274	278	280	285	294	302	305	307	308

Peptides were arranged in pools using the Deconvolution program (Mario Roederer, Vaccine Research Center, NIH). There were a total of 320 peptides arranged in 40 pools; each pool consisted of 32 peptides and each peptide appeared four times. Pools were tested singly.

Table B2 Peptide pool configuration for the PTE peptide set in Nef

Pool #	Peptides in pool																			
1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
2	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
3	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
4	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
5	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
6	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
7	121	122	123	124	125	126	127													
8	1	2	3	21	22	23	41	42	43	61	62	63	81	82	101	102	121	122		
9	4	5	6	24	25	26	44	45	46	64	65	83	84	103	104	123	124			
10	7	8	9	27	28	29	47	48	49	66	67	85	86	105	106	125	126			
11	10	11	12	30	31	32	50	51	52	68	69	87	88	107	108	127				
12	13	14	15	33	34	35	53	54	55	70	71	89	90	109	110					
13	16	17	18	36	37	38	56	57	58	72	73	91	92	111	112					
14	19	20	39	40	59	60	74	75	76	93	94	95	113	114	115					
15	77	78	79	80	96	97	98	99	100	116	117	118	119	120						
16	1	4	7	21	24	27	44	47	50	68	70	77	89	91	109	111				
17	2	5	8	22	25	30	45	48	51	66	69	78	90	92	110	112				
18	3	6	9	23	26	31	46	49	52	67	71	79	87	93	101	116				
19	10	13	19	28	32	33	41	56	59	61	64	72	83	94	105	117				
20	11	14	16	29	34	39	42	53	57	65	74	80	84	95	106	118	121	127		
21	12	15	20	35	36	40	43	54	58	62	73	85	88	96	103	113	125			
22	17	37	55	60	63	75	81	86	97	102	104	107	119	123	126					
23	18	38	76	82	98	99	100	108	114	115	120	122	124							

Peptides were arranged in pools using the Deconvolution program (Mario Roederer, Vaccine Research Center, NIH). There were a total of 120 peptides arranged in 23 pools; each pool consisted of seven to 20 peptides and each peptide appeared three times. Pools were tested singly.

Table B3 ELISPOT plate layout for PTE peptide set

	1	2	3	4	5	6	7	8	9	10	11	12
A	Gag Pool 1	Gag Pool 9	Gag Pool 17	Gag Pool 25	Gag Pool 33	Nef Pool 1	**Nef Pool 9**	Nef Pool 17	Media	QC Cells+Media		
B	Gag Pool 2	Gag Pool 10	Gag Pool 18	Gag Pool 26	Gag Pool 34	Nef Pool 2	**Nef Pool 10**	Nef Pool 18	**Cells+Medi**	QC Cells+Media		
C	Gag Pool 3	Gag Pool 11	Gag Pool 19	Gag Pool 27	Gag Pool 35	Nef Pool 3	**Nef Pool 11**	Neff Pool 19	**Cells+Medi**	QC Cells+Media		
D	Gag Pool 4	Gag Pool 12	Gag Pool 20	Gag Pool 28	Gag Pool 36	Nef Pool 4	**Nef Pool 12**	Nef Pool 20	**Cells+Medi**	QC Cells + CEF		
E	Gag Pool 5	Gag Pool 13	Gag Pool 21	Gag Pool 29	Gag Pool 37	Nef pool 5	**Nef Pool 13**	Nef Pool 21	**Cells + CEF**	QC Cells + CEF		
F	Gag Pool 6	Gag Pool 14	Gag Pool 22	Gag Pool 30	Gag Pool 38	Nef Pool 6	**Nef Pool 14**	Nef Pool 22	**Cells + CEF**	QC Cells + CEF		
G	Gag Pool 7	Gag Pool 15	Gag Pool 23	Gag Pool 31	Gag Pool 39	**Nef Pool 7**	**Nef Pool 15**	Nef Pool 23	**Cells + PHA**	QC Cells + PHA		
H	Gag Pool 8	Gag Pool 16	Gag Pool 24	Gag Pool 32	Gag Pool 40	**Nef Pool 8**	**Nef Pool 16**	Media	**Cells + PHA**	QC Cells + PHA		

			Reagent number	Initials		Start Time	End Time	Initials
PtID		Coating ab			Blocking	:	:	
		Secondary ab			O/N incubation	:	:	
Cells/well		Streptavidin			2nd Ab	:	:	
		NovaRed			Streptavidin	:	:	
QC ID		Blocking media			Nova Red	:	:	
		FCS			Plate Number			
		Coating date			Plate Read By		Date	

Comments _____

This table shows the ELISPOT layout utilised for those individuals screened for PTE Gag and Nef and HIV-specific T cell responses. Subsequent confirmatory assays were performed on single predicted reactive peptides.

Appendix B4. The correlation between the consensus and PTE ELISPOT responses

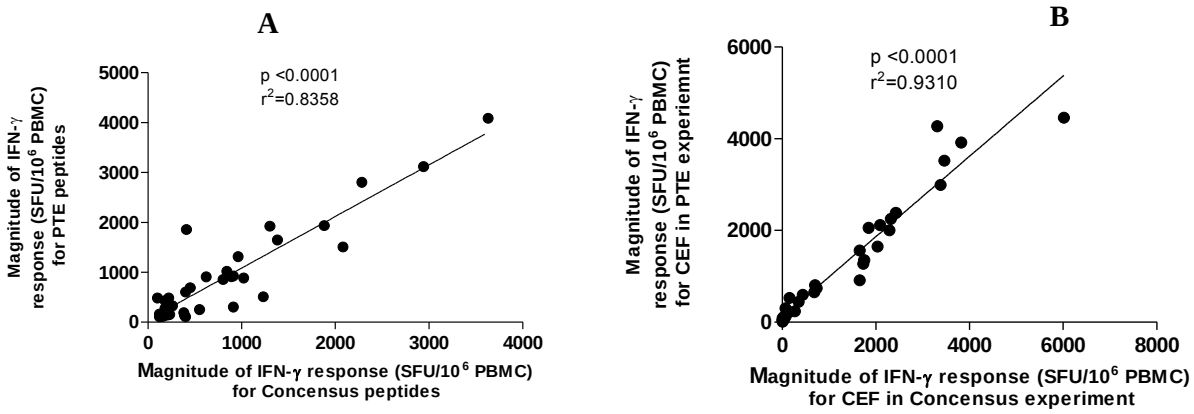

Figure B4 The correlation between the consensus and PTE ELISPOT responses when exactly matched peptides, or peptides shifted by one amino acid, between the two sets were compared PBMC (A) and when PBMC were stimulated with the CEF control peptide pool (B).