



# **Genetic and immunological characterization of new subtype G envelope expressing HIV-1 pseudoviruses**

A thesis submitted by

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Department of Clinical and Laboratory Sciences

Faculty of Health Sciences

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## **DECLARATION**

I, Essomba René Ghislain, hereby declare that this thesis is my own original work. It is being submitted for the degree of Doctor of Philosophy (PhD) at the University of Cape Town. It has not been submitted before for any degree or examination at this or any other University.

Signed by candidate

**Essomba René Ghislain**

**Cape Town, 14 February 2014**

*I dedicate this thesis*

*To the Almighty God,*

*To my wonderful parents, Atangana Joseph and Atangana Madeleine, I hope that this achievement will complete the dream that you had for me all those many years ago when you chose to give me the best education you could,*

*To the special creatures that came from me, Scott and Jane,*

*To my wife to be, Valentina*

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## LIST OF ABBREVIATIONS

µg	Microgram
µl	Microliter
µM	Micromolar
ADCC	Antibody Dependant Cell-mediated cytotoxicity
ADCVI	Antibody Dependant Cell mediated Viral Inhibition
AIDS	Acquired Immune Deficiency Syndrome
AnAbs	Autologous Neutralizing Antibodies
ARRRP	AIDS Research and Reference Reagent Program
BnAbs	Broadly Neutralizing Monoclonal Antibodies
CCR5	C-C Chemokine Receptor 5
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic Acid
CDR	Complimentarity Determining Regions
CDRH3	Complimentarity Determining Region 3
Cpx	Complex
CRFs	Circulating Recombinant Forms
CTL	Cytotoxic T-Lymphocyte
CXCR4	C-X-C Chemokine receptor 4
DEAE-dextran	Diethyleminoethyl-dextran
DMEM	Dulbecco's Modified Eagle's Media
DNA	Deoxyribonucleic Acid
<i>env</i>	envelope gene
Env	envelope glycoprotein
ER	Endoplasmic Reticulum
gp	Glycoprotein
gp120, gp41	Glycoprotein 120kda, 41kda
HAART	Highly Active Anti-Retroviral Therapy
HIV-1/HIV-2	Human Immunodeficiency Virus Type 1/2
IC <sub>50</sub>	50% Inhibitory Concentration
ID <sub>50</sub>	50% Inhibitory Dilution
IgG	Immunoglobulin
IQR	Inter-Quartile Range

LANL	Los Alamos National Library Database
LLP-1, LLP-2, LLP-3	Lentivirus Lytic Peptide -1, -2, -3
LTR	Long Terminal Repeat
mAb	Monoclonal antibody
MEGA	Molecular Evolutionary Genetic Analysis
mg	Milligram
MLV	Murine Leukemia Virus
MPER	Membrane Proximal External Region
MUSCLE	Multiple Sequence Comparison by Log-Expectation
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institute of Health
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NSI	Non –Syncytium-Inducing
°C	Degrees Celcius
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PNGS	Potential N-linked Glycosylation Site
RDP	Recombination detection Program
RLU	Relative Light Units
RNA	RiboNucleic Acid
RNase H	Ribonuclease H
SCD4	Soluble CD4
SGA	Single Genome Amplification
SHIV	Simian-Human Immunodeficiency Virus
SI	Syncytium-Inducing
SIV	Simian Immune Deficiency Syndrome
TM	Transmembrane
URFs	Unique Recombinant Forms
V1, V2, V3, V4, V5	Variable Regions 1-5
WebPSSSM	Web Position Specific Scoring Matrix

## AMINO ACID ABBREVIATIONS

<b>Amino Acid</b>	<b>Three letter code</b>	<b>One-Letter code</b>
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	

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# **ABSTRACT**

## **Background**

One of the greatest challenges for the development of an effective HIV-1 vaccine is the high genetic diversity of this pathogen and the complex escape mechanisms employed by the envelope gp120 and gp41 glycoprotein that form the envelope spike. An ideal vaccine would elicit the production of broadly neutralizing antibodies (nAb), capable of having potent activity against worldwide viral isolates. This thesis encompasses a series of studies on characterizing the genetic diversity of envelope genes of subtype G and the neutralization antibody responses to these viruses. HIV-1 subtype G accounts for 5% of HIV-1 infection worldwide, centered on West Africa, with spread to European countries, primarily Spain and Portugal, presumably with immigration of infected individuals. Small satellite subtype G epidemics have been documented in Cuba and among Russian intravenous drug users. In addition, 80% of the recombinant strains circulating in Cameroon contain segments attributed to subtype G. However, until recently, little research has been done on the neutralization sensitivity and vulnerabilities of subtype G viruses, particularly those that circulate in its main reservoir, Central and West Africa.

## **Methods and Objectives**

We studied plasma derived viruses from eight HIV-1 subtype G infected individuals. Five samples were collected for donation by the Yaoundé Central Hospital Blood Service from individuals who were subsequently found to be HIV-infected. Presumably, these donors were unaware of their HIV status. The remaining three samples were collected at the CIRCB Research Institute in Yaoundé during testing for antiretroviral resistance. These donors were failing therapy, and had sufficiently high viral loads that HIV-1 envelope clones could be isolated.

The objectives of my thesis were: 1) to examine the molecular and functional characteristics of the HIV-1 envelope glycoproteins of subtype G viral variants which is crucial to improving strategies to prevent transmission; 2) to evaluate the neutralization sensitivity subtype G viruses and the neutralizing capacities of antibodies induced by the viruses by determining the neutralization antibodies titers against autologous and heterologous HIV-1 viral isolates; 3) to

characterize the sensitivity of HIV-1 subtype G viral isolates against broadly neutralizing antibodies, to gain insight into the neutralization vulnerabilities of the subtype G viruses.

## **Results and Discussion**

Using the single genome amplification technique (SGA), we characterized 42 full length envelope genes (Chapter two) who had been typed subtype G for *gag* and *nef* or for *pol*. We found that all our envelope sequences clustered within the expected subtype G and were apparently not recombinants. The sequences from our donors were highly diverse within the subtype G node of sequences: Sequences from three individuals grouped with sequences predominantly from Nigeria with few from Spain and Cameroon, sequences from three other individuals fell into a cluster comprising only sequences from Cameroon, that themselves appears linked to a major cluster of most of the sequences collected in Europe and Cuba. Sequences from two individuals clustered from near the root of the subtype G subtree in a poorly populated branch with reference virus from Cameroon, Kenya, Russia, and CRF06\_cpx.

The median intra-patient genetic distance samples was high (> 1.5%) in 4 out of 8 subjects, including all three patients failing ART Therapy and low (<1.5%) in the naïve ART patients perhaps because subjects with higher genetic distance may be infected for a longer period. There was a variation in the number and location of Potential N-linked glycosylation sites (PNGS) as well as variable domain amino acid lengths between clones. In general, our subtype G clones exhibit a shorter V1V2 and V4 region sequences compared to the well characterized subtype B. All envelope clones exhibited CCR5 co-receptor usage as measured by inhibition of invasion of pseudoviruses by CCR5 binding inhibitor TAK-779. This is consistent with the predictions made by web PSSM and geno2pheno. This suggests that CCR5 inhibitors may be useful for treatment for subtype G-infected individuals. However, conclusions about CCR5-based treatment approaches in West and Central Africa will have to also account for more such data from other subtypes that are prevalent in that region,

In chapter three, we characterized the neutralization sensitivity of subtype G pseudoviruses to autologous neutralization (own plasma), and plasma pools. Of particular note, all four BS12-derived viruses exhibited substantial sensitivity to neutralization by their own plasma (geometric mean ID<sub>50</sub>= 564). Autologous neutralization from a contemporaneous sample is rarely seen, and its significance in study participant BS12 is unclear. We cannot measure virus

and anti-HIV-1 antibody evolution in this donor because this donor was an anonymous blood bank donor and no subsequent samples can be collected.

We next evaluated the ability of subtype G plasma to neutralize a panel of 14 HIV-1 *env* pseudoviruses comprising representatives of subtypes A, B, C, and CRF02\_AG which were from tier 2 (moderately resistant viruses) to tier 3 (highly resistant viruses). Three of the samples were from patients undergoing testing for antiretroviral resistance, and thus may have antiretroviral drugs in their blood plasma despite having detectable viral loads. However, plasma from two of these three patients failed to neutralize negative control pseudovirus (Murine Leukemia Virus envelope, MLV) or most other viruses tested, suggesting that levels of antiretroviral drugs in these two samples were too low to interfere with the neutralization assay. It may be that these donors were not taking their antiretroviral. One donor may have antiretroviral drugs in the plasma, and was excluded from the analysis. Blood plasma from two out of the remaining seven study participants, including BS12, neutralized more than 50% of panel virus tested at ID<sub>50</sub>>50, indicating at least limited neutralization breadth in these two samples. Interestingly, BS12 serum, the samples that showed a substantial potent autologous activity, also neutralized 3/5 of the tier 3 isolates from the panel viruses (278-50 and 257-31 not neutralized; 33-7, 253-11, and PV0.4 neutralized). Mapping of the epitope targeted by neutralizing antibodies in BS12 plasma indicated that the dominant neutralizing antibody for virus CAP45.2.00.G3 in sample BS12 targeted the PG9/16 site. This site is the target in the V2 and V3 regions of the mAbs PG9 and PG16. However, these viruses were not unusually sensitive to the PG9 and PG16 monoclonal antibodies themselves. BS12 plasma also exhibited substantial neutralization of an HIV-2 chimeric virus displaying the MPER of 253-11, a CRF02\_AG virus, although we did not examine if these anti-MPER antibodies are capable of neutralizing HIV-1 viruses by recognizing MPER.

We further characterized (chapter four) the susceptibility of these viruses to neutralization by a panel of eleven (11) first and second-generation of broadly neutralizing monoclonal antibodies, and three HIV-1 entry inhibitors: TAK-779, a CCR5 inhibitor, soluble CD4 (sCD4), and T20, a fusion inhibitor. We found that subtype G viruses were highly sensitive to the CD4-binding site mAbs NIH45-46<sup>G54W</sup> and VRC01 as well as MPER mAb 10E8, neutralizing all (100%) viral variants. In addition, subtype G viruses showed sensitivity to 4E10 which was able to neutralized 25 of 27 (92%) of viruses and median IC<sub>50</sub> of 0.42ug/ml. These viruses were more sensitive to 4E10 than a large panel of worldwide viruses published

last year ( $IC_{50}=1.93\mu\text{g/ml}$ ) and of all subtype G viruses with published sensitivity to 4E10 (median  $IC_{50} =17\mu\text{g/ml}$ ). However, neutralization by the mAbs B12, 2G12, Z13e1 was generally poor, while 2F5, PG16, PG9 and VRC03 have moderate activity neutralizing 42%, 65%, 27%, and 50% of viral variants respectively. Subtype G viruses were highly sensitive to TAK-779 and T20 but exhibit more variable sensitivity to sCD4, a phenotype that have been observed frequently among freshly isolated viruses because sensitivity to sCD4 protein is thought to be associated with exposure of the CD4 binding site.

## **Conclusion**

Although there are more than 100 subtype G *env* sequences in the database, some of our sequences showed relatively poor similarity to them. This suggests that the full extent of the diversity of Subtype G has not yet been described, and provides additional evidence for the hypothesis that subtype G itself may be very old. Within patient diversity corresponded to other measures of time since infection. One sample, BS12, was unusual in that the plasma sample itself neutralized viruses derived from sequences found in the same sample. Despite the mapping hit in BS12 plasma for PG9/PG16 site antibodies against CAP45.2.00.G3, BS12 derived viruses were not highly sensitive to neutralization against PG9 and PG16. Moreover, these viruses were not more sensitive to PG9 and PG16 compared to other viruses. This group of subtype G viruses could also be useful for screening T20 and CCR5 inhibitors that are being tested as potential microbicides in regions where HIV-1 subtype G predominate. In that regard, the relative sensitivity of the viruses examined here to T20 and CCR5 inhibitors is encouraging, because freshly isolated viruses will be a critical target for such interventions. Last, our characterization of these viruses added detailed information about subtype G, which is historically understudied.

**This study has contributed to the following conference papers:**

- 1- **E Rene Ghislain**, M Tongo, A Fatima, E Ngolle, W Burgers and J Dorfman  
Genetic characterization of HIV-1 subtype G envelope sequences by single genome analysis  
*Retrovirology* 2012, 9 (Suppl. 2): P152
  
- 2- **Essomba Rene Ghislain**, M Tongo, A Fatima, E Ngolle, W Burgers and J Dorfman  
Immunological characterization of subtype G expressing viruses  
Federation of African Immunology Society (FAIS), and South African Immunology Society (SAIS) conference 2012, Durban, South Africa (Poster)
  
- 3- **Essomba Rene Ghislain**, M Tongo, A Fatima, E Ngolle, W Burgers and J Dorfman  
HIV-1 subtype G envelope variants have similar sensitivity to new generation of anti-CD4-binding site and anti-MPER broadly neutralizing monoclonal antibodies, but sensitivity to inhibitors of viral entry varies  
HIV Vaccine 2014, Keystone symposia, Calgary, Canada (Poster).

# **CHAPTER 1:**

## **INTRODUCTION**

## **1. 1 Background**

### **1.1.1 HIV-1 and the global AIDS pandemic**

The first cases of patients with the acquired immunodeficiency syndrome (AIDS) were reported in 1981 in the United States following the observation of opportunistic infections that usually occur in strongly immune compromised individuals (Hymes et al., 1981). AIDS is caused by infection with Human Immunodeficiency Virus.1 (HIV-1) (Barre-Sinoussi et al., 1983), and also by HIV-2 (Clavel et al., 1986). AIDS is characterized by a failure of the immune system permitting the emergence of any of a variety of opportunistic infections that may kill the patient. The transmission of HIV can occur through sexual contact by unprotected vaginal, oral or anal intercourse with HIV-1 infected individuals (CDC, 2003), through blood transfusion or exposure to HIV-contaminated needles, syringes, and other equipment (CDC, 2003), or through mother-to-child transmission (MTCT) during pregnancy, labor and delivery, or breastfeeding (McGowan and Shah, 2000).

HIV infects dendritic cells, macrophages, and CD4<sup>+</sup> T cells of the immune system (Alimonti et al., 2003). During HIV infection, the number of CD4 T cells progressively declines. When the CD4 count falls below 200/mm<sup>3</sup>, the person infected with HIV become vulnerable to opportunistic infections caused by bacteria, viruses, fungi and parasites that are normally controlled by the immune system (Holmes et al., 2003) .

Since the discovery in 1983 by the team of Françoise Barré-Sinoussi and Luc Montagnier (Pasteur Institute, Paris, and all the two Nobel Prize for Medicine 2008) of the human immunodeficiency virus of type I (HIV-1), the scientific community has been mobilized to implement a large number of research programs to fight against that disease. Despite significant scientific progress such as the development of reliable diagnostic tests, sequencing of thousands of viral genomes and the development of highly active antiretroviral therapy (HAART), HIV-1 has continued to spread largely unabated. Importantly, the global prevalence of HIV infection seems to have been stable since around the end of the 20<sup>th</sup> century (figure 1.1).

In 2011, UNAIDS and WHO estimated that there were approximately 34 million people living with HIV with over 2.5 million people who became infected with HIV-1 and 1.7 million people who died from HIV (WHO, 2013). The majority of people living with HIV live in developing countries. Sub-Saharan Africa supports the greater part of the burden of the global epidemic, accounting for 69% of the people living with HIV worldwide (figure 1.2). In South and South East Asia, the spread is high with almost 5 million people estimated living

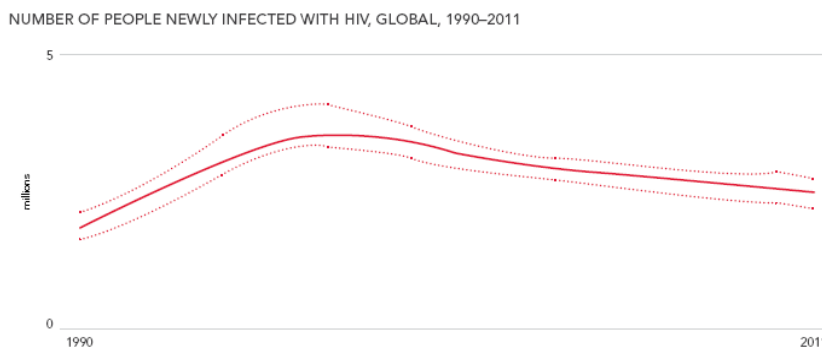
with the disease. After Sub-Saharan Africa, regions most heavily affected are the Caribbean, Eastern Europe and Central Asia, where 1% of adults were living with HIV in 2011(UNAIDS, 2012).

#### **1.1.1.1 HIV in Sub-Saharan Africa**

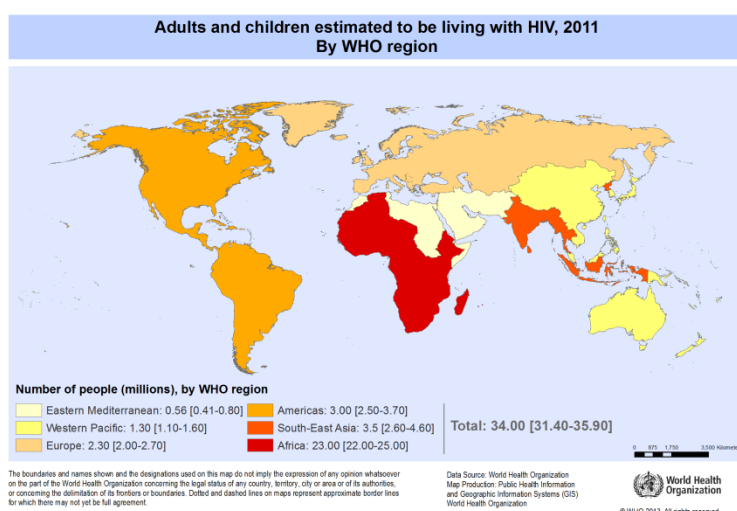
In Africa, Sub-Saharan Africa is the most affected region in the global AIDS epidemic with 25% of new infections in 2011 and 70% of all deaths due to AIDS (WHO, 2013). In Southern Africa, the prevalence of HIV infection in the general population exceeds 15% and South Africa alone has more than 10% of the general population infected with HIV.

In Cameroon, the HIV prevalence rate is estimated at 5.1%, the highest rate for the West and Central Africa Sub-region (NACC/CTG, 2010). The prevalence is higher in urban areas than in rural areas and prevalence rates vary from one province to another with highest rates in the North-Western, Eastern provinces, and Yaoundé (figure 1.3). HIV transmission is mainly heterosexual, and women are more vulnerable. The most vulnerable groups include sex workers, truck drivers, mobile populations and military personnel (NACC/CTG, 2010).

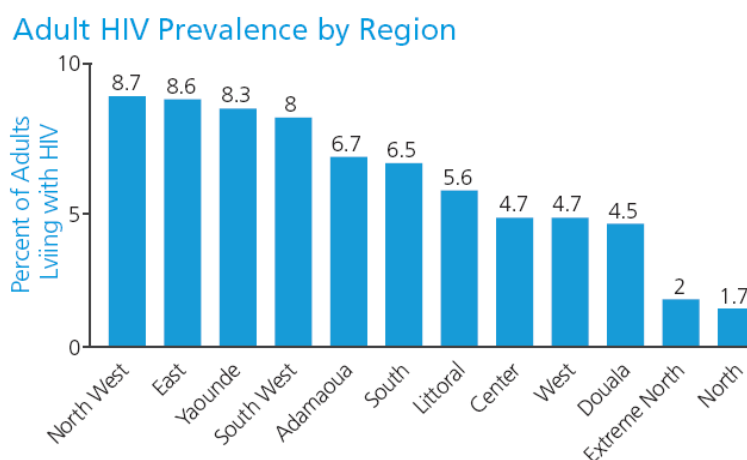
The development of antiretroviral drugs targeting the viral enzymes reverse transcriptase and protease, the use of combination of inhibitors targeting these enzymes, and the development of new antiretroviral agents targeting not only these two enzymes but novel targets as well helped to significantly improve the conditions of life of the HIV-infected individuals, and especially to increase their life expectancy (Reeves and Piefer, 2005). However, due to poverty-related barriers, access to antiretroviral therapy remains limited in Africa and an effective vaccine is hoped to be a practical and cost effective intervention to control the spread of the HIV/AIDS pandemic.



**Figure1.1: Estimated number of people living with HIV globally during 1990-2011.** (Figure from UNAIDS, 2011)



**Figure1.2: Estimated number of adults and children to be living with HIV in 2011.** (Figure from WHO, 2013)

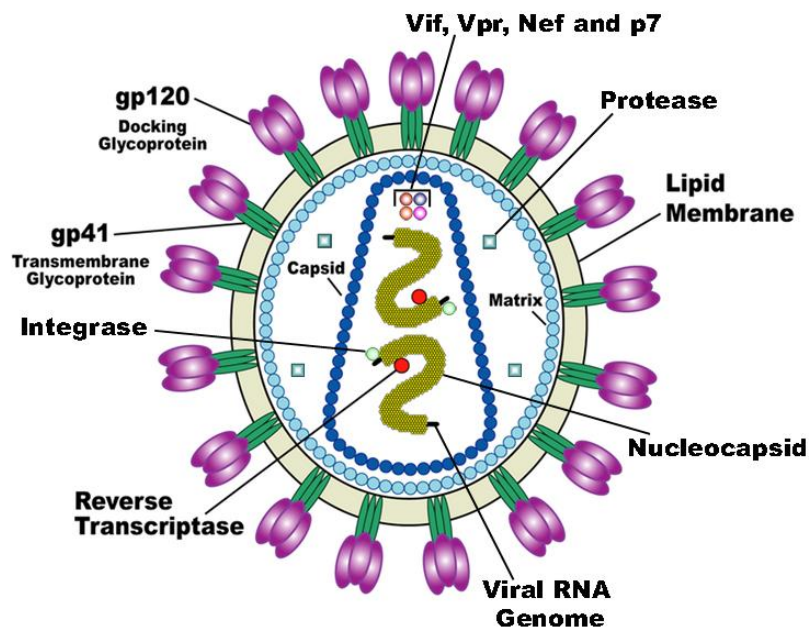


**Figure1.3: Adult HIV prevalence by region in Cameroon.** (Figure from NACC/CTG, 2010)

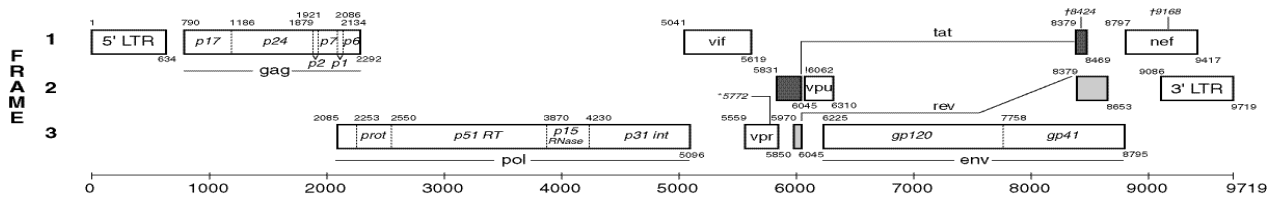
## 1.1.2 HIV-1 structure, genome organisation and replication cycle

### 1.1.2.1 Viral structure

HIV-1 is a lentivirus belonging to the retroviridae family. A common feature of all retroviruses is that they synthesize DNA from their RNA genome via the reverse transcriptase enzyme. The mature HIV virion, as illustrated in figure 1.4, is roughly spherical with a diameter of around 120 nanometer (nm). The virion is composed of two identical copies of positive single-strand RNA together with the reverse transcriptase (RT), integrase (IN), RNase H and protease (PR) enzymes encapsulated within the viral core (CA; p24), which is enclosed within the matrix (MA; p17) (Leis, Baltimore et al. 1988). The HIV virion is enveloped by a lipid bilayer that is derived from the membranes of the host cell, into which the gp41 is embedded, which noncovalently anchors the gp120 in a trimer of heterodimers.



**Figure 1.4 : Schematic representation of a mature HIV-1 virion illustrating major viral components.** Figure from [http://commons.wikimedia.org/wiki/File:HIV\\_Virion.en.png](http://commons.wikimedia.org/wiki/File:HIV_Virion.en.png) (active April 2009).



**Figure 1.5: Organization and landmarks of the HIV-1 DNA genome.** Open reading frames are shown as rectangles. The gene start is indicated by number in the upper left corner of each rectangle (ATG start codon). The number in the lower right indicates the position of the stop codon. The *tat* and *rev* spliced exons are shown in dark grey and grey, respectively. The numbering positions in relative to HXB2 strain. (Figure from Los Alamos National Library <http://www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html>).

### 1.1.2.2 Genome organisation

HIV-1 virus consists of three structural genes: *gag*, *pol*, and *env* with flanking long terminal repeat (LTR) sequences at each end of the genome. In addition, HIV possesses regulatory genes (*tat*, *rev*), and accessory genes (*vif*, *vpr*, *vpu*, and *nef*). The organization and landmarks of HIV-1 genome is shown in figure 1.5.

The *gag* codes for the internal structural proteins of the virus: matrix (MA, p17), capsid (CA, p24), and nucleocapsid (NC, p7). *pol* codes for the viral enzymes: reverse transcriptase (RT), which contains both DNA polymerase and associated ribonuclease H (RNase H) activity, integrase (IN), and protease (PR) (Coffin, 1990). *env* codes for viral envelope glycoproteins as a precursor (gp160), which is then processed to a surface glycoprotein, gp120 and a trans-membrane glycoprotein, gp41. The mature gp120.gp41 proteins are bound by non-covalent interactions and are associated as a trimer on the surface of virions. The envelope (Env) protein is responsible for recognition of cellular receptors and viral entry into cells.

The regulatory genes are *tat* and *rev*. They modulate transcriptional and post-transcriptional steps of virus gene expression and are essential for virus propagation. *Tat* acts by binding to the trans-activation response (TAR) RNA element and activates transcription initiation and elongation from the LTR promoter (Roy et al., 1990). *Rev* acts by binding to Rev response element (RRE) and promotes the nuclear export, stabilization, and utilization of the viral mRNAs containing RRE (Pollard and Malim, 1998). RRE is located at position 7709-8063 within the Env coding region of HIV-1 (Cullen, 2003).

The accessory genes are *vif*, *vpr*, *vpu*, and *nef*. They are called accessory genes because they not always required for viral infection in in vitro cell culture systems. Instead, these proteins

enable pathogenesis *in vivo* by allowing viruses to evade antiviral responses (Collins and Collins, 2014).

Virion infectivity factor (*Vif*) a 23 kDa accessory protein found in HIV-1 and other retroviruses is essential for viral replication either *in vivo* or in culture for nonpermissive cells such as peripheral blood lymphoid cells, macrophages, and H9 T cells (Zhang et al., 2000). *Vif* inactivates the antiretroviral activity of the host APOBEC3 (Apo-lipoprotein B mRNA-editing enzyme, catalytic polypeptide) cytidine deaminases, including APOBEC3G (A3G) and A3F (Chiu and Greene, 2008, Goila-Gaur and Strebel, 2008). APOBEC3G is a human enzyme encoded by the APOBEC3G gene that belongs to the APOBEC superfamily of proteins (Sheehy et al., 2002). This family of protein has been suggested to play an important role in innate anti-viral immunity (Takaori, 2005). A3G (Sheehy et al., 2002) and related human APOBEC3 proteins are potent inhibitors of HIV-1 in the absence of viral *Vif*. APOBEC3G exerts its antiviral effect during reverse transcription to trigger G-to-A hypermutation in the nascent retroviral DNA (Mangeat et al., 2003). *Vif* hijacks the cellular Cullin5 E3 ubiquitin ligase, which is composed of ElonginB, ElonginC, Cullin5, and Rbx2 (Stanley et al., 2008), in order to target APOBEC3G for degradation.

Viral protein R (Vpr) is a 14 kDa protein. Vpr plays an important role in regulating nuclear import of the HIV-1 pre-integration complex, and is required for virus replication in non-dividing cells such as macrophages (Miller and Sarver, 1997). Vpr also accelerates the production of HIV proteins by arresting infected cells at the G2 phase of the cell cycle, inhibiting cell division by mitosis (Kim et al., 2012). Viral protein U (Vpu) is a 17kDa protein which is not present in HIV-2, is involved in the assembly of new virus particles and facilitates budding (Deora and Ratner, 2001). The formation of Env-CD4 complexes interferes with viral assembly. Vpu down-modulates CD4 in the endoplasmic reticulum therefore reducing the likelihood of superinfection and is also involved in Env maturation. This reduces the formation of the Env-CD4 complexes (Estrabaud et al., 2007).

*Nef* is a small 27–35 kDa myristoylated protein encoded in the genomes of primate lentiviruses (HIV-1, HIV-2 and SIV). *Nef* localizes primarily to the cytoplasm of infected cells and is partially recruited to cellular membranes and expressed from the earliest stage of viral gene expression (Fackler and Baur, 2002). *Nef* reduces cell surface expression of receptors, including CD4, the primary receptor for HIV and SIV and MHC class I and class II complex, facilitating HIV immune evasion and thus increases viral pathogenesis (Fackler and Baur, 2002). *Nef* retards HIV replication by downregulation of transcription factors natural

factor kappa B (NF- $\kappa$ B) and activator protein one (AP-1) (Abraham et al., 2012). *Nef* also induces downregulation of CD4, the primary receptor for HIV and SIV and MHC class I and class II complex which impairs T cell function, thereby facilitating HIV immune evasion and thus increases viral pathogenesis (Fackler and Baur, 2002).

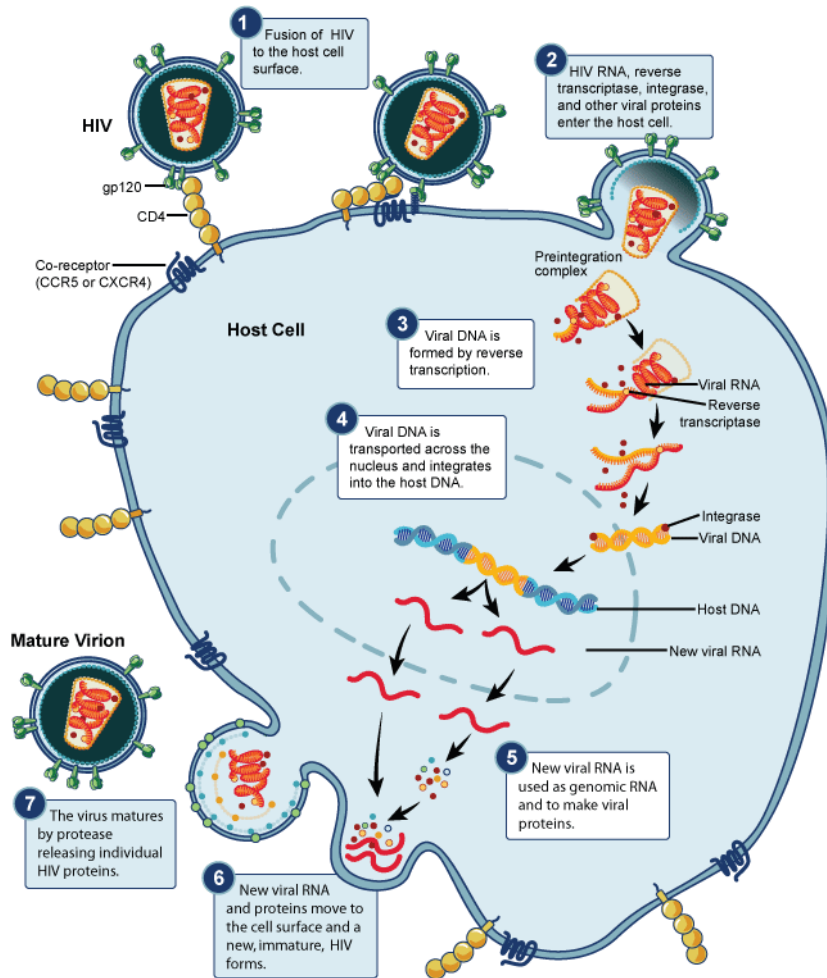
Vpx is found in HIV-2, but not in HIV-1. This accessory gene is a homolog of HIV-1 *vpr*, both are incorporated into virions at levels comparable to gag proteins through interactions with Gag p6. Vpx enhances HIV-2 replication in humans by counteracting the host factor SAMHD1 (SAM domain and HD domain-containing protein 1) (Laguet et al., 2012). Vpx-mediated degradation of SAMHD1 therefore decreases deoxynucleoside triphosphate hydrolysis, thereby increasing the availability of dNTPs for viral reverse transcription in the cytoplasm. Vpx is also involved in the nuclear import of the HIV-2/SIV genomes and associated proteins (Bouzar et al., 2003), but the specific mechanisms and interactions are currently unknown.

### **1.1.2.3 Replication cycle**

The life cycle of HIV can be described in different phases. Figure 1.6 graphically depicts the events described below:

- **Virus entry (binding and fusion)**

Entry is the first step in the process of HIV infection and requires binding of HIV gp120 to host cells expressing CD4 with extremely high affinity (Hladik and McElrath, 2008). This results in conformational changes in gp120, which then exposes co-receptor binding sites. The co-receptors required for entry of HIV-1 are CCR5 and CXCR4, depending on the viral tropism. Immediately following gp120 and co-receptor binding, further conformational changes take place in gp41, which allows it to expose of the fusion peptide. The fusion peptide is inserted into the target cell membrane.



**Figure 1.6: Schematic representation of the life cycle of HIV.** (Figure from <http://www.niaid.nih.gov/topics/HIVAIDS/Understanding/Biology/pages/hivreplicationcycle.aspx>)

- **Reverse transcription**

Following membrane fusion the virus core enters and uncoats into the cytoplasm of the target cell. The virus RNA genomes are converted into double-stranded DNA. Reverse transcriptase has two distinct enzymatic activities: it is a DNA polymerase capable of copying either RNA or a DNA template into a complementary DNA sequence; and it is an RNase H, capable of degrading the RNA strand of an RNA.DNA duplex into small pieces once it has been used as a template for the first DNA strand (Arnold and Sarafianos, 2008). The reverse transcription is initiated and viral RNA is converted into proviral DNA by the action of reverse transcriptase, followed by the generation of a DNA/RNA hybrid. The template RNA is degraded by the RNase H activity, generating a DNA fragment known as the minus-strand

strong stop DNA (-sssDNA). The identical sequences known as the repeated (R) sequences mediated the -sssDNA jumps from 5' to the 3' end of the genome. This step is referred to as the first strand transfer. Minus-strand DNA synthesis occurs, using 3' end of the -sssDNA as a primer, accompanied by RNase H digestion of the template strand. This degradation is not complete, because the RNA genome contains a short polypurine tract (PPT) that is relatively resistant to RNase H degradation. The PPT serves as a primer for plus-strand DNA synthesis. A central PPT serves as an additional primer for plus strand DNA synthesis. The tRNA bound to the PBS is removed by RNase H, allowing a second-strand transfer of plus-strand strong stop DNA (+sssDNA) to take place between PBS sequences. Elongation continues along both DNA strands. A central termination signal (CTS) located at 3' of the central PPT allows termination of plus strand DNA synthesis in the center, resulting in the formation of a DNA flap. The final product is a double stranded (ds) HIV-1 proviral DNA that contains the long terminal repeats (LTRs), U3-R-U5 sequences, at both end. This entire process utilizes only the virion-associated RT without host enzyme.

- **Integration**

The integration of HIV DNA into the host DNA is a critical step in the HIV life cycle. HIV's enzyme for inserting the DNA version of its genome into the host cell DNA is called integrase (IN). HIV-1 integrase catalyzes the "cut-and-paste" action of clipping the host DNA and joining the proviral genome to the clipped ends. The first step of the integration process occurs in the cytoplasm of the host cell following the completion of reverse transcription of the HIV RNA into cDNA. This step involves the binding of integrase most likely in the dimer form to each end of the newly formed HIV cDNA. The integration process begins when IN clips off several nucleotides from the 3' termini of both strands of linear viral DNA. This reaction, known as 3'-end processing, generates a molecule of DNA with 3'-recessed ends. The preintegration complex is transported into the nucleus of the host cell, entering through one of the nuclear pore complexes. This nuclear translocation occurs despite the preintegration complex having a size that is more than twice the size of the central channel of the nuclear pore complex. In the nucleus, IN makes a staggered cleavage in the cellular target DNA. The 3'-recessed ends of viral DNA formed in the 3'-end processing reaction are joined to the ends of the cleaved cellular DNA. This reaction is known as strand transfer. The sites for integration into cellular DNA are random. The integration process is completed when cellular repair enzymes fill in the gaps between the integrated viral DNA and the host target DNA.

- **Transcription**

Regulation of HIV gene expression involves a complex interplay between chromatin-associated proviral DNA, cellular transcription factors and the viral encoded trans-activator of transcription, Tat. The process of viral transcription can be divided into two distinct phases (Wu, 2004). The first phase occurs early in transcription and is mediated by direct interaction between cellular transcription factors and cis-acting elements located in the HIV promoter region. The second phase immediately follows the first one, and relies on the accumulation of sufficient amounts of Tat from the first phase (Jordan et al., 2003) . Following integration, the HIV promoter is under the control of local chromatin environment, which determines the basal transcriptional activity. Independent of the site of integration, HIV 5' LTR is assembled into three unique nucleosomes: nuc-0, -1 and -2. Nuc-1 is positioned immediately downstream of the transcription start site (Steger and Workman, 1997), and is rapidly disrupted upon transcriptional activation of the HIV-1 promoter. Interestingly, the region between nuc-0 and 1 appears to remain nucleosome-free although it is large enough to accommodate an additional nucleosome. The viral core or basal promoter (nt -78 to -1) contains a TATAA box and three consensus SP1 binding sites. The enhancer (nt -105 to -79) carries a duplication of the 10-bp NF- $\kappa$ B binding sites. Regions upstream from the NF- $\kappa$ B sites also influence viral gene expression and are designated the modulatory region (-454 to -104). This region has been proposed to contain a negative regulatory element (NRE). Sequences near the RNA initiation site also contain regulatory elements such as the putative inducer of short transcripts (IST) (Sheldon et al., 1993, Ratnasabapathy et al., 1990), the initiator and the trans-activation response (TAR) element (nt +1 to +60) which interacts with Tat and plays an important role in Tat mediated trans-activation. In the absence of Tat and cellular stimulation, the nucleosome packed LTR is almost silent. Low levels of transcription are mediated by available cellular transcription factors. Efficient activation of the LTR promoter is largely driven by Tat, and is concomitant with an acetylation-dependent rearrangement of the nucleosome positioned at the viral transcription start site (Jordan et al., 2003, Jordan et al., 2001). Additionally, Tat appears to be able to directly interact with some transcription factors such as Sp1 (Jeang et al., 1993) to promote transcription. One unique feature of Tat mediated trans-activation is the ability of Tat to interact with RNA rather than with DNA (Berkhout et al., 1989). This interaction occurs specifically between Tat and a specific 59-residue stem-loop structure, TAR, on the RNA leader sequence. In general, the current model suggests that Tat causes a dramatic increase in transcriptional levels upon binding to TAR. Successful transcription leads to the generation of approximately 30 different

viral transcripts from the provirus. All these transcripts are derived from a single full-length transcript by alternative splicing, which generates mRNA with common 5' and 3' ends. The spliced viral RNA can be grouped into three classes: the multiply spliced mRNA encoding early regulatory proteins such as Tat, Nef and Rev; the singly spliced mRNA encoding Vpu, Vpr, Vif and Env; the un-spliced, full-length mRNA encoding the Gag-Pol poly protein. HIV gene expression is also regulated at a second level by the nuclear export of intron-containing transcripts. This process is mediated by the viral encoded Rev protein. Specific interaction between REV and RRE permits nuclear export of incompletely spliced viral transcripts in infected cells (Malim et al., 1989).

- **Viral assembly, maturation and release**

The HIV Env glycoprotein is synthesized in the rough endoplasmic reticulum (ER) to generate the Env precursor protein, gp160 and then transported to the golgi complex, where it is cleaved by a host protease (furin) into gp120 and gp41. The Env protein is incorporated in the plasma membrane of the cell, while Gag and Gag-Pol are assembling into capsids in the cytoplasm. The viral RNA genomes bound by p7 Gag product form a nucleoprotein complex. The newly formed nucleoprotein particle migrates to the plasma membrane at the site of Env insertion, since each particle has two viral RNA with associated Gag and Gag-Pol precursors. Immature virus particles then are assembled at the plasma membrane and are released by budding through the plasma membrane, acquiring a portion of the plasma membrane that contains gp41 and gp120. Pol, the protease, and the Gag proteins are generated by proteolytic cleavage of the precursor polypeptides upon release of the particles from the cell, thereby producing mature virus particles. HIV-1 budding and release are essential for spreading viral infection, and it is therefore not surprising that innate immune pathways have evolved to interfere with these processes. It is now well established that the antiviral protein tetherin blocks HIV-1 dissemination by tethering newly budded viral particles to the cell surface (Neil et al., 2008). In the absence of Vpu, and the presence of tetherin, HIV-1 particles are assembled normally, their lipid envelopes undergo endosomal sorting complexes required for transport (ESCRT)-protein-mediated fission from the plasma membrane, and they adopt a mature morphology. However, tetherin causes virions to remain trapped at the surface of the infected cell from which they are derived and to accumulate thereafter in endosomes following internalization (Neil et al., 2006). The HIV-1 Vpu protein is used by HIV-1 strains as an antagonist of tetherin by causing cytoplasmic tail domain-dependent sequestration into internal compartment and causing downregulation and degradation in only a subset of cell

types, even though all cells tested support Vpu activity (Neil et al., 2008, Van Damme et al., 2008).

## **1.2 HIV envelope glycoproteins**

HIV-1 Env glycoproteins are assembled as Env spikes. They are responsible for interacting with cellular receptors and initiating the fusion of the viral and cell membranes, and therefore are a potential target for drugs aimed at blocking the first step of the viral replication cycle. Furthermore, HIV-1 Env spikes are the only viral target available for neutralizing antibodies. The functional envelope spike consists of a trimer of heterodimers formed by two glycoproteins, gp120 (the exterior envelope glycoprotein) and gp41 (the transmembrane glycoprotein). Three gp120 molecules interact non-covalently with three gp41 units forming an oligomer, where the trimeric structure is maintained by the interactions between the gp41 domains.

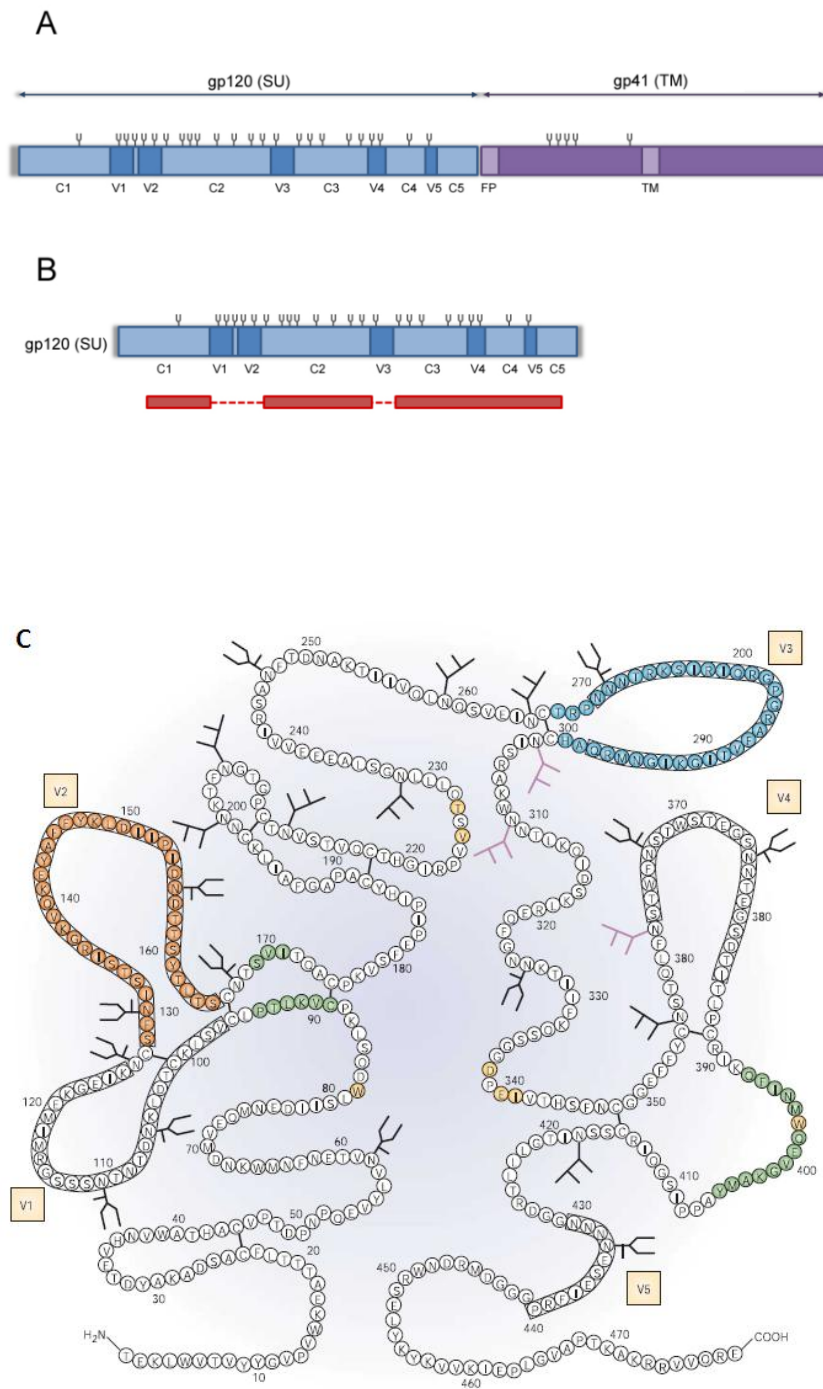
### **1.2.1 The gp120 molecule**

#### **1.2.1.1 Structural domains of gp120**

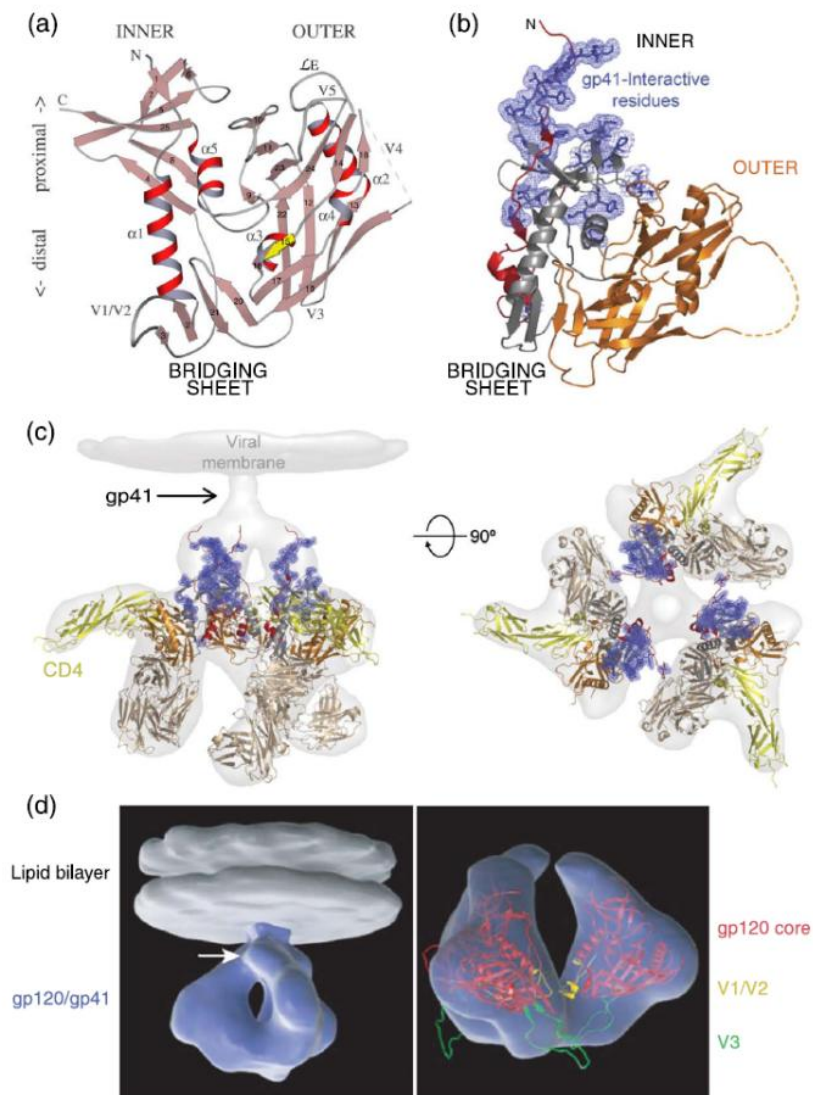
The gp120 molecule (figure 1.7) consists of five relatively conserved regions (C1.C5), interspersed between five variable regions (V1.V5), which are bracketed by cysteine forming disulfide bonds (Leonard et al., 1990). Gp120 is a highly glycosylated protein with half of its mass being N-linked glycans (Allan et al., 1985) with a small proportion being O-linked sugars (Bernstein et al., 1994). X-ray crystallography has solved a certain number of gp120 structures (Checkley et al., 2011). To obtain crystal structures that diffracted with sufficient resolution, HIV-1 and SIV gp120s have been deglycosylated and the N. and C terminals, V1/V2 and V3 regions deleted, to generate what is commonly referred to as the “gp120 core” (Kwong et al., 1998). The final deglycosylated  $\Delta V3$ ,  $\Delta V1.V2$  gp120 core retains 67% of the envelope amino acid content of the full-length molecule and has a molecular weight of 35 kDa. The gp120 core is composed of three general areas: the inner domain, the outer domain, and the bridging sheet (figure 1.8). The inner domain is formed mainly by the C1 and C5 regions which interact with the gp41 trans-membrane unit (Helseth et al., 1991). The inner domain surface is devoid of glycosylation (Wyatt et al., 1998). The outer domain is heavily glycosylated to shield its antigenic surface, protecting it from antibody recognition. The proximal end of the outer domain includes V4 and V5 variable loops, whereas the distal end includes the base of the excised V3 loop, which interacts through hydrogen-bonds with the V1/V2 stem emanating from the inner domain. In between the outer and inner domains is the

bridging sheet region, formed by four antiparallel  $\beta$ -sheets:  $\beta 2$  and  $\beta 3$ , which constitute the stem of the deleted V1/V2 loop; and the  $\beta 20$  and  $\beta 21$  of the C4 region.

Many studies have provided information on the structure and the mechanisms by which the gp120 interacts with the CD4 and co-receptor, the gp41, as well as neutralizing antibodies. These studies include the simian immunodeficiency virus (SIV) “unliganded” gp120 (not bound to CD4). This unliganded gp120 structure is presumed to represent the native state conformation (Chen et al., 2005). In contrast to the outer domain, which has a similar conformation as the liganded form, the inner domain and the bridging sheet revealed a much altered structure, suggesting that binding of CD4 induces radical conformational changes in these regions. A third gp120 structure was resolved using an HIV-1 gp120 core that included the V3 loop, bound to CD4 and the X5 antibody (Huang et al., 2005). More recently, other gp120 structures include: the gp120 core stabilized in the CD4 bound state associated with a CD4 binding site specific neutralizing antibody (Zhou et al., 2007), the gp120 core in complex with two CD4 binding site antibodies F105 and b13 (Chen et al., 2009), and the gp120 core bound to the gp120.interacting portion of gp41(Pancera et al., 2010).



**Figure 1.7: Organization of gp120 in linear and two-dimensional diagrams.** A) Linear representation of the structure of HIV-1 envelope. The gp120 and gp41 domains are indicated in blue and violet boxes, respectively. N-linked glycosylation sites are indicated by U-shaped branches. B) The gp120 core protein that was crystallized (red). It corresponds to the almost entire gp120 that was deleted of the V1.V2 and V3 regions (adapted from Chen et al 2005). C) Diagram of the structure of HIV-1 gp120 envelope glycoprotein. The gp120 molecule with the location of the variable regions indicated in boxes (V1.V5). The glycosylation sites containing high mannose-type and/or hybrid-type oligosaccharide structures are indicated by the branched structures, and glycosylation sites containing complex-type oligosaccharide structures are indicated by the U-shaped branches. Epitopes in gp120 that induce neutralizing antibodies are highlighted in color: the highly conformational CD4-binding domain (yellow), the CD4-induced epitope (green), an epitope composed of  $\alpha 1 \rightarrow 2$  mannose residues (purple), the V2 loop (orange) and the V3 loop (blue). [Figure from (Zolla-Pazner, 2004)].

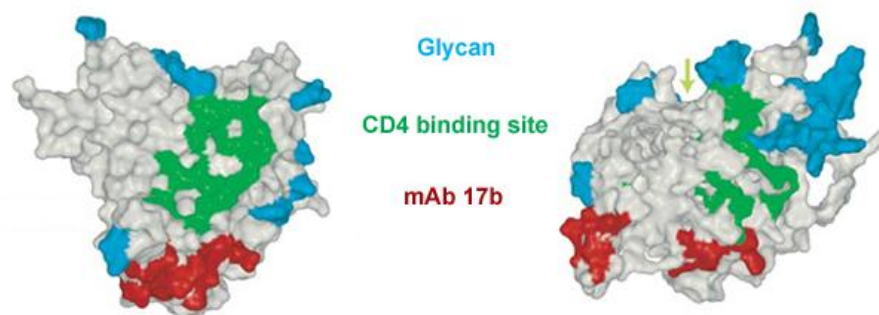


**Figure 1.8: HIV-1 gp120 and gp41 structures.** (a) Ribbon diagram of gp120 core containing  $\alpha$ -helices ( $\alpha 1$ – $\alpha 5$ ),  $\beta$ -strands (1–25), with relative positions of variable loops (V1–V5) and N- and C-termini shown. The orientation of gp120 in this diagram places the viral membrane toward the top and the cell membrane toward the bottom. When gp120 is bound to CD4, it forms a bridging sheet consisting of four  $\beta$ -strands, which separates the inner and outer domains of gp120 relative to their orientation in the trimeric complex. (b) Ribbon diagram of gp120 core [as in (a)] with N-terminus (red) and gp41 interaction site (blue) shown. The inner domain is shown in red and gray, and the outer domain is shown in orange. The bridging sheet, which shares elements from both inner and outer domains, is in gray and orange. (c) Trimeric gp120 [same colors as in (b)] bound to three molecules of CD4 (yellow) and Fab from neutralizing antibody 17b (brown), used to stabilize the gp120 structure, superimposed onto the electron density observed by cryoelectron tomography (light gray). The orientation of this structure that is rotated 90°, which places the viral membrane in the plane of the page, is also shown on the right. (d) Three-dimensional representation of HIV-1 Env in its CD4-bound conformation. (Left) A trimeric Env spike (blue) anchored in the lipid bilayer of the viral membrane (gray) is shown. The white arrow indicates the predicted location of gp41. (Right) Ribbon diagram of the gp120 core (red) superimposed on the density map (blue) with the V1/V2 loop (yellow) and the V3 loop (green) shown. (Figure from Checkley et al., 2011).

### 1.2.1.2 Functional sites of gp120

- **CD4 binding site (CD4bs)**

The CD4bs of gp120 constitutes a conformational region suggested to be only apparent in the context of the liganded structure of gp120 (figure 1.9). The CD4 binding loop projects away from the centre of the outer domain and the  $\beta 20.\beta 21$  segment of bridging sheet. The  $\alpha$ -helices of the inner domain, the CD4 binding loop and the  $\beta 20.\beta 21$  segment of bridging sheet create a long, narrow cavity, lined principally with hydrophobic side chains, in which many of the residues that are presumed to contact CD4 are located near or within this long cavity (Chen et al., 2005). The binding of CD4 induces large conformational changes in the inner domain, which leads to the formation of the bridging sheet and co-receptor binding site (Pancera et al., 2010).



**Figure 1.9: The binding sites of CD4 and monoclonal antibody 17b.** Molecular surface representations of gp120 core structures in liganded HIV (left) and unliganded SIV (right) states are shown from similar views. Residues in direct contact with CD4 are in green; residues contacting human monoclonal antibody 17b, in red; carbohydrate, in light blue. (Figure from Chen et al., 2005)

- **Co-receptor binding site**

Neither the receptor (CD4) nor the co-receptor (CXCR4 or CCR5) site is properly formed in the unliganded conformation of the gp120 core. In the unliganded conformation, the bridging sheet can close up to create the co-receptor binding surface, which is flanked by the V1–V2 and V3 loops (Chen et al., 2005). The V3 loop has been shown as the major determinant of

co-receptor switching, which demonstrates its involvement in the co-receptor-binding site (Chen et al., 2005).

### **1.2.2 The gp41 molecule**

The gp41 molecule is a transmembrane glycoprotein that is less variable and less glycosylated than gp120. It interacts non-covalently with gp120 and is responsible for maintaining the trimeric structure of the envelope glycoprotein, although its structure in the native conformation is unknown. The HIV-1 envelope glycoprotein gp41 is a homotrimeric structure formed by three gp41 monomers, with each monomer non-covalently associated with gp120. Each gp41 molecule consists of three domains: an extracellular domain (ectodomain), a transmembrane domain (TMD), and a C terminal cytoplasmic tail (figure 1.10.A).

#### **1.2.2.1 The extracellular domain and the fusion process**

The ectodomain is the primary structure involved in membrane fusion (Figure 1.10.B). At the amino end terminus of the ectodomain is a hydrophobic fusion peptide sequence, which has been proposed to act as an insertional sequence that penetrates the cell membrane. On the carboxyl side of the fusion peptide, there are two hydrophobic heptad repeat sequences: HR1, adjacent to the amino terminus and HR2, at the carboxyl terminus and adjacent to the TM domain. These HR sequences have a leucine zipper motif suggested to form an alpha-helix, and are also known as N-helix (near the amino terminus) and C-helix (near the carboxy terminus). Prior to co-receptor binding the N-helices form a central three stranded coiled-coil, and are surrounded by three anti-parallel C-helices that bind to conserved grooves on the coiled-coil surface. This formation can be blocked by peptides derived from either HR1 or HR2 (Baldwin and Berkhout, 2008). One such HR2 derived peptide, known as enfuvirtide (T-20), is approved for use in HIV-1 infected patients.

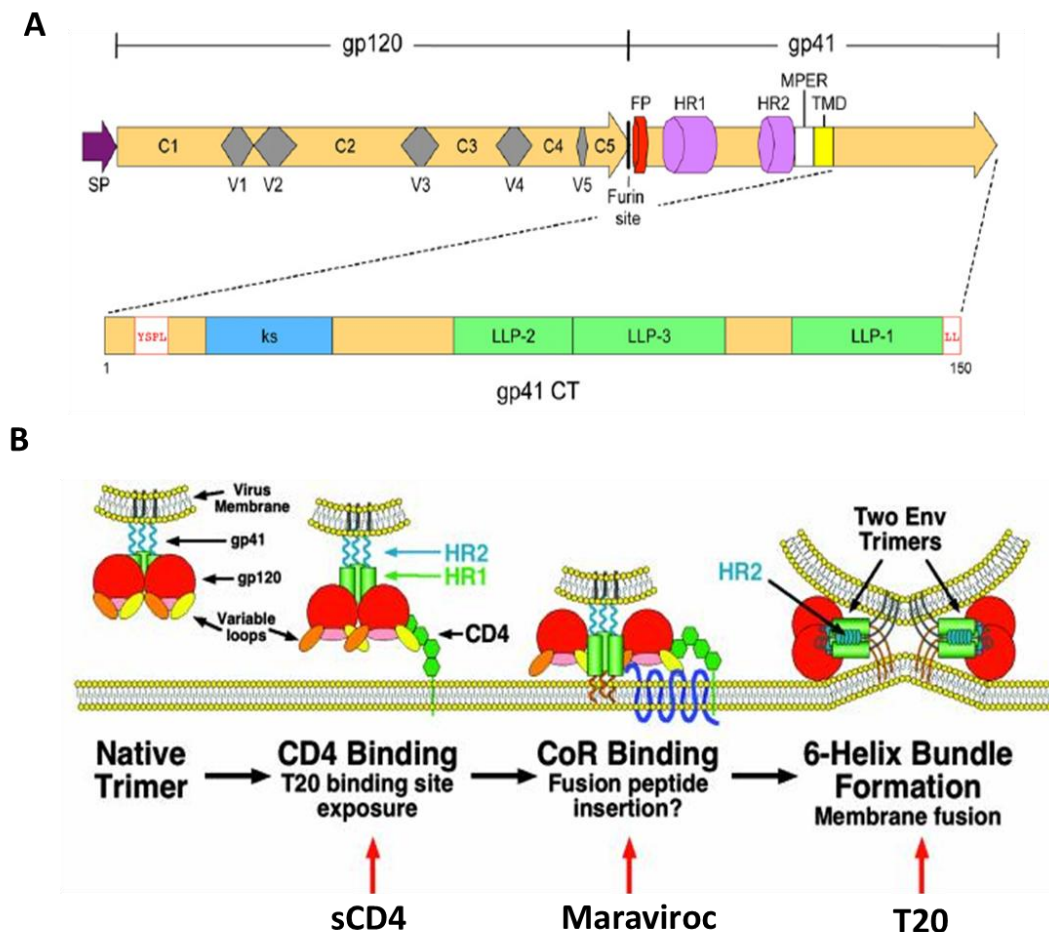
#### **1.2.2.2 The transmembrane domain (TMD)**

The TMD of gp41 consists of approximately 25 highly conserved amino acids suggesting that it might play specific roles in envelope function. Mutations in the core region of the TMD influence fusion (reviewed by Checkley et al 2011).

#### **1.2.2.3 The cytoplasmic tail (CT)**

HIV and SIV have very long CT of approximately 150 amino acids compared to other members of the Retroviridae family. Several properties of the gp120/ gp41 glycoprotein complex are mediated by the CT such as virus infectivity, gp120 shedding, Env incorporation into virus particles, and cell-surface Env expression (reviewed by Checkley et al 2011). The

HIV and SIV cytoplasmic tails contain a number of functional domains (figure 1.10A) including: the membrane-proximal tyrosine-based motif with the consensus (YxxΦ) and the dileucine motif at the C terminus mediate binding to AP2 chains, clathrin-dependent endocytosis, alter intracellular localization, and regulate envelope expression and incorporation in the virion (Boge et al., 1998, Wyss et al., 2001, Byland et al., 2007, Berlioz-Torrent et al., 1999). Three conserved amphipathic  $\alpha$ -helical segments that are referred to “Lentivirus Lytic Peptides” domains (LLP.1, LLP.2, and LLP.3) are present in the central and C-terminal regions of the gp41 CT. LLP domains have been implicated in interacting with the plasma membrane, decreasing bilayer stability, altering membrane ionic permeability, and mediating cell killing (Chen et al., 2001, Chernomordik et al., 1994, Eisenberg and Wesson, 1990, Kalia et al., 2003, Miller et al., 1993).



**Figure 1.10 : Organization of gp41 and fusion process.** A) Schematic representation of the functional domains of gp41. Gp41 is formed by three domains: an extracellular domain, containing the fusion peptide (FP), heptad repeats (HR1 and HR2), and the MPER; a TMD and a CT. An enlarged representation of the gp41 CT is shown to highlight several motifs: the internalization signal YSPL; the Kennedy sequence (ks); the amphipathic  $\alpha$ -helices LLP.1, LLP.2, and LLP.3; and a C-terminal dileucine motif (LL) (Figure from Checkley et al., 2011). B) Schematic representation of a model of HIV entry. The gp120 binds to the CD4 receptor on a target cell to initiate viral entry resulting in a conformational change which exposes the co-receptor binding site, allowing binding to a co-receptor. Following co-receptor binding gp41 undergoes conformational changes which expose an N-terminal hydrophobic peptide. This peptide inserts into the membrane of the target cell and following subsequent conformational changes causes fusion of cell and viral membranes. The stages of entry at which three HIV-1 entry inhibitors are believed to act are indicated. Figure adapted from (Moore and Doms, 2003).

## **1.3 HIV co-receptor usage and tropism**

### **1.3.1 HIV co-receptor usage**

Soon after the discovery of the CD4 molecule as the major receptor for HIV (Dalglish et al., 1984), new evidence started to accumulate indicating that CD4 alone was not sufficient for HIV to enter the target cells. In 1986, a study showed that CD4 expressed on mouse cells allowed virus to bind but did not confer virus entry (Maddon et al., 1986). These results supported the conclusion that this restriction was due to the requirement for a cofactor of unknown identity that was specific to human cells (Berger et al., 1999). The second conclusion of great importance was the observations that HIV-1 isolates fell into two distinct groups depending on their phenotypes. Some HIV-1 isolates showed efficient infectivity for continuous CD4<sup>+</sup> T cell lines, but poor infectivity for primary macrophages. Thus, such viruses were designated as T-cell line-tropic (or T-tropic) and they were generally syncytium-inducing 1 (SI) in assays using a highly permissive T-cell line. Other HIV-1 isolates showed the ability to infect primary macrophages much more efficiently than those T-cell lines. They were designated as macrophage-tropic (-) or non-syncytium-inducing 1 (NSI) isolates. Asjo et al. (1986) described the two groups as rapid-high (SI) and slow-low (NSI) depending on their replication rates in PBMCs. The first HIV-1 co-receptor was identified in 1996 and was named fusin, because it mediated HIV-1 fusion (Alkhatib et al., 1996) and was thereafter renamed CXCR4 (Moriuchi et al., 1997). The second HIV-1 co-receptor was identified based on the finding that the CC-chemokines (i.e. RANTES, MIP.1 $\alpha$ , and MIP.1 $\beta$ ) that are the natural ligands of CCR5 could block the infection of NSI HIV-1 isolate. Several groups reported CCR5 as the co-receptor for NSI viruses (Alkhatib et al., 1996, Deng et al., 1996, Dragic et al., 1996), while HIV-1 strains able to use both co-receptors were termed dual-tropic (D-tropic). Both CCR5 and CXCR4 belong to the superfamily of seven transmembrane (7TM) G protein-coupled receptors. More than fourteen other 7TM receptors or structural-related molecules have been identified to act as co-receptors for entry of HIV-1 *in vitro* (Dejucq et al., 1999). Currently, there is little evidence to suggest that co-receptors other than CCR5 and CXCR4 are used significantly *in vivo*.

### **1.3.2 HIV tropism**

HIV tropism is now commonly defined based on the co-receptor usage which is defined as the ability of a particular HIV-1 virus to infect a target cell using a specific co-receptor, either CCR5, or CXCR4 or both. The HIV-1 viruses can be characterized into three classifications: R5, X4, and R5X4 viruses based on the type of co-receptor that they bind. After the

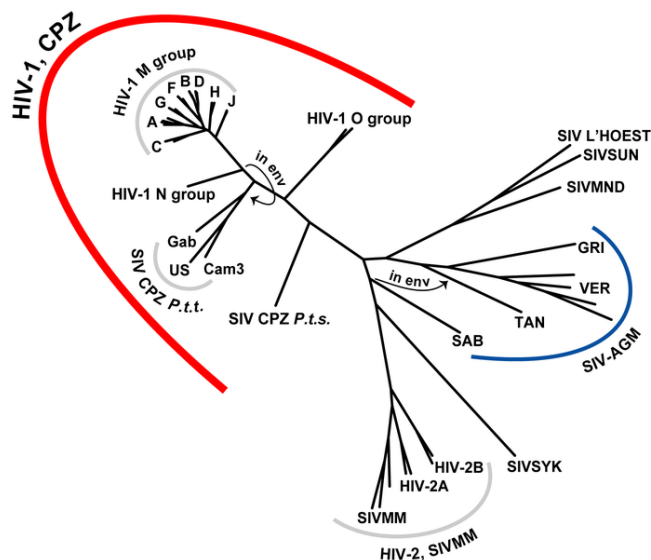
identification of the co-receptors, CCR5 and CXCR4, it became clear that the NSI/M-tropic and SI/T-tropic phenotypes were linked to their differential use of chemokine receptors for HIV-1 entry. NSI/M-tropic viruses preferentially use CCR5, while SI/T-tropic viruses use CXCR4. Therefore, NSI/M-tropic virus has been classified as R5, SI/T-tropic virus as X4, and dual-tropic virus as R5X4. The major genotypic determinant for HIV-1 co-receptor usage is the V3 variable loops of the gp120 envelope glycoprotein (Hwang et al., 1991). Different bioinformatics tools have been developed to predict HIV-1 co-receptor usage from the amino acid sequence of V3, taking into account the key amino acids at positions 11 and 25, plus other sites in V3 that differ between CCR5 and CXCR4-using strains (Briggs et al., 2000).

## **1.4 HIV genetic diversity**

### **1.4.1 HIV classification**

HIV is a member of the lentivirus genus of the *Retroviridae* family. The name lentivirus refers to slowly replicating viruses, because these viruses take a long time before it induces the full-blown disease. Two types of HIV have been characterized: HIV-1 and HIV-2 and both share 40% to 50% genetic homology, with the greatest sequence divergence localized in the envelope gene (De Cock et al., 1991). HIV-1 is thought to be the result of cross-species transmission of simian immunodeficiency viruses (SIVs) isolated from chimpanzees (SIVcpz) (Peeters et al., 1989, Gao et al., 1999), while HIV-2 is most closely related to a virus found in sooty mangabeys (SIVsm) (Chen et al., 1997).

HIV-1 is divided into group M (main), the group O (outlier), the group N (non-M/ non-O), and the recently identify group P. A phylogenetic tree representing the HIV-1 genetic diversity is shown in Figure 1.11. Group M is responsible for the worldwide HIV-1 epidemic and Group O has been endemic in Cameroon and neighboring countries in West Central Africa, but even there the group O represents a minority of HIV-1 strain, with prevalence less than 10% of HIV-1 infections in Cameroon (Yamaguchi et al., 2004). Group N has been also identified in Cameroonian patients and it is only represented by a limited number of isolates from Cameroonian patients (Ayoub et al., 2000, Simon et al., 1998). HIV group M is classified into 9 recognized subtypes: A, B, C, D, F, G, H, J and K and presumed recombination of those subtypes (McCutchan, 2006).



**Figure 1.11: Phylogenetic tree illustrating comparative relationship between SIV, HIV-1 (group M, N and O) and HIV-2.** Figure constructed utilizing *pol* gene sequences with small arrows indicating where sequences would branch in *env* gene reconstruction. (Figure from <http://www.hiv.lanl.gov/content/sequence/HIV/COMPENDIUM/99compendium.html>)

Studies have shown that intra-subtype genetic distance can differ by up to 30% in the *env* gene and inter-subtype genetic distances can reach up to 42% (Robertson et al., 2000, Korber et al., 2001, Gaschen et al., 2002). Sequencing full-length genomes have led to the identification of inter-subtype recombinants known as circulating recombinant forms (CRFs). These are presumed to be the result of recombination between different subtypes within an individual patient concurrently infected with HIV-1 of two or more subtypes. The inter-subtype recombinant genomes become designated as CRFs, if: i) the identical recombinant viruses are identified in at least three epidemiologically unlinked people, ii) are characterized by full-length genome sequencing that share the same recombinant structure, and iii) form a monophyletic cluster in all regions of the genome; and as URFs, if only a single or two sequences are available (Robertson et al., 2000). Recombinants are currently estimated to be responsible for at least 20% of HIV-1 infections worldwide (Hemelaar et al., 2011a). The CRFs are named with a number sequential in the order in which they are reported in the literature and followed by the letters of the subtype involved, starting with CRF01\_AE (Figure 1.12). If the recombinants consist of more than two subtypes involved, they are therefore replaced by designation “cpx”, meaning complex, e.g. CRF04\_cpx (A, G, H, K, and U). Taxonomically, the CRFs are at the same level as the subtype. Currently, 55 CRFs for HIV-1 (CRF01 to CRF55) are found in the HIV database at Los Alamos National Laboratory

([www.hiv.lanl.gov](http://www.hiv.lanl.gov)), most of them having been described in Africa. Some CRFs are major strains circulating in certain regions and responsible of newly emerging epidemics. For example CRF01\_AE and CRF02\_AG are dominant in Thailand, Asia (Xiridou et al., 2007) and West Africa respectively (Fischetti et al., 2004).

As of 2010, HIV-2 can be divided into 8 different groups (A, B, C, D, E, F, G and H), with only one CRF (CRF01\_AB) (Ibe et al., 2010) and a novel HIV-2 variant recently identified in the Ivory Coast (Ayouba et al., 2013). The majority of HIV-2 sequences in the database are groups A and B, which are circulating in the human population, while groups C to H represent only few detected infections. The geographical distribution of HIV-2 is less extensive than that of HIV-1. It is concentrated primarily to West Africa, although the prevalence of HIV-2 is a growing concern in certain parts of Europe and in the South-Western region of India.

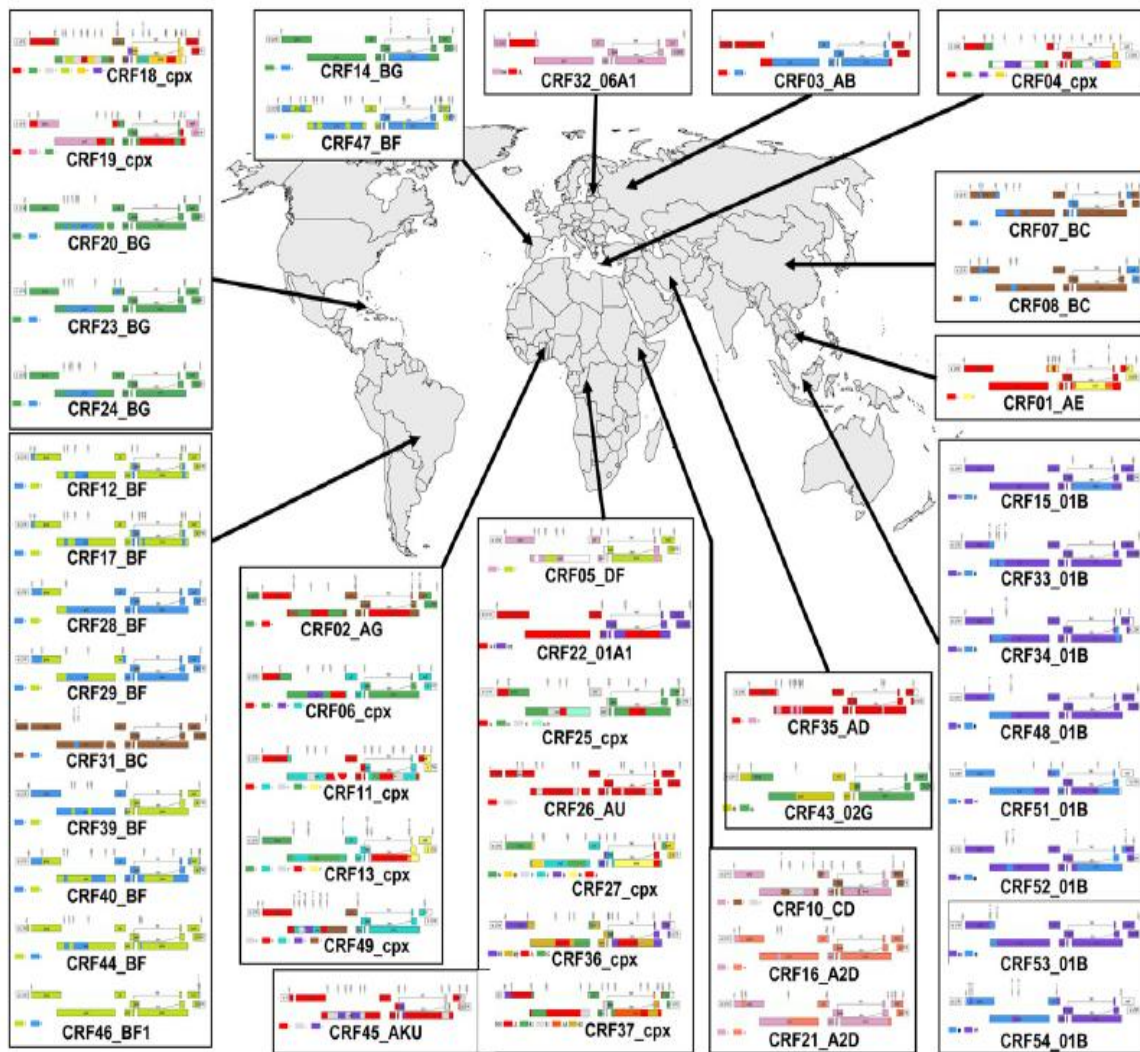
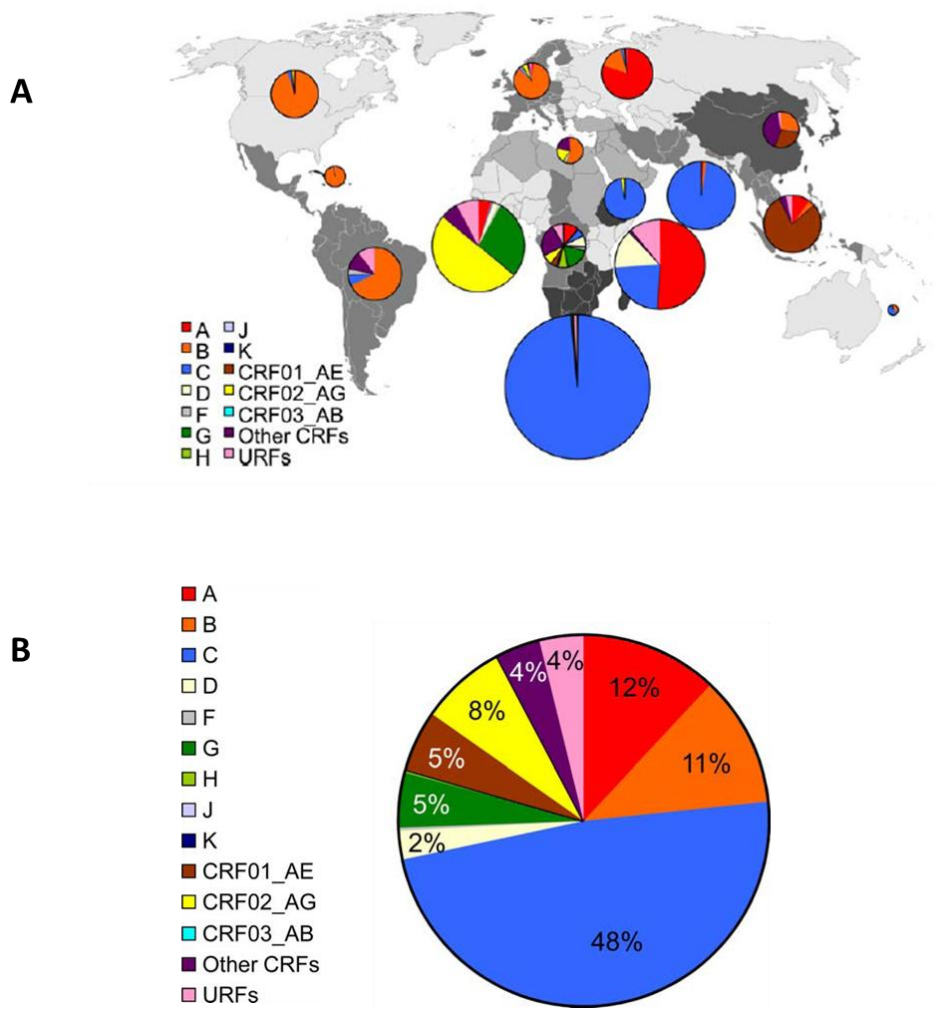


Figure 1.12: Global spread of circulating recombinant forms of HIV-1. All currently identified CRFs are shown with arrows pointing to the geographical location where each CRF has been found (Figure from Hamelaar et al., 2013).

#### 1.4.2 Global distribution of HIV subtypes

HIV-1 genetic subtypes are unevenly distributed in different geographical locations. According to recent studies, the most prevalent HIV-1 subtypes are subtypes A, B and C. Subtype C accounts for nearly half (48%) of all HIV-1 infections in 2004-2007, while subtypes A, B, D and G account for 12%, 11%, 2%, and 6%, respectively (Hamelaar et al., 2011a). The subtypes F, H, J, and K all together account for approximately 1% of infections. The circulating recombinant forms CRF02\_AG and CRF01\_AE are responsible for 8% and 5% of cases respectively, and CRF03\_AB for 0.1%. Other recombinants account for the

remaining 16% of infections. All recombinant forms (all CRFs and URFs) are responsible for over 20% of infections worldwide (Hemelaar et al., 2011a). The global distribution and prevalence of HIV-1 worldwide are shown in Figure 1.13A and 13B. This distribution reflects the present situation and might be susceptible to modifications in the next years. Subtype A viruses are predominant in Central and Eastern Africa (Kenya, Rwanda, Uganda, and Tanzania) and in Eastern European countries formerly constituting the Soviet Union. Subtype B, which is the most widely disseminated subtype, is predominant in North and Latin America, the Caribbean, Europe, and Australia. It is also common in several countries of Southeast Asia, North Africa, Middle East (Israel), and among South-African and Russian homosexual men. Subtype C is predominant in southern Africa, Ethiopia and India. Subtype D viruses are found principally in East Africa and to a lesser extent in West Africa. CRF01\_AE and subtype B co-circulate in South-East Asia, whereas CRF02\_AG, along with other recombinants, dominates in West and West Central Africa. In South America, the epidemic is a mixture of subtype B and BF recombinants, with a small proportion of subtype C infections. In East Asia subtypes B, C and BC recombinant strains dominate. Central Africa harbors a complex mixture of rare subtypes (F, G, H, J and K) and recombinants, without any predominant strain.



**Figure 1.13: Global distribution and prevalence of HIV-1 subtypes and recombinant forms.**

- A) Regional distribution of HIV subtypes and recombinants. The colors representing the different HIV-1 subtypes are indicated in the legend on the left-hand side of the figure.
- B) World global distribution. (Figure from Hamelaar et al., 2011).

### 1.4.3 Mechanisms of genetic diversity

The high genetic variability and rapid evolution of HIV are important factors in its worldwide spread. HIV genetic heterogeneity originates from the high mutation and recombination rates of the reverse transcriptase enzyme combined with a high turnover rate. This results in genetically diverse populations of viral species in each infected individual, referred as “quasispecies” (Eigen, 1993, Eigen, 1996), each with diversity comparable to that of the entire influenza population of any one year (Korber et al., 2001).

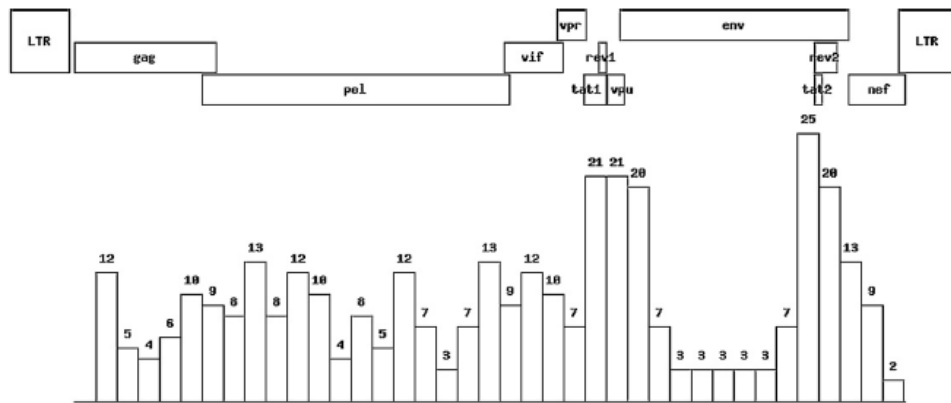
### 1.4.3.1 Mutations

As for all RNA viruses, the inability of the reverse transcriptase (RT) to correct misincorporations leads to a high error rate. Most HIV mutations are introduced during reverse transcription stage of the viral life cycle. The most prominent source is the reverse transcriptase enzyme itself, which is error prone due to the lack of a proofreading capability. It does not contain a 3' exonuclease activity capable of excising misincorporated nucleotides. The rapid viral turnover in an infected individual with the production of up to  $10^{10}$  particles per day (Ho et al., 1995), and the poor fidelity of the replication process with the generation of  $3 \times 10^{-5}$  mutations per nucleotide per cycle (Mansky and Temin, 1995), indicate that on average  $3 \times 10^9$  mutations are generated each day in the viral population present in a patient. A more recent study indicates that the HIV-1 mutation rate is in fact around the retroviral average of  $1.4 \times 10^{-5}$  errors per base pair, per replication cycle (Abram et al., 2010).

### 1.4.3.2 Recombination

Detectable recombination of HIV may occur when a cell becomes infected with two or more genetically distinct HIV virions. These virions may come from the same subtype (intra-subtype recombination) (Rousseau et al., 2007, Philpott et al., 2005) or from different subtypes (inter-subtype recombination) (Charpentier et al., 2006, Yirell et al., 2002). Dual infection (similar to double infection) occurs when an individual is infected with strains derived from two different individuals. The dual HIV infections can be divided into co. and superinfections. Co-infection is defined as simultaneous infection with two heterologous strains before an immune response has developed and antibodies are detectable in the blood before seroconversion. Thus, co-infection would occur within the first month of infection. Superinfection is defined as infection with a second strain after the initial infection and the immune response to it has been established, after initial seroconversion. The HIV-1 recombination rate was approximately two to three recombination events per genome per replication cycle, and crossovers or breakpoints were identified throughout the viral genome (Jetzt et al., 2000). A recent study found that recombination breakpoints are non-randomly distributed across the genomes of HIV-1 inter-subtype recombinants by analyzing the position of the breakpoints found in sequences of the Los Alamos Database (<http://www.hiv.lanl.gov/>) (Fan et al., 2007). Two recombination prone regions or “hot spots” were identified. They are located at the borders of the *env* gene around the first exon of *tat*, *vpu* and the beginning of *env*, and the second exon of *tat*, *rev*, and the 3' end of *env* (Figure 1.14). However, comparing

the recombination breakpoints across gp120 indicated that that the C2 region is also a hotspot for recombination (Baird et al., 2006, Galetto et al., 2004).



**Figure 1.14: The distribution of breakpoints across HIV-1 genome.** The diagrammatic representation of HIV-1 genome is shown in upper panel. The bars and the number of breakpoints detected in sequences of the Los Alamos Database (<http://www.hiv.lanl.gov/>) are shown in lower panel. (Figure from Jun Fan *et al.* 2007)

#### 1.4.4 Consequences of HIV-1 genetic diversity

##### 1.4.4.1 Impacts of HIV-1 genetic diversity on transmission and disease progression

Although earlier studies found an association between CRF01\_AE and heterosexual transmission as well as between subtype B and intravenous drug use (Gao et al., 1996, Soto-Ramirez et al., 1996), a more recent longitudinal study performed in Thailand found an increased probability of CRF01\_AE transmission among IDUs compared with subtype B (Hudgens et al., 2002). A recent study performed in HIV-discordant couples in Uganda found that subtype A was associated with a significant higher rate of heterosexual transmission than subtype D (Kiwanuka et al., 2009). The rate of transmission may reflect differences in subtype-specific co-receptor tropism. The HIV strains capable of using CCR5 are more frequently transmitted than strains that use CXCR4, however CXCR4 viruses emerge later in infected patients and are associated with more rapid disease progression (Berger et al., 1998). It was found that HIV-1 subtype D used CXCR4 more frequently in early infection which may in part explain their reduced heterosexual transmissibility when compared to other genetic forms (Huang et al., 2007, Kaleebu et al., 2007, Kiwanuka et al., 2009) whereas subtype A mostly used CCR5 even in late infection. This may explain why HIV-1 subtype D-

infected patients had more rapid progression than those infected with subtype A in Uganda, Kenya, and Tanzania. The percentage of CXCR4 viruses appears lower in subtype C than in subtype B, even when the viruses are obtained from patients with advanced AIDS (Cilliers et al., 2003). A previous study in Tanzania suggested that subtypes A, C and recombinants are more likely to be perinatally transmitted than subtype D (Renjifo et al., 2001), and that pregnant women infected with subtype C were more frequently susceptible to transmit HIV to their children than those infected with subtype B (Renjifo et al., 2003).

HIV-1 subtype differences in disease progression have been studied in several cohorts. A retrospective cohort study (1996-2007) reported that African patients infected with HIV-1 non-B subtypes (A, C, F.K, CRF01\_AE, CRF02\_AG,) had slower rates of disease progression compared to Haitians and Canadians infected with subtype B viruses (Keller et al., 2009). A study in Senegal reported that women infected with non-A subtypes were 8 times more likely to develop AIDS than those infected with subtype A (Kanki et al., 1999). A study of a Kenyan cohort showed that patients infected with subtype D had a higher mortality rate and a faster decline in CD4+ count than those infected with subtype A or C (Baeten et al., 2007). The propensity of subtype D to exhibit a greater degree of using dual co-receptor than other subtypes (Huang et al., 2007) may help to explain the observation that subtype D appears to be associated with a more rapid rate of disease progression than other subtypes. In contrast, no difference in disease progression was found between patients infected with subtypes B and CRF01\_AE in Thailand (Amornkul et al., 1999) or subtypes CRF02\_AG or other subtypes in Cameroon (Laurent et al., 2002).

#### **1.4.4.2 Impacts of HIV-1 genetic diversity on diagnostics**

HIV-1 fourth-generation immunoassays are able to detect all known HIV-1 group M subtypes, group O and HIV-2 positive samples with 100% sensitivity and >98% specificity (Kwon et al., 2006). These assays provide an advantage for detection of infection during the window period prior to seroconversion since the diagnostic window may be reduced by an average of 5 days relative to an IgM-sensitive EIA (Fiebig et al., 2003, Weber et al., 1998). However, such advanced assays are often not available in resource limited countries where most new infections occur. In field situations with a high diversity of circulating HIV strains, such as in Cameroon, the performance of rapid diagnostic tests is much less satisfactory with sensitivities ranging from 94.1 to 100% and specificities ranging from 88.0% to 98.8%. In particular, group O infections are poorly detected (Aghokeng et al., 2009). PCR-based assays for viral load measurements also have difficulty detecting and reliably quantifying HIV-1

RNA when testing diverse genetic variants of HIV-1 from Africa, especially group O. Different assays also frequently yield discordant viral load results (Rouet et al., 2010). Group P infections may not be efficiently detected by the current HIV screening tests due to the absence of group P-specific reagents for antibody detection (Vallari et al., 2011).

#### **1.4.4.3 Impacts of HIV-1 genetic diversity on antiretroviral therapy**

The development of resistance to antiretroviral drugs continues to be an important problem in the treatment of HIV-infected individuals. Studies around the world have demonstrated that different group M subtypes have similar susceptibilities to currently used antiretroviral drugs, which were originally developed based on subtype B viruses (Kantor, 2006). Different HIV genetic forms carry in their genomes genetic signatures and polymorphisms that could alter the structure of viral proteins which are targeted by drugs, thus impairing ARV drug binding and efficacy. In general, several mutations are generally required for the virus to become resistant to protease inhibitors (PI), whereas a single amino acid substitution can induce resistance to the non-nucleoside reverse transcriptase inhibitor (NNRTIs) (Beerenwinkel et al., 2005). The NNRTI resistance mutation V106M occurs in subtype C and CRF01\_AE, but not in subtype B, and the protease inhibitor (PI) mutation L89IV occurs in subtypes C, F and G, but not in B (Martinez-Cajas et al., 2009). Among non-B subtypes differences are also notable, as Nevirapine resistance mutations developed more frequently in subtype D than A in a mother-to-child transmission prevention study using single-dose Nevirapine (Eshleman et al., 2005). Of note, a high proportion of group O viruses are naturally resistant to NNRTIs due to the presence of the C181Y substitution in RT (Depatureaux et al., 2011).

## **1.5 Neutralizing antibodies against HIV infection**

### **1.5.1 Antibody responses to HIV**

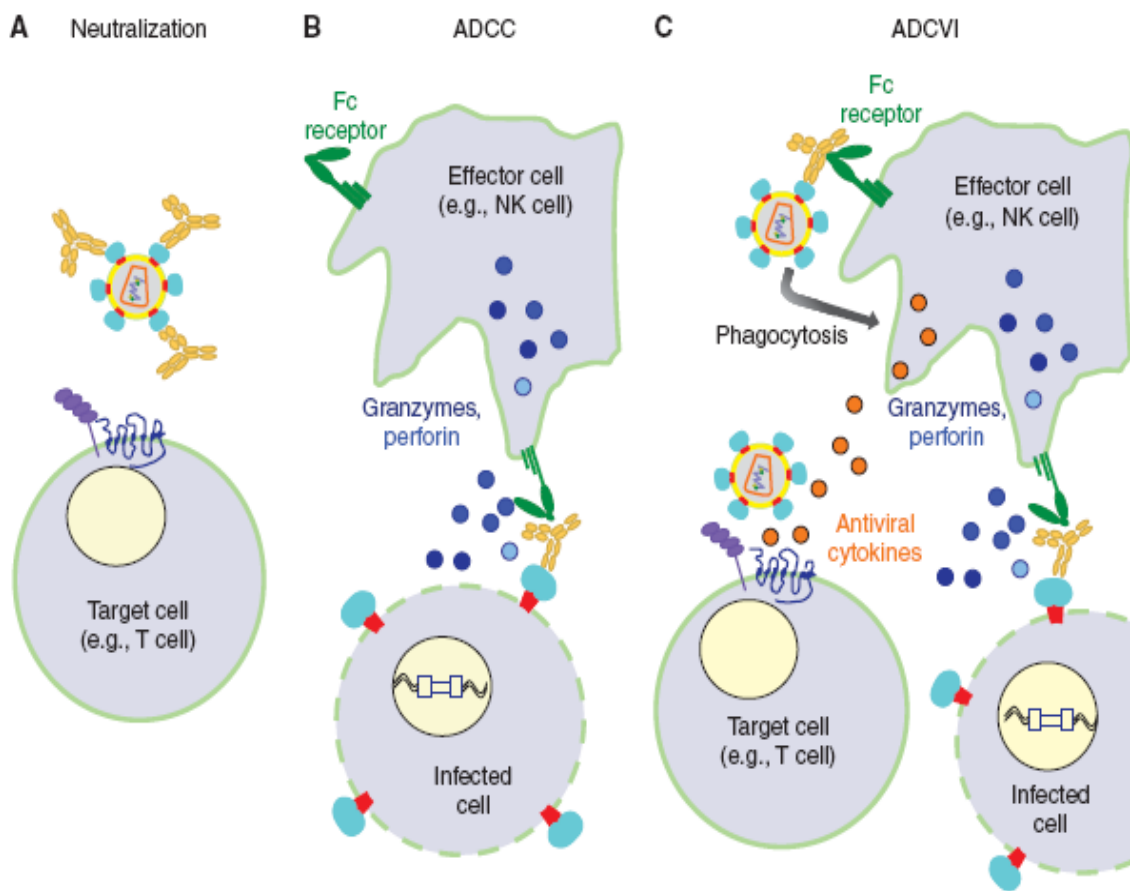
Following acute HIV infection an abundance of antibodies are elicited. Antibodies have the ability to inhibit HIV-1 infection through multiple pathways: they can bind cell-free virus and prevent the infection or they can complex with Fc $\delta$  receptor to block HIV-1 through effector cell mechanisms (Overbaugh and Morris, 2012). The progression of these antibodies include: i) binding antibodies that first develop within 8 days after plasma virus detection and initially exist as antigen–antibody complexes, followed a few days later by circulating anti-gp41 antibodies, and further few weeks later with anti-gp120 antibodies (Tomaras et al., 2008); ii) non-neutralizing antibodies that act together with innate immune cells to kill off virus infected cells known as antibody dependent cell-mediated cytotoxicity (ADCC) and antibody

dependent cell-mediated viral inhibition (ADCVI) (Sarmay et al., 1992); iii) neutralizing antibodies (Nabs) (Figure 1.15). Neutralizing antibodies aim to block viral entry and subsequent infection by binding to exposed regions on the envelope, however, little or no role for these antibodies is known once HIV-1 has entered its target cell.

#### **1.5.1.1 Non-neutralizing antibodies (ADCC and ADCVI)**

Non-neutralizing antibodies can clear the virus by binding to the infected cells and initiate the recruitment of activated effector cells, which in turn induces cytolysis or apoptosis of infected cells. ADCC is the result of the formation of a complex between the IgG Fab portion of the antibody with the viral protein on the cell surface and binding of the Fc portion to the Fc receptors of the antibody (Sarmay et al., 1992). Fc receptors are expressed on natural killer cells, monocytes, macrophages, dendritic cells and neutrophils (Sarmay et al., 1992). The binding to the Fc receptors can lead to release of antiviral cytokines (Russell and Ley, 2002), resulting to the killing of the infected target cell (Baum et al., 1996) (Figure 15B). ADCVI also involves the interaction between a target cell, ADCVI antibody and an effector cell. However, rather than causing cell death, ADCVI antibodies aim to reduce the viral output from infected target cells (Overbaugh and Morris, 2012) (Figure 1.15C).

Vaccine studies in both humans and non-human primate model systems have brought some evidence that non-neutralizing antibodies may provide protection from infection (Mascola et al., 1999; Mascola et al., 2000; Barouch et al., 2012; Baba et al., 2000; Parren et al., 2001). In human vaccine studies, a correlation was observed between both antibody binding activity and ADCVI antibody activity and the incidence of HIV-1 infection in vaccine recipients in the Vaxgen Phase III efficacy trial (Gilbert et al., 2005, Forthal et al., 2007).



**Figure 1.15: Schematic representation of the mechanism of action of nAbs and antibodies that act through ADCC and ADCVI.** (A) Antibody neutralization of cell-free virus. Neutralizing antibodies bind to HIV-1 envelope glycoproteins and block the interaction of viral particles with CD4 and CCR5, essential receptors on target cells required for infection. (B) Antibody-dependent cellular cytotoxicity leads to the killing of infected cells. In the case of ADCC, a complex between the IgG Fab portion of antibody bound to envelope protein on the cell surface and the Fc portion to the Fc receptors on effector cells leads to lysis of the infected cell. (C) Antibody-dependent cell-mediated virus inhibition. ADCVI measures the effects of ADCC-mediated cell killing, which lead to reduced virus production, as well as virus inhibition by antiviral cytokines and other secondary effects of FcR-virus interactions such as phagocytosis (Figure from Overbaugh and Morris, 2012)

### 1.5.1.2 Neutralizing antibodies (nAbs)

- **Autologous neutralizing antibodies**

In HIV-1 infection, nAbs can block the virus-cell interaction by inhibiting the binding of virion to CD4 and co-receptors on the cell surface, therefore preventing conformational changes of the virus envelope that are required for subsequent steps in the virus life cycle. The earliest neutralizing antibodies can be detected within months of infection in most HIV-1 infected individuals (Gray et al., 2007, Richman et al., 2003, Wei et al., 2003). Most HIV-1

infected individuals produce antibodies capable of inhibiting their own virus (autologous virus) and are known as autologous neutralizing antibodies (Arendrup et al., 1992, Moore et al., 1994, Moog et al., 1997, Pilgrim et al., 1997, Richman et al., 2003, Wei et al., 2003, Frost et al., 2005, Deeks et al., 2006, Gray et al., 2007). These antibodies target immunogenic exposed regions of the HIV-1 virion; however their neutralization capacity is transient due to viral mechanisms to escape antibody recognition (Arendrup et al., 1992, Richman et al., 2003). The appearance of neutralization escape variants soon after the autologous response supports the notion that these antibodies exert immunological pressure on the virus (Wei et al., 2003, Richman et al., 2003, Deeks et al., 2006, Frost et al., 2005). Recent studies suggested that autologous antibody response primarily target variable regions rather than the conserved regions of the HIV-1 envelope, explaining the strain specificity of these antibodies (Moore et al., 2009a). The V1V2 region was therefore shown to be a frequent target of autologous nAbs in HIV-1 and Simian–Human Immunodeficiency Virus (SHIV) (Moore et al., 2008, Laird et al., 2008). A study reported on the detection of autologous nAbs as early as 52 days after detection of HIV-specific antibodies in acutely infected patients (Wei et al., 2003). Gray et al, (2007) have evaluated autologous and heterologous neutralizing antibody responses in 14 HIV-1 subtype C acutely infected individuals (Gray et al., 2007). Envelope clones were used which were obtained within the first 2 months of infection. Their results revealed that potent autologous neutralizing antibodies are produced within 3 to 12 months post-infection with an increase in autologous antibody production observed within the first 6 months (Gray et al., 2007). Interestingly, it was also noted that potent autologous neutralization correlated with shorter Env variable region lengths as well as the presence of fewer glycosylation sites especially in the V1V2 region of Env (Gray et al., 2007). Another study suggested that the C3V4 region, specifically the C3  $\alpha$ .2-helix is a major target of autologous neutralization antibodies in subtype C infections (Moore et al., 2008). V4 does not appear to be a significant autologous nAb target, although changes in this region may mediate neutralization escape (Sato et al., 2008) while the role of V5 is less clear. Studies on the control of superinfection have also revealed the role of autologous neutralizing antibodies. Basu et al (2012) have shown that superinfected individuals infected with subtype C mount low or undetectable autologous nAb responses prior to intra-subtype superinfection in contrast to singly-infected matched controls who presented HIV-1 specific antibodies with very potent neutralizing features (Basu et al., 2012). Another study from Mayr et al (2012), reported evidences that patient superinfected by discordant subtypes who exhibits broad nAbs against heterologous viruses, mount low autologous nAbs response against their original

discordant autologous viruses (Mayr et al., 2012). These results were discordant with the description of six untreated female sex workers who became superinfected despite a broad and potent nAbs responses prior to the infection with a second HIV-1 strain (Blish et al., 2008). These superinfected individuals were able to mount autologous nAbs responses to their superinfecting variant following superinfection (Blish et al., 2008).

- **Heterologous neutralizing antibodies**

In contrast to the early autologous Nab responses, antibodies capable of neutralizing heterologous viruses (heterologous neutralization) develop later in infection and can be remarkably potent and cross reactive (Richman et al., 2003, Wei et al., 2003, Gray et al., 2007). Only a small percentage of chronically infected patients can develop broadly cross-reactive nAbs against multiple HIV-1 viruses (Braibant et al., 2006, Donners et al., 2002, Pilgrim et al., 1997, Moore et al., 1996, Moog et al., 1997, Simek et al., 2009, Gray et al., 2011b). The reasons why some individuals develop broadly cross-reactive nAbs as well as why breadth develops so rarely is unclear but is related to the duration of infection and viral levels suggesting that years of persistent viral stimulation are necessary for their generation (Piantadosi et al., 2009b, Sather et al., 2009, Euler et al., 2010, Gray et al., 2011b). Broadly cross-reactive nAbs response was initially thought to be quite uncommon (Mascola and Montefiori, 2010), but recent studies have described the presence of broadly cross-reactive nAbs in different cohorts (Doria-Rose et al., 2009, Sather et al., 2009, Simek et al., 2009, Gray et al., 2009a). These have been important studies as they indicate that such sera and antibodies are not rare (Stamatatos et al., 2009, Gray et al., 2009a, Sather et al., 2009), providing evidence that the natural B cell response can generate broadly cross-reactive nAbs against HIV-1. This is obviously the type of antibody response that we would like to elicit with a preventative vaccine.

### **1.5.2 Mechanisms of evasion from neutralizing antibodies**

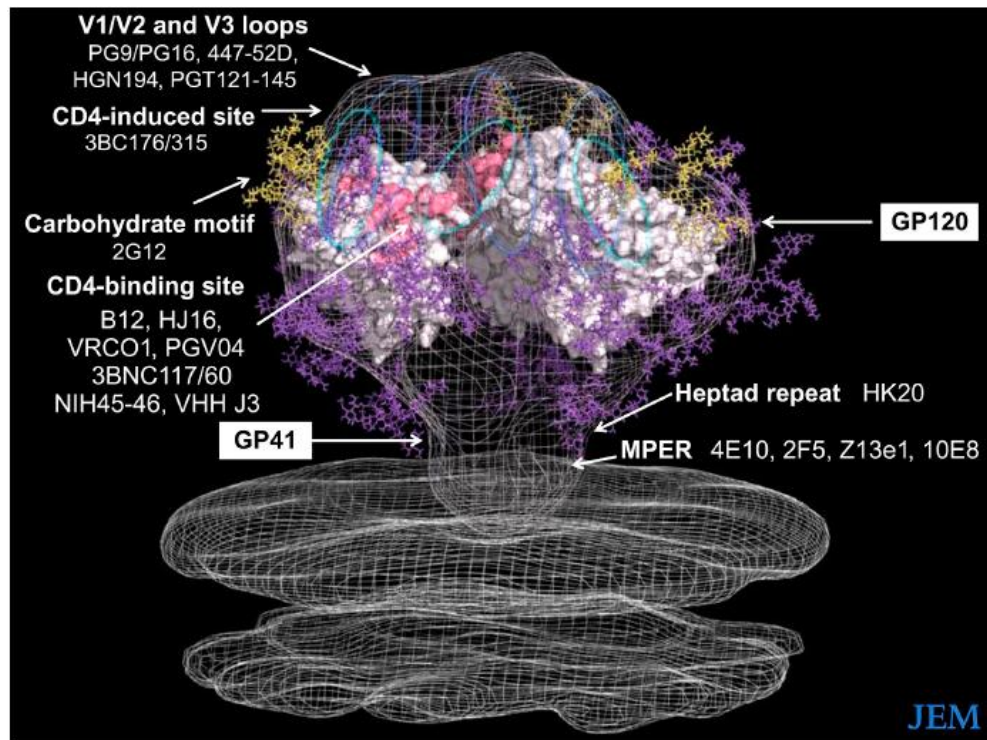
HIV-1 has developed multiple escape mechanisms to avoid neutralization. Such mechanisms include the inaccessibility of relevant epitopes due to the trimeric structure of envelope, nucleotide substitutions, insertions and deletions, modification of envelope potential N-linked glycosylation sites (PNGS), (Mc Cann et al., 2005). HIV envelope is heavily glycosylated, with almost 50% of the total mass consisting of poorly or non-immunogenic glycans (Leonard et al., 1990) shielding antibody access to the epitope. It has been reported that changes in glycan packing yields viruses resistant to the autologous neutralizing antibody response (Wei et al., 2003, Wibmer et al., 2013). This neutralization escape mechanism is referred as an

“evolving glycan shield” (Wei et al., 2003). Moore et al. (2012) suggested that a change in glycan at amino acid 332 result in resistance to BnAb PGT128 (Moore et al., 2012). The trimeric nature of the envelope glycoprotein shields conserved regions, while exposing relative amorphous highly glycosylated loop structures. These regions tolerate high levels of variation and therefore can easily escape from neutralizing antibodies (Wyatt et al., 1998). Multiple studies have suggested that the V1/V2 loops cover conserved epitopes involved in the co-receptor binding site (Kwong et al., 2000), as deletion of these variable loops confers sensitivity to antibodies targeting this region (Sullivan et al., 1998, Wyatt et al., 1995). Van Gils et al (2011) have shown that an increase in the length of the V1V2 loop and the number of PNGS in that same region of the HIV-1 envelope glycoprotein is directly involved in the protection of HIV-1 against HIV-specific neutralizing antibodies (van Gils et al., 2011).

### **1.5.3 Broadly neutralizing antibodies (BnAbs)**

Until 2009, only a small number of first-generation BnAbs have been isolated from HIV-1 subtype B infected individuals (Zolla-Pazner, 2004). These BnAbs neutralize many primary isolates from different genetic subtypes, indicating some conserved structures on the envelope glycoproteins. The mapping of the sera of the HIV-1 individuals exhibiting broadly neutralizing activity against different strains revealed five predominant neutralizing antibody specificities: a) the CD4 binding site on gp120; b) glycan-dependent epitopes on gp120 that are N332A sensitive; c) an epitope in the vicinity of the CD4-induced site; d) a quaternary epitope on gp120 that is sensitive to the loss of glycosylation at position N160; and e) the conserved gp41 membrane proximal external region (MPER) (Figure 1.16). Passive immunization of primates challenged with chimeric SHIV strains has shown that human BnAbs can protect against infection and are effective against intravenous (Baba et al., 2000, Mascola et al., 1999), oral (Hofmann-Lehmann et al., 2001) or intravaginal challenges (Mascola et al., 2000, Parren et al., 2001, Veazey et al., 2003).

Since 2009, several second-generation BnAbs have been identified with improve breadth and potency activity against several different HIV-1 strains (Hraber et al., 2013) (Figure 1.16). These new BnAbs were derived from donors infected with different HIV-1 subtypes and the success of this effort was based on the combination of three strategies: a) the selection of chronically infected individuals with potent and cross-subtype reactive serum antibodies; b) the use of novel selection of screening approaches; and c) the development of efficient methods to isolate human monoclonal antibodies (Corti and Lanzavecchia, 2013).



**Figure1.16: Neutralizing monoclonal antibodies targeting HIV envelope.** (Figure from McCoy and Weiss 2013 adapted from Burton and Weiss 2010)

### 1.5.3.1 First-generation of HIV-1 broad neutralizing monoclonal antibodies

- **IgG1b12 (b12)**

Before 2009, the mAb with the best anti-HIV potency and breadth was b12 (Burton et al., 1994). It was selected by phage display of an antibody library constructed from the bone marrow of an HIV-1 infected donor. B12 recognizes the highly conserved CD4 binding site on gp120 and acts as a competitive inhibitor of CD4 (Roben et al., 1994). B12 interacts with its epitope via its unique extended long complementarity-determining region (CDR3 loop) from the variable domain of its heavy chain (Roben et al., 1994) allowing it to access the CD4 binding pocket (Saphire et al., 2001). Upon binding to its epitope, b12 induces little entropic and conformational changes suggesting a favourable fit into the CD4 binding site of gp120 (Kwong et al., 2002). The mAb b12 neutralized approximately half of the virus panel tested by Binley et al (Binley et al., 2004) and is thus reasonably broad.

- **2G12**

2G12 was selected from hybridomas of human peripheral blood B lymphocytes from HIV-1 positive volunteers; it targets a conserved cluster of oligo-mannose glycans on gp120 (Trkola et al., 1996). Binding of 2G12 could be abolished when the N-linked carbohydrates in the C1, C2, C3, C4, and V4 were removed from gp120 (Trkola et al., 1996). Similar to b12, 2G12 induces little conformational changes when bound to its epitope (Kwong et al., 2002). More recently, modifications in V1/V2 and V3 have been linked to 2G12 sensitivity (Chaillon et al., 2011). Mutagenesis studies have implicated the glycans at positions 295, 332, 339, 386 and 392 in gp120 as being the most critical for 2G12 binding (Sanders et al., 2002, Scanlan et al., 2002). 2G12 was tested in a neutralization assay against pseudoviruses representing subtypes A, B, C, D, F, AC and CRF01\_AE as well as 25 primary viral isolates in order to determine the neutralization breadth of the antibody (Binley et al., 2004). Results revealed that 2G12 could effectively neutralize 41% of the tested viruses none of which were from subtype C or E (Binley et al., 2004). The fact that 2G12 does not exhibit cross neutralization against subtype C isolates may be due to the lack of a glycan at position 295 at the N-terminal base of the V3 loop in subtype C viruses (Binley et al., 2004).

- **2F5 and 4E10**

The two most studied mAbs 2F5 (Barbato et al., 2003) and 4E10 (Stiegler et al., 2001) recognizes two adjacent highly conserved epitopes in the extreme C-terminal of the gp41 ectodomain, the MPER, and also have a lipid binding ability (Alam et al., 2009). This region is particularly attractive for vaccine design because it mediates the viral entry process and is highly conserved between viral strains.

The 2F5 epitope has been mapped to the motif ELDKWA at the end of the HR2 region of gp41 (Muster *et al.*, 1993), where the core residues D664, K665 and W666 are indispensable for antibody recognition (Zwick et al., 2005). 2F5 neutralized 67% of the isolates however shows poor neutralization capacity against subtype C isolates; possibly due to the fact that most subtype C viruses have a DSW motif instead of DKW (Purtscher et al., 1996, Stiegler et al., 2001).

4E10 recognizes the epitope containing the NWFDIT motif. Mutagenesis experiments have demonstrated that the residues W672, F673 and W680 are indispensable for recognition by 4E10 (Zwick et al., 2005). 4E10 exhibits potent cross-reactive neutralization activity against 90 viruses from subtypes A, B, C, D, F, G, J and AC, AE, AG, BF and BG recombinants (Binley et al., 2004).

- **Z13e1**

Z13 was isolated in 2001 from an antibody phage display library and was shown to recognize an epitope similar to that of 4E10 but with the ability to neutralize a limited set of primary viruses (Zwick et al., 2001). Recently, a high affinity variant of Z13 named Z13e1 was generated by mutagenesis and recognizes an epitope overlapping that of 2F5 and 4E10 (WASLWNWFDITN). Z13e1 exhibited about 100-fold better affinity for the MPER as well as enhanced neutralization capacity against sensitive strains of HIV-1 (Nelson et al., 2007).

- **447.52D**

The anti-V3 neutralizing antibody 447.52D shows moderate neutralization breadth by interacting with a conserved GPGR motif in subtype B isolates via its long CDR H3 loop in a sequence independent manner (Conley et al., 1994). However, for CCR5-utilizing viruses, the V3 loop is poorly immunogenic before CD4 binding therefore 447.52D has limited neutralization breadth against these isolates (Lusso et al., 2005). Interestingly, 447.52D has been shown to neutralize 45% of subtype B viruses but only 7% of other subtypes (A, B, C, D, F, AC and AE as well as 25 primary viral isolates) (Binley et al., 2004). Neutralization of non-B subtypes largely depends on the presence of the GPGR motif (Binley et al., 2004).

### **1.5.3.2 Second generation of HIV-1 broad neutralizing monoclonal antibodies**

Although much was learnt from the first-generation of mAbs described above, there was relatively limited breadth and potency, especially with respect to non-subtype B viruses, which account for the majority of HIV-1 infections. These mAbs are unable to completely eliminate viruses from infected individuals and the use of immunogens based on their epitopes has also failed to elicit broadly neutralizing immune responses (Mehandru et al., 2007, Trkola et al., 2005). Therefore, novel approaches to isolate new BnAbs with more breadth and potency are needed.

- **PG9 and PG16**

Recently in 2009, PG9 and PG16 have been identified by the adaptation of single B cell cloning to a functional high-throughput screening of about 30,000 activated memory B cells from a subtype A-infected African donor (Walker et al., 2009). PG9 and PG16 neutralize between 70 and 80% strains of HIV tested (Walker et al., 2009) by binding preferentially to trimeric Env via the V1/V2 loops of the gp120 subunit (McLellan et al., 2011, Walker et al., 2009). Mutagenesis analysis revealed that their epitopes are primarily located in the conserved regions of the V2 and

V3 loops of gp120 and the preferential binding to trimeric gp120 is due to gp120 subunit presentation in the context of trimeric viral spike rather than gp120 cross-linking (reviewed by Chen et al., 2012).

- **VRC01 and VRC02/VRC03**

In 2010, two novel CD4-binding sites mAbs were identified named as VRC01 and its somatic variants VRC02 and VRC03 (Zhou et al., 2010, Wu et al., 2010b). VRC01 and VRC02 were identified by isolating single mAb-producing B cells with an antigenically resurfaced HIV-1 gp120 core protein (Wu et al., 2010b). Both antibodies neutralizes with high potency *in vitro* up to 90% of circulating HIV-1 strains tested (Wu et al., 2010b, Zhou et al., 2010, Li et al., 2011). VRC03 showed less neutralization potency than VRC01 and VRC02, with the ability to neutralize 57% of HIV-1 diverse primary isolates, including subtypes A, B, C, D, G and CRF01\_AE and CRF07\_BC (Wu et al., 2010b). VRC01 binds to a highly conserved portion of the CD4 binding site. Interestingly there is a significant correlation to the residues on gp120 involved in CD4 binding as well as VRC01 binding (Zhou et al., 2010). This unique antibody partially mimics the CD4 molecule with 73% homology with the CD4 N-terminal domain (Zhou et al., 2010). An interesting feature of the VRC01 antibody is that it targets the outer domain of the CD4 binding site which is occluded by glycan shields (Zhou et al., 2010). The VRC01 light chain makes contact with the N-linked glycan at residue 276 on gp120; therefore VRC01 uses the glycan for binding rather than being occluded by it (Zhou et al., 2010).

- **HJ16**

HJ16 is another mAb isolated in 2010, which recognize an epitope in the CD4-binding site (Corti et al., 2010). It competes with sCD4 and b12 in binding to gp120s, and exhibits potent and selective neutralization of Tier.2 viruses (reviewed by Chen et al., 2012).

- **PGT121.145**

The PGT121 antibody family was identified from African donor 17 of the IAVI Protocol G cohort (Walker et al., 2011). It consists of three primary members (PGT121, PGT122 and PGT123), and additional antibodies from this donor described recently (125.131) (Pejchal et al., 2011). This family of BnAbs neutralizes 65–80% of HIV-1 isolates and are the most potent anti-HIV-1 BnAbs identified to date, with a median IC<sub>50</sub> ranging between 0.03–0.05 µg/mL (Walker et al., 2011). This family of antibodies competes with PG9/PG16 and

interacts with two glycans at positions N301 and N332, as well as with the base of the gp120 V3 loop (Pejchal et al., 2011, Walker et al., 2011, Diskin et al., 2011).

- **NIH45.46**

NIH45.46 is a new CD4-binding site mAb identified in 2011 (Diskin et al., 2011). It is a more potent clonal variant of VRC01 that was isolated from the same donor with substantial advances in terms of potency and breadth of the antibody. This antibody did not have a large aromatic residue to contact the hydrophobic pocket between the CD4 binding loop and the bridging sheet on Env, as does CD4 itself (Diskin et al., 2011). By mutating a phenylalanine into this position on NIH45.46, an extremely potent CD4bs mAb was generated which could provide protection against approximately 75% of circulating strains at levels as low as 0.1 µg/ml (Diskin et al., 2011).

- **10E8**

Recently, 10E8 was isolated from an HIV-1 infected individual with high neutralization titers (Huang et al., 2012). 10E8 is an anti-MPER mAb and is among the broadest and most potent of antibodies described so far, neutralizing 95% of viruses tested with a median IC<sub>50</sub> of 0.35 µg/ml. 10E8 lacks many of the characteristics previously thought to limit the usefulness of MPER-specific antibodies in vaccines or passive therapies, including lipid binding and auto-reactivity (Huang et al., 2012). The crystal structure of 10E8, along with biochemical binding studies, demonstrates that the breadth of 10E8 is mediated by its unique mode of recognition of a structurally conserved site of vulnerability within the gp41 MPER (Huang et al., 2012).

## **1.6 Vaccine development**

Most vaccines work by stimulating an antibody response against the disease of interest. These antibodies then allow the immune system of the individual to fight off the pathogen. Normally, the immune system only makes large amounts of antibodies after an infection has taken place. Vaccination allows the antibodies to be made before a person has even been exposed to the disease, so the body will be ready to protect itself when it is actually exposed. After 30 years of HIV-1 pandemic, no vaccine exists for clinical use to prevent HIV-1 infection. The reasons are multiples and include the remarkable high HIV-1 diversity; and the host's inability to mount antibodies to targets within conserved envelope regions that confer broad neutralization. Recent insights of ways that vaccines can potentially stimulate protective T- and B-cell immunity, the identification of new targets for BnAbs, and the

discovery of new mechanisms of host control of HIV-BnAbs induction offer renewed hope for the development of a well-tolerated and effective preventive HIV-1 vaccine (Haynes and McElrath, 2013).

Traditional vaccine strategies have focused on live attenuated viruses, whole killed viruses and protein subunits (Baba et al., 1999, Learmont et al., 1999). These approaches have proven successful against other viruses such as the influenza virus but raise great safety concerns with regard to HIV-1 (Giri et al., 2004). More recent vaccine strategies have made use of gene delivery technologies such as plasmid DNA vaccines, recombinant viral vectors (attenuated or replication-incompetent viruses) such as adenoviruses (Priddy et al., 2008) or poxviruses (Harari et al., 2008).

A vaccine trial by Merck made use of a candidate which comprised a recombinant adenovirus vector that expressed HIV-1 subtype B *gag*, *pol* and *nef* genes (Priddy et al., 2008). However participants with pre-existing antibodies against the adenovirus vector showed suppressed immune responses to the vaccine (Kostense et al., 2004). Phase II b clinical trial studies were subsequently initiated to evaluate 3000 subjects for HIV-1 specific cellular immune responses elicited by this vaccine regimen. The trial was co-funded by the National Institute of Allergy and infectious disease (NIAID) and Merck & co. This was known as the STEP study and was conducted in America, Caribbean and Australia (McElrath et al., 2008). Unexpectedly, this study was brought to an early end due to safety concerns. It was hypothesized that the recombinant adenovirus vector may have increased the acquisition of HIV-1 infection in some individuals (Sekaly, 2008, Watkins et al., 2008). The results of the trial have caused scientists to call for a re-examination of vaccine development strategies.

The first efficacy trial (Vax004) began in 1998. The Vax004 trial was aimed at determining the efficacy of a recombinant bivalent subtype B gp120 vaccine (AIDSVAX) in preventing sexual transmission of HIV-1 in North America and the Netherlands, where subtype B infections are prevalent (Berman, 1998). The trial included 5108 men who have sex with men (MSM) and 309 high risk women. Although the AIDSVAX vaccine elicited strong antibody responses, it failed to protect against HIV-1 infection and had no effect on the viral loads of participants who acquired HIV-1 infection after vaccination (Gilbert et al., 2005). This same vaccine was retested in Thailand within a vaccine regimen called RV 144. The vaccine was named as "ALVAC.AIDSVAX B/E" that uses ALVAC.HIV from Aventis-Pasteur with a combination of genetic elements of several different HIV strains encapsulated in a harmless canary pox virus vector in addition to elements of the HIV strains circulating in Thailand

(subtype E) and in US (Subtype B). The latest results from the RV144 trial showed, for the first time, a statistically significant 30% protective efficacy (Rerks-Ngarm et al., 2009). It was reported that subjects in the study in which antibodies recognizes the V1V2 loop in the HIV envelope protein gp120 were 43% less likely to become infected (Rolland et al., 2012) but those who produced envelope specific IgA were 54% more likely to become infected. This suggests that an effective HIV-1 vaccine should include components that elicit responses in both arms of the immune system. In summary, the major strategies that are being pursued include several approaches for the induction of broad nAbs, the augmentation of the quality and the quantity of non-neutralizing V1V2 antibodies as seen in the RV144 immune correlates study, and the development of vaccine vectors that better represent critical T-cell epitopes and the viral diversity of circulating strains (Haynes and McElrath, 2013).

### 1.7 Importance to study HIV-1 subtype G

HIV-1 subtype G is responsible for an estimated 1,500,000 HIV-1 infections worldwide, making it the sixth most prevalent subtype of HIV-1, after subtypes A, B, C, CRF01\_AE and CRF02\_AG (Hemelaar et al., 2006). Subtype G is endemic in West and Central Africa (Figure 1.17). A few studies describe the prevalence of subtype G (30.54%) in Nigeria (Ajoge et al., 2012, McCutchan et al., 1999, Peeters et al., 2000, Agwale et al., 2002), in Cameroon (4.5.12%) (Brennan et al., 2008, Tongo et al., 2013, Teto et al., 2013, Ndembi et al., 2008), in Congo (21%) (Niama et al., 2006), in Central African Republic (11%) (Marechal et al., 2006), and Democratic Republic of Congo (10%) (Vidal et al., 2000). At lower prevalence, subtype G is also found in Spain (Thomson et al., 2001, Delgado et al., 2002), in Portugal (Esteves et al., 2002), and Cuba (Perez et al., 2006, Sierra et al., 2007).



**Figure 1.17: Distribution of HIV-1 subtype G in 2004.2007.** (Figure from Hemelaar et al., 2001)

Segments of subtype G are present in 19 of the 43 (44%) classified CRFs in Los Alamos National Library (LANL) database ([www.lanl.org](http://www.lanl.org)). In Cameroon, the majority (84%) of HIV-1 infection among blood donors were caused by CRFs and unique recombinant forms (URFs) and 80% of the recombinant strains containing segments of subtype G (Yamaguchi et al., 2009).

A great debate has recently risen on whether subtype G is a pure subtype or CRF. Abecasis et al (2007) proposed that subtype G is actually a recombinant derived from parental strains CRF02\_AG, and subtype J, and that CRF02\_AG is a non recombinant subtype (Abecasis et al., 2007). Since the different subtypes are assumed to have evolved independently, different genome regions are expected to have the same evolutionary history. However, this was not apparently the case for subtype G (Abecasis et al., 2007). The subtype G designation was based on a classification of partial genome sequences from *gag*, *pol*, and *env* regions (Robertson et al., 2000). This was a preliminary classification because without a complete genome sequence, inter-subtype recombination may not be recognized (Yamaguchi et al., 2009). This was the case when analysis of six HIV-1 isolates from Saudi Arabia were designated as subtype G based on partial *gag*, *pol* and *env* sequences and found to be CRF25\_cpx and CRF43\_02G when the complete genome sequences were obtained (Yamaguchi et al., 2008). However, Yamaguchi et al (2009) reported recently five full length subtype G genome from Cameroon as pure subtype G based on the current classification of HIV-1 strains (Yamaguchi et al., 2009).

## **1.8 Rationale of the study**

Antibody that neutralizes HIV is likely be effective in preventing establishment of new infections and thus may be a critical product of an effective prophylactic HIV vaccine. The study proposed for this thesis is designed to provide some of the basic knowledge that should be helpful in developing such a vaccine. In particular, a vaccine should be a global vaccine, effective against any of the major subtypes of HIV-1 found in the world today.

HIV-1 subtype G accounts for 5% of HIV-1 infection, making it the sixth most prevalent subtype of HIV-1, after subtypes A, B, C, CRF01\_AE and CRF02\_AG (Hemelaar et al., 2006). Subtype G is concentrated in West and Central Africa (Hemelaar et al., 2011a) with spread to European countries, primarily Spain and Portugal, presumably with immigration of infected individuals and small satellite subtype G epidemics have been documented in Cuba

and among Russian intravenous drug users. In addition, 80% of the recombinant strains circulating in Cameroon contain segments attributed to subtype G (Brennan et al., 2008). However, until recently, little research has been done on the neutralization sensitivity and vulnerabilities of subtype G viruses, particularly those that circulate in its main reservoir. Systematic analyses of a wide range of HIV-1 envelope sequences (Brown et al., 2005) and neutralization patterns (Brown et al., 2008, Seaman et al., 2010) have been performed. However, subtype G viruses are severely underrepresented in these works. Brown et al (2005) studied only two subtype G-associated sequences while Brown et al (2008) did not study the neutralization patterns of any subtype G viruses and Seaman et al (2010) evaluated the intrinsic neutralization resistance of only one putative subtype G virus. A recent study has one neutralization study of Subtype G viruses (Revilla et al., 2011). In this work, only two of the subtype G sequences were derived from individuals from West or Central Africa and no testing of intrinsic neutralization capacity was performed, which is critical for evaluating which viruses should be included in test panels for vaccine evaluation.

Understanding the role of nAbs and the molecular characteristics of the subtype G HIV-1 variants involved are important for the development of effective HIV/AIDS vaccine and passive immunization approaches to HIV-1.

There is thus an urgent and important need to study subtype G envelope sequences and neutralization capacity of antibodies to subtype G envelope viruses. This will allow immunity to subtype G viruses to be evaluated, both in infected donors and those immunized with test vaccines in the future.

That is why our study focused on both molecular characterization of subtype G viruses and the role of nAbs to subtype G HIV-1 viruses. We restricted our analysis to subtype G HIV-1 variants circulating in Cameroon which accounts for the most predominant HIV-1 subtype circulating in Cameroon, with the hope that our work would benefit to the development of strategies for prevention of HIV-1 infection, especially in Cameroon.

### **Objectives of the study**

The goal of this project was to generate and characterise HIV-1 subtype G envelope clones and to define the role of neutralizing antibodies to subtype G viruses expressing these envelopes. The ultimate purpose for pursuing this project was to contribute to immunogen design of an effective HIV vaccine that elicits broadly neutralizing antibodies to prevent infection.

This was achieved by the following specific objectives:

**Specific objective 1:** To examine the molecular and functional characteristics of the HIV-1 envelope glycoproteins of subtype G viral variants that are crucial to improving strategies to prevent HIV-1 transmission.

**Specific objective 2:** To evaluate the neutralization sensitivity of subtype G viruses and the neutralizing capacities of antibodies induced by the viruses by determining the neutralization antibodies titers against autologous and heterologous HIV-1 viral isolates.

**Specific objective 3:** To characterize the sensitivity of HIV-1 subtype G viral isolates against broadly neutralizing antibodies and HIV-1 entry inhibitors, to gain insight into the neutralization vulnerabilities of the subtype G viruses.

## **CHAPTER 2**

### **RESULTS**

# Genetic, functional and co-receptor properties of HIV-1 subtype G envelope genes circulating in Cameroon

## 2.1 Abstract

### Background

HIV-1 subtype G circulates mainly in West and Central Africa and infects an estimated 1.5 million individuals. Characterization of subtype G strains has been very limited so far; it is not clear that the full diversity of subtype G is reflected in available sequences. There is a need to better understand the genetic and biological properties of HIV-1 subtype G isolates because 80% of the recombinant strains circulating in Cameroon contain segments attributed to subtype G. We characterized 47 full length envelope genes isolated from plasma RNA by single genome amplification (SGA) obtained from 8 HIV-1 subtype G-infected individuals. Five study participants were blood donors later determined to be HIV-infected and the remaining three were undergoing testing for antiretroviral drug resistance.

### Results

Samples were identified as subtype G-infected by prior sequencing of segments of *gag* and *nef* genes or in *pol* genes, and all *env* sequences obtained clustered more closely with subtype G reference sequences than other reference sequences. The five blood donors were classified as likely to be recent infections (<6 months, n=3) and not-recent infection (n=2) using a BED assay. All sequences from each individual donor clustered together, suggesting infection derived from single or highly related viruses. Phylogenetic trees of viruses from three individuals showed structures suggestive of infection with distinct but related viruses with evidence of recent recombination within related sequences, although other explanations are possible. One donor's sequences (12541) clustered in a subnode with CRF06\_cpx sequences and sequences from Russia and Cameroon. Genetic distance between *env* sequences from isolates from the same donor ("within-donor genetic distance") was uniformly high in samples from donors undergoing antiretroviral resistance testing. One sample from the blood donor group had unusually low within-donor genetic distance; equivalent to that seen in very recent infection (<90 days), and had unusually high V4 lengths. In general, our subtype G clones exhibit lower V1V2 and V4 region lengths compared to the well characterized subtype B. All envelope clones exhibits CCR5 co-receptor usage predicted by web PSSM and geno2pheno and confirmed in TZM-bl cells by inhibition of invasion of pseudoviruses with CCR5 binding inhibitor TAK-779.

## **Conclusion**

Although there are more than 100 subtype G *env* sequences in the database, some of our sequences showed relatively poor similarity to them. This suggests that the full extent of the diversity of Subtype G has not yet been described, and provides additional evidence for the hypothesis that subtype G itself may be very old. This study highlights the identification of apparent recombination events in some individuals likely due to infection with two highly related sequences from the same donor, or the existence of a recombination product, one of whose parents is present and the other which was not present in the sequences obtained. The majority of HIV-1 subtype G infections are caused by viruses that exclusively utilize CCR5 co-receptor, as previously found in the literature for a small number of tested subtype G samples. These findings are likely to provide useful information for the design and the evaluation of HIV-1 vaccine and for a better understanding of the HIV-1 epidemic.

## 2.2 Introduction

Cameroon is located in West Central Africa, the epicenter of the HIV epidemic, where many type and subtypes of HIV-1, as well as HIV-2, co-circulate (Peeters et al., 2003, Vergne et al., 2003). Cameroon has one of the most genetically diverse HIV epidemics in the world and the widest variety of circulating recombinant forms (CRFs) of HIV-1 despite relatively low HIV prevalence (Takehisa et al., 1998, Machuca et al., 2007, Ndembi et al., 2008). HIV-1 subtype G is responsible for an estimated 1,500,000 HIV-1 infections worldwide, making it the sixth most prevalent subtype of HIV-1 worldwide (Hemelaar et al., 2011a). Subtype G has a complex relationship with CRF02\_AG, which was originally presumed to be a recombinant derived partly from subtype G (Abecasis et al., 2007). CRF02\_AG is one of the mostly commonly found subtypes in Cameroon (Agyingi et al., 2013, Vidal et al., 2013, Machuca et al., 2007), and 80% of the recombinant strains circulating in Cameroon contains segments attributed to subtype G (Brennan et al., 2008). Nonetheless, subtype G sequences and viruses are incompletely characterized. The *env* gene of HIV is the only target for an antibody-based vaccine. Due in part to the unique pressure of antibody-mediated immunity, it exhibits the highest degree of genetic variation due to recombination, mutation and escape from host immune response making challenging the development of a vaccine (Wei et al., 2003). Changes in response to immune pressure in *env* include changes in the length of variable loops, the number and position of glycosylation sites, and the net charge value due to amino acid substitutions (Burton et al., 2005, Moore et al., 2012, Wei et al., 2003).

Several studies in well characterized subtypes such as subtype B and C *env* genes have shown that some of these changes may impact the neutralization phenotype and can also change the cellular tropism (Mascola and Montefiori, 2003, Reitter and Desrosiers, 1998, Bunnik et al., 2008, Cao et al., 1997, Moore et al., 2009a, Rong et al., 2007b, Rong et al., 2009, Sagar et al., 2006, van Gils et al., 2011, Cilliers et al., 2003, Briggs et al., 2000, Cann et al., 1992, Fouchier et al., 1992). In addition, in contrast to subtypes B and C, there are few studies of subtype G clones with respect to co-receptor use (Revilla et al., 2011, Tebit et al., 2002, Zhong et al., 2003, Brandful et al., 2007).

In this study, the genetic properties of full length *env* from 8 individuals infected with HIV-1 subtype G were investigated. Five were blood donors subsequently found to be HIV-1 infected and three were individuals who submitted samples for antiretroviral resistance testing. The latter three were included because we do not expect antiretroviral drug resistance selection to exert pressure upon the *env* gene. In analyzing these samples, substantial diversity

was found in within-donor diversity, and thus presumably also in time since infection. Some of our samples include similarity to CRF06\_cpx *env* sequences within the subtype G phylogenetic tree that nonetheless cluster better with subtype G reference sequences than any other available reference sequences showing that the HIV-1 subtype G database plausibly does not reflect the full diversity of HIV-1 *env*.

## 2.3 Methods

### 2.3.1 Study participants

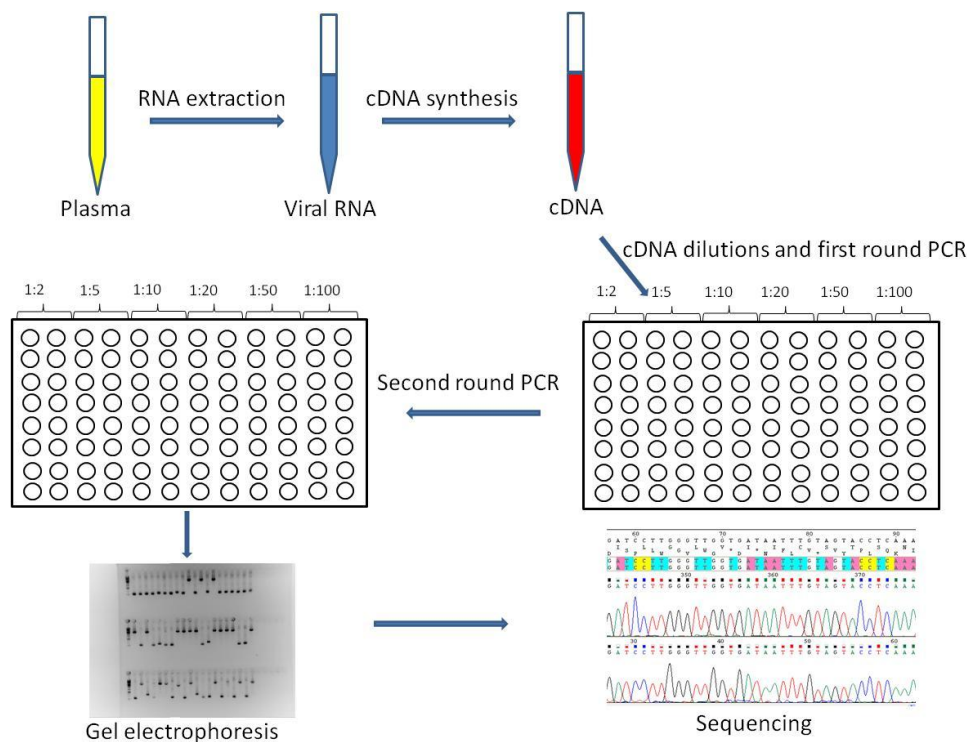
Anonymous blood samples found to be HIV-1 infected were obtained from Yaoundé Central Hospital Blood Service, Yaoundé, Cameroon (n=64) between December 2006 and August 2007 and were subtyped by sequencing and phylogenetic analysis of full length sequences of *gag* and *nef* (Jacob et al., 2012, Tongo et al., 2013). Five samples (8%) contained subtype G sequences in both *gag* and *nef* and were included in the present study. An additional ninety-seven samples from CIRCB Research Institute in Yaoundé were collected for antiretroviral resistance testing, presumably because they were failing therapy, and these donors were recruited for research studies at CIRCB, including the present study. Five were found to have subtype G sequences in the fragment of the *pol* gene analyzed for antiretroviral resistance, and three of those five were included in the current study. These three samples were collected between June 2009 and April 2010. CD4<sup>+</sup> T cells counts and viral load were also measured. The latter three were included because we do not expect antiretroviral drug resistance selection to exert pressure upon the *env* gene. This study received approval from the CIRCB Ethics Committee and the Human Research Ethics committee of the University of Cape Town, Faculty of Health Sciences (REC NO: 107/2011).

### 2.3.2 Viral RNA extraction and cDNA synthesis

RNA was extracted from the plasma (250µl) using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Complimentary DNA (cDNA) was synthesized from the entire extracted RNA using the SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Following the completion of the reverse transcription step, the reverse transcriptase was inactivated by incubation at 70°C for 15 minutes followed by digestion of the RNA by RNase H (Invitrogen) for a digestion at 37°C for 20 minutes. The cDNA was therefore either used immediately for PCR or stored at -20°C until analysis.

### 2.3.3 Single genome analysis (SGA)

Single genome analysis (Simmonds et al., 1990, Salazar-Gonzalez et al., 2008) is a method for amplifying single genomes within a multigenome population, such as the cDNA we synthesised from plasma. By diluting the cDNA we increase the likelihood that a PCR amplification will contain only a single genome from the population. This approach has been used extensively (Keele et al., 2008, Salazar-Gonzalez et al., 2009, Abrahams et al., 2009). Figure 2.1 illustrates the process of single genome amplification in order to achieve the desired dilution for viral quasispecies.



**Figure2. 1: Experimental overview of single genome analysis.** Several dilutions of the cDNA solutions are subject to PCR amplification of the *env* gene. PCR products from dilutions >20% positive PCR reaction were excluded from the analysis. If for example the 1:50 dilution resulted in a <20% positive PCR reaction rate, for that particular participant, the 1:50 dilution of cDNA was then repeated in a larger number of wells. To further confirm single-genome amplification, the entire envelope gene was sequenced in forward and reverse directions and any sequences containing double peaks were excluded from the analysis (This figure was drawn for the thesis).

Several dilutions of the cDNA solutions are subject to PCR amplification of the *env* gene. The least dilute sample with <20% of wells positive was then repeated in a larger number of wells. The cDNA dilution where less than 20% of reactions yield a positive PCR amplification product is likely to contain one single amplifiable cDNA template. Single genome analysis

was confirmed when a directly sequenced PCR product gave rise to a sequence with a single peak.

#### **2.3.4 Full length *env* PCR amplification and sequencing**

Two primer sets were tested for the 2-stage PCR used for the SGA-based amplifications. The first set has been used extensively to amplify envelopes from other subtypes (Abrahams et al., 2009, Salazar-Gonzalez et al., 2008). The second set of primers was modified to reflect as best as possible the commonly found polymorphisms where these primers annealed in the database subtype G sequences available at the start of this project. Amplifications were performed using FastStart Taq DNA polymerase (Roche Diagnostics). Second round PCR products were run on a 0.8% agarose gel and visualized under UV light with UVP system (UVP, Upland, CA). Bands between 2500-3000 bp were marked to be positive for full length HIV-1 envelope. Before sequencing, post PCR clean-up was performed to each PCR products using either DNA clean and concentrator kit (Zymo Research) or the Wizard SV Gel and PCR clean-up system (Promega). Sequencing was done at the University of Stellenbosch core sequencing facility using a standard set of twelve overlapping primers to generate sequence encompassing the entire *env* open reading frame.

#### **2.3.5 Sequence analysis**

Full length *env* gene sequences were assembled and edited using Chromaspro in the software package Bioedit and first aligned by MUSCLE in the software MEGA 5 (Tamura et al., 2007), followed by manual adjustment to optimize the alignment. All chromatograms were carefully inspected for sites of ambiguous sequences (double peaks), and those that displayed doubled peaks were excluded from further analysis. Sequences containing premature stop codons or frame shifts were also excluded. Reference sequences were obtained from the Los Alamos National Library (LANL) HIV sequence database ([www.hiv.lanl.gov](http://www.hiv.lanl.gov)) and used to determine the HIV-1 subtype of all nucleotide sequences obtained from our patients together with the REGA HIV Subtyping Tool Version 2.0 (<http://www.bioafrica.net>). Maximum likelihood phylogenetic trees were constructed from these sequences with 100 full maximum likelihood bootstrap replicates (implemented in PHYML(Guindon et al., 2005)), and using the maximum likelihood method implemented in MEGA 5. The reliability of the branching orders was tested by bootstrap analysis of 1,000 replicates when using MEGA 5. Potential N-linked glycosylation sites (PNGS) were identified using the web-based program N-Glycosite ([www.hiv.lanl.gov](http://www.hiv.lanl.gov)). For each patient, the pairwise DNA distances (genetic distance) were calculated using MEGA 5 and differences were visualized using highlighter plots generated

by Highlighter ([www.hiv.lanl.gov](http://www.hiv.lanl.gov)). Consensus sequences were generated for each individual using consensus maker tool ([www.hiv.lanl.gov](http://www.hiv.lanl.gov)). Recombination analysis was performed using an advanced recombination detection program (RDP4) (Martin et al., 2005) to detect intra-patient recombination events in DNA sequences, and a bootstrap value equal to or greater than 70% was considered as significant.

### **2.3.6 Co-receptor usage prediction and co-receptor usage assay**

The co-receptor usage of all *env* sequences was predicted based on V3 amino acid sequences using various approaches: the 11/25 rule which is taking into account the key residues 11 and 25 and total net charge of V3 loop (De Jong et al., 1992, Fouchier et al., 1992), Geno2pheno which is the bioinformatics tool based on the statistical model support vector machines (SVM) (Lengauer et al., 2007), and WebPSSM which is a tool based on the statistical model position specific scoring matrix (PSSM) (Jensen et al., 2006). Co-receptor usage assay of the newly derived *env* pseudoviruses was determined in TZM-bl cells by using two blocking agents: TAK-779 (NIH HIV reagent repository), a CCR5 receptor antagonist, and AMD-3100 (NIH HIV reagent repository), a CXCR4 antagonist and was adapted as previously reported (Fouda et al., 2013, Wang et al., 2013, Li et al., 2006b). 100 µl of freshly trypsinized TZM-bl cells were distributed into 96-well plates (10,000 cells/well in DMEM.10% FBS containing HEPES and 7.5 µg/ml of DEAE-dextran) and incubated for 1 hour with either 50 µl of TAK-779 (3.3µM), AMD-3100 (430nM), a combination of the two reagents, or no inhibitor (control wells). 50 µl of Pseudoviruses were added to wells with the different treatments in duplicate. The volume of each pseudoviruses added (50 µl) was normalized by titration to 50.000 relative luminescence units (RLU) which is directly proportional to the number of infectious virus particles present in the initial inoculums. This standard protocol is followed by the National Institute for Communicable Disease (NICD) and by our laboratory (Montefiori, 2009). After 48 hours of incubation, luminescence was measured (Bright-Glo, Promega) and the fold drop in infectivity due to the addition of inhibitor was calculated. Wells containing co-receptor inhibitors were compared to control wells to determine if either agent led to a reduction in infectivity. Viruses known to use either CCR5 (III B) or CXCR4 (Bal PS) were used as controls.

Virus percentage neutralizations were determined by the following calculation

$$\left( \frac{\text{Difference in average RLU between virus control and sample}}{\text{Difference in average RLU between virus control and cell control}} \right) \times 100\%.$$

## 2.4 Results

### 2.4.1 Study participants

The five blood bank samples were screened for longer duration of HIV-1 infection using the BED™ HIV-1 incidence test kit (Parekh et al., 2011) (CALYPTE Biomedical, Portland, Oregon, USA) and the median BED value was 0.67 (IQR=0.26-1.2). The BED assay detects increasing levels of anti-HIV IgG after seroconversion and can be used for detecting recent infections (Parekh et al., 2011). The BED assay values > 0.8 were associated with being infected for more than 5 months, and BED assay values < 0.8 were associated with a recent infection. The BED assay was not an appropriate test for the remaining three samples because ART substantially affects antibody levels independently of time since infection (Binley et al., 2000, Morris et al., 1998). All ART testing patients had viral loads, and the median viral loads and CD4+ T cell counts of study participants at the enrolment was 105796 Copies/ml (IQR= 58305-242775) and 463 cells/μl (IQR= 225-463) respectively (Table 2.1).

**Table2. 1:** Epidemiological and clinical information of participants

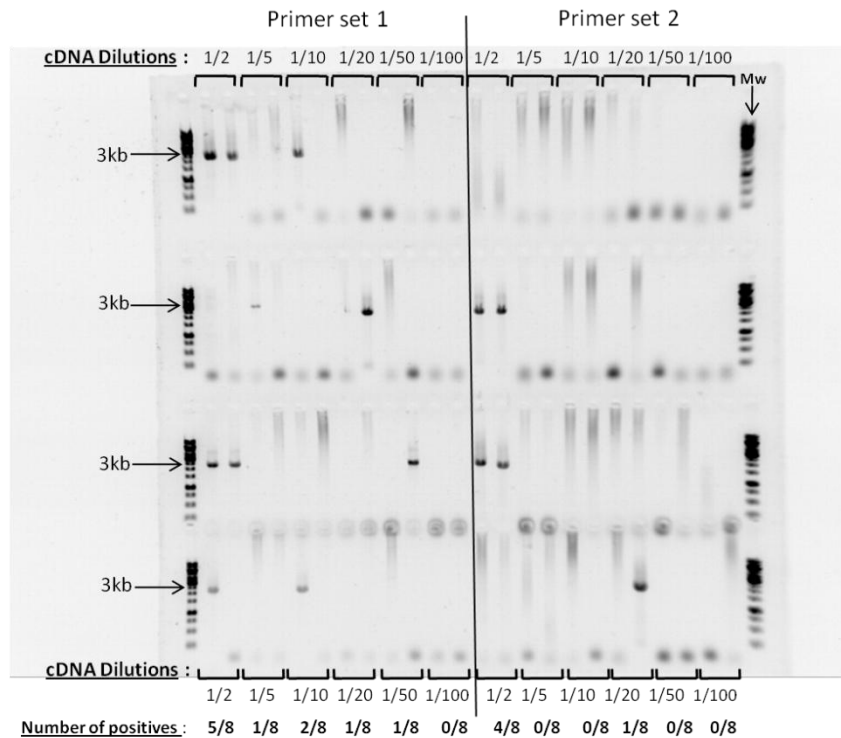
Sample ID	Subtype	Age	Sex	Viral load (Copies/ml)	CD4+ cell count (Cell/ul)	BED Assay	year of sample collection
BS03	G	Unk	M	346,463	Unk	0.67	2007
BS12	G	36	M	69,244	334	1.28	2007
BS46	G	46	F	79,555	230	0.36	2007
BS48	G	29	M	132,037	878	0.15	2007
BS51	G	31	M	257,039	278	1.12	2007
10056	G	36	F	54,658	225	ND	2009
11439	G	39	F	15,000	216	ND	2009
12541	G	7	F	199,982	463	ND	2010

Unk= unknown; Samples under ART: 10056, 11439 and 12541.

### 2.4.2 Primer sets

A previously used primer set, and a similar primer set we optimized for use in subtype G were tested on two samples (BS03 and BS51) (Figure 2.1). We found that the primer set modified to match known subtype G sequences (primer set 1) at the point at which the

primers are intended to anneal gave PCR products of the correct size. Subsequently, only the primer set optimized for subtype G (primer set 1) was used in this study.



**Figure 2. 2:** Example of a PCR gel showing a test of dilutions of cDNA (SGA) from one subtype G sample (BS03) using 2 sets of primers. Subtype G primers (primer set 1) and subtype C primers (primer set 2) are indicated on top of the figure. The expected size of a PCR product from HIV-1 envelope is also indicated on the left of the figure (3kb).

### 2.4.3 Phylogenetic analysis

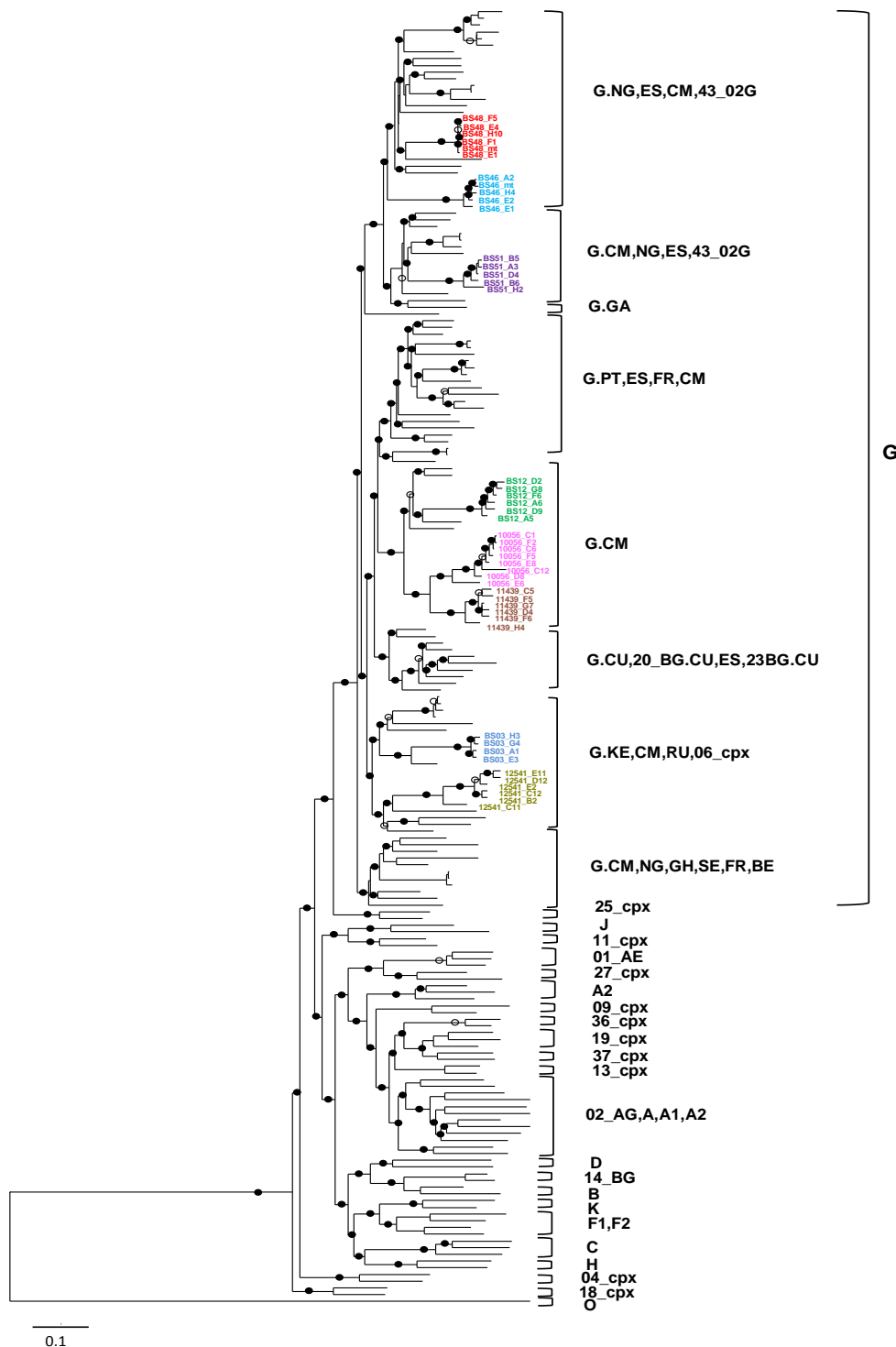
We analyzed in total 47 *env* sequences from which, 45 full length gp160 SGA *env* sequences generated from this study, each from an independent limiting dilution RT.PCR followed by sequencing (range 4.8 for each of eight study participants), and an additional two *env* sequences generated previously (Tongo et al., 2013). To determine the subtype classification, a maximum likelihood phylogenetic tree was constructed including group M reference sequences from LANL database. Sequences were screened for recombination and all our *env* sequences showed no evidence of inter-subtype recombination, suggesting that they are all homogeneously subtype G derived and any sequence derived from other subtypes is in a patch small enough to remain undetected. All sequences clustered into three major groups within the expected subtype G with 100 bootstrap values (Figure 2.2). Sequences from three individuals (BS48, BS46, and BS51) grouped with sequences predominantly from Nigeria

with few from Spain and Cameroon, sequences from three individuals (BS12, 10056, and 11439) that fell into a cluster comprising only sequences from Cameroon, that themselves appears linked to a major cluster of most of the sequences collected in Europe and Cuba. Sequences from two other individuals (BS03 and 12541) clustered with reference virus from Cameroon, Kenya, Russia, and CRF06\_cpx. Moreover, all *env* sequences from the 8 participants clustered into respective individual monophyletic lineages suggestive of infection with a single subtype.

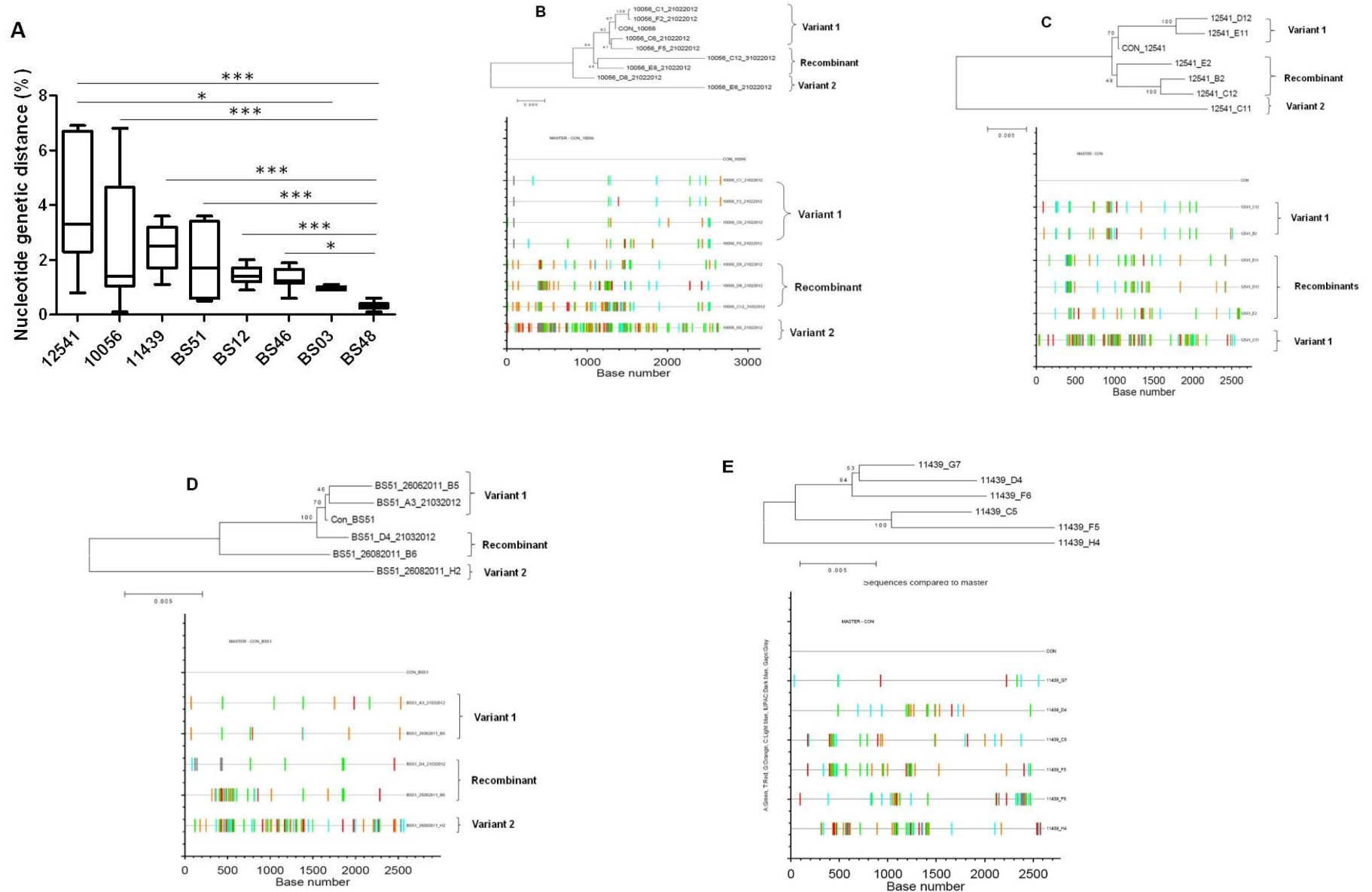
#### **2.4.4 DNA sequences divergences and highlighter plot analysis**

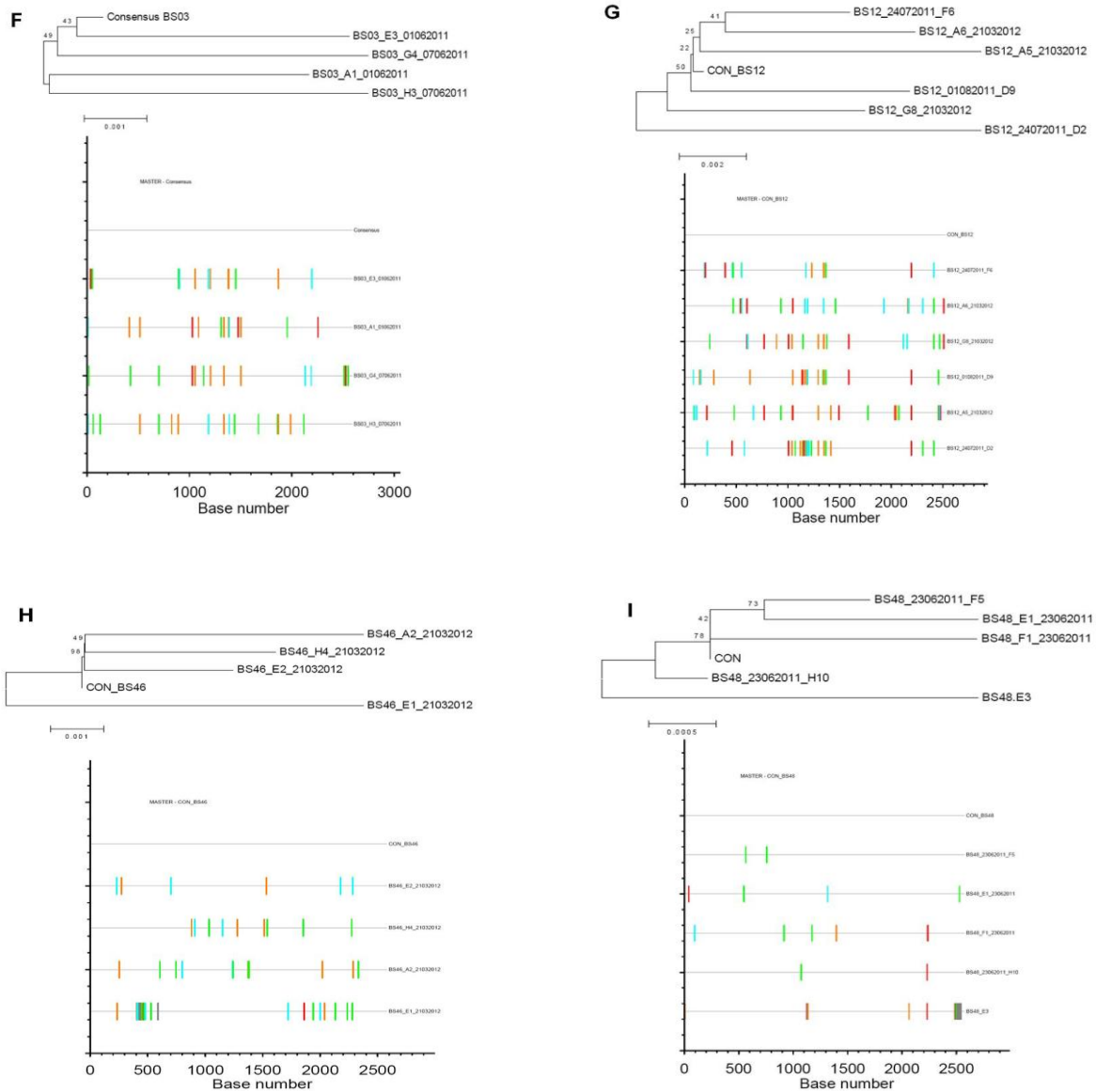
Intra-patient genetic distances varied substantially among the patients (Figure 2.3.A). The median intra-patient distance was unusually low in one patient, BS48, with values similar to those normally observed in the first 90 days following infection with a single viral variant (Keele et al., 2008). Consistent with a short time since infection, BS48 had the lowest BED assay value (Table 1). The median intra-patient distance was high (>1.5%) in 4 individuals; 10056, 11439, BS51, and 12541 with 3.45% (IQR= 0.1-6.8%), 2.1% (IQR=1.3-2.9%), 1.7% (IQR= 0.6-3.4) and 3.3% (IQR=2.3-6.7%) respectively, indicating a likely long period of time since infection and/or infection with multiple variants. Consistent with a long time since infection, BS51 had a high BED assay value (Table 2.1). The other three individuals were infected long enough to be on antiretroviral treatment.

Sequences from each single donor grouped together on the phylogenetic tree, suggesting that each donor was infected with one virus or closely related viruses (Figure 2.3.B-I). However, the tree structure of three donors (10056, 12541 and BS51) showed substantial structure with one sequence from each donor appearing on the tree as an outlier (Figure 2.3.B-D). Several possibilities could explain this observation, including infection with two highly related sequences from the same donor, or the existence of a recombination product, one of whose parents is present and the other which was not present in the sequences obtained.



**Figure2. 3: Maximum likelihood tree indicating the phylogenetic relationships between 193 *env* sequences of HIV-1.** The tree was constructed using 47 sequences generated in this study and HIV-1 reference sequences available in LANL with 100 bootstrap replicates. Sequences from each individual are displayed with different colors. The tree was rooted using HIV-1 group O isolate. Solid and open circles indicate branches with greater than 70% and 50% bootstrap support, respectively. Country codes are as follow: Cameroon=CM, Nigeria=NG, Spain= ES, Gabon=GA, Portugal= PT, France= FR, Cuba= CU, Kenya= KE, Russia= RU, Ghana= GH, Senegal= SE and Belgium= BE.



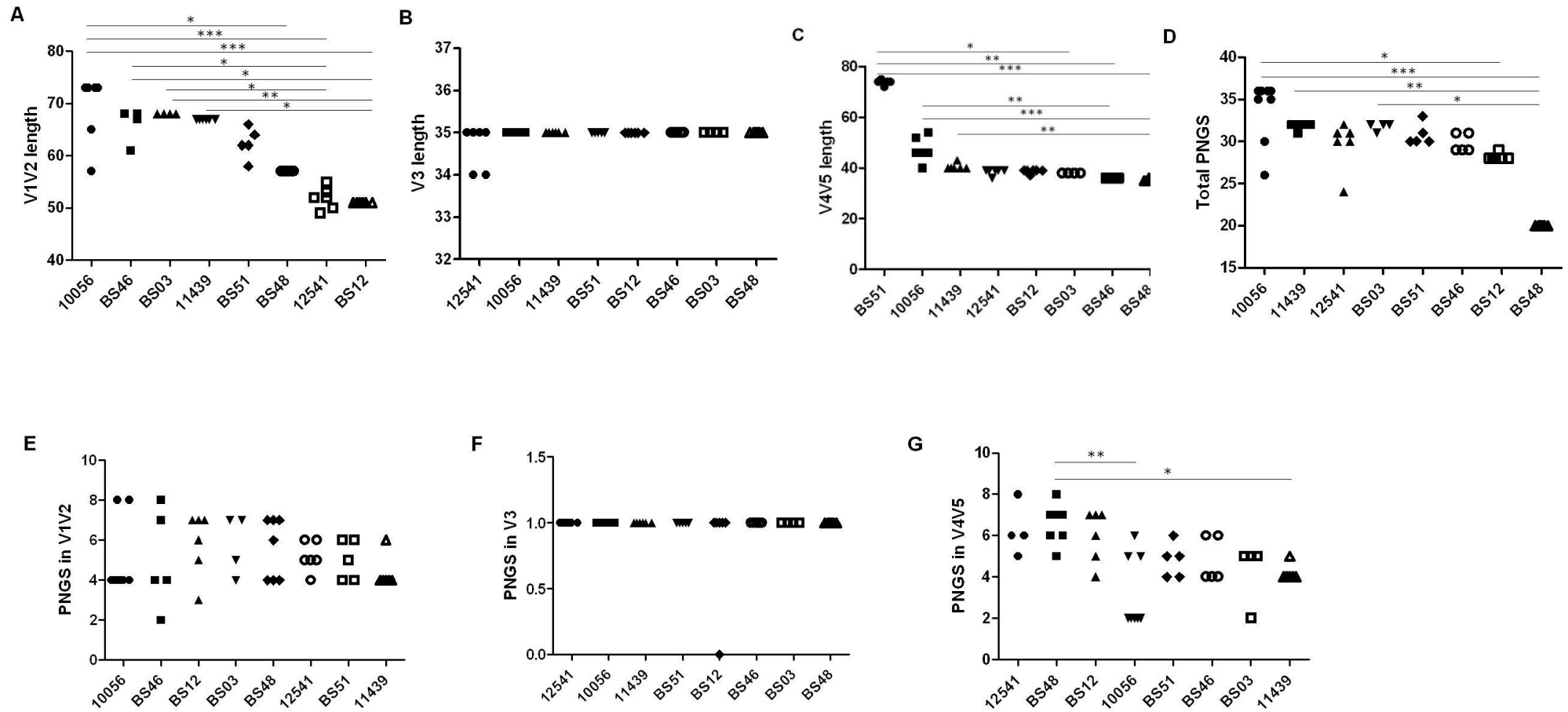


**Figure 2. 4: DNA genetic distances and highlighter analysis of the gp160 env sequences.** (A) Box plots of DNA genetic distances of the gp160 env sequences obtained from each donor. The line within each box represents the median value for each group. The  $P$  value was calculated by the one-way ANOVA, and individual differences were calculated using Dunn's multiple comparison test; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . (B-D), Neighbor joining phylogenetic tree and highlighter analysis of individuals exhibiting one sequence that appears as an outlier and (E-I), Individuals with no multiple variant events. The ticks in the highlighter plot represent the following: A: green, T: red, G: yellow, C: light blue, Gaps: gray.

#### **2.4.5 Lengths and potential N-linked glycosylation sites of the V1/V5 env sequences**

The degree of length polymorphism was analyzed in regions V1/V2, V3 and V4/V5. All sequences showed high conservation of the cysteine residues that form the V1, V2, V3 and V4 loops of gp120. Another consistent observation was the considerable length variations in V1/V2 and the combined V4/V5 loop lengths, whereas the V3 loop remains constant with 35 amino acid residues and exhibits only few sequences variability with 2 sequences containing 34 amino acids each (Figure 2.4.A-C). The V1/V2 which ranged from 49 to 73 (median= 62) amino acids, contains the largest number of residues and the highest degree of length variation ( $P<0.0001$ ) compared to the combined V4/V5 ranging from 35 to 46 (median= 39) amino acids ( $P<0.0001$ ). The patients 12541 and BS12 exhibits the lowest V1V2 length, whereas the patient 10056 was having the highest V1/V2 length (Figure 2.4.A).

A similar intra-patient analysis was performed for the degree of variation in the number and location of potential N-linked glycosylation sites (PNGS). The number of PNGS in gp120 range from 18.31 (mean= 26) and was more than the number in gp41 with a range of 2.5 (mean= 5). The total number of PNGS in gp160 for all the 47 sequences range from 20 to 36 (mean= 30.5) and was significantly different ( $P<0.0001$ ) indicating a variation in the glycosylation pattern in viral quasispecies in these patients (Figure 2.4.D). The patient 10056 had the greater number of total PNGS than the rest of patients in which, there were no statistically significant changes in the number of PNGS. Similarly, a significant difference was observed in the combined V4/V5 region ( $P<0.0009$ ) (Figure 2.4.G), however no significance difference was found in the V1/V2 ( $P=0.6174$ ) and V3 region ( $P=0.4464$ ) (Figure 2.4.E and F). One patient (BS12) had a lower distribution of PNGS in V1V2 as well as the combined V4V5 regions. Most of these glycosylation sites are located within the V1/V2 and the V4/V5 regions. Several sites were quite conserved; however, 5 PNGS in gp120 and 3 PNGS in gp41 have showed 100% conservation in all our sequences.



**Figure 2.5: Longitudinal analysis of changes in the length of gp160 and the number of PNGS.** Each dot represents one envelope virus variant. (A-C), Comparison of the number of amino acid residues in the variable loops and (D-G), Comparison of the PNGS in the variable loops. The *P* value was calculated by the one-way ANOVA, and individual differences were calculated using Dunn's multiple comparison test; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

#### **2.4.6 Genetic comparison between subtype G with subtype B and C sequences**

Amino acid sequences generated in this study were used in aggregate named as “subtype G.agg” and compared to the well characterized subtypes C (n=206), subtype B (n=211) reference sequences, but also to subtype G (n=71) reference sequences (subtype G-db) available in LANL database (Table 2). The comparisons were focused on the gp120 length of amino acids and the number of PNGS since both factor seems to play a role in neutralization phenotype (Mascola and Montefiori, 2003, Reitter and Desrosiers, 1998, Bunnik et al., 2008, Cao et al., 1997, Moore et al., 2009b, Rong et al., 2007b, Rong et al., 2009, Sagar et al., 2006, van Gils et al., 2011). The comparison showed that difference in gp120 length between subtype G-agg and subtype B was significant ( $P<0.005$ ) with a considerable size variation in the V1/V2 and V4 regions, however, V3 and V5 did not vary in length (Table 2.2). The median lengths of gp120 and V4 for subtype G-agg were shorter than for subtype B. Although a considerable length variation was observed in the V1/V2 and V4, there was no significant difference between subtype G-agg and subtype C. When subtype G-agg was compared to subtype G-db reference sequences there was no statistical difference between the two groups.

Fewer PNGS were present in V1/V2, V3, V4 and V5 and no significant difference was found in the number of PNGS in gp120 length, V1/V2, and V3 between all groups. However, there were two statistical differences: one in the V4 region ( $P<0.05$ ) between Subtype G-agg and subtype B and the other one in the V5 region ( $P<0.05$ ) when we compared PNGS in the two subtype G groups (Table 2.2). Many PNGS were common in all subtypes. Taken together, we found that a significant difference was only found in gp120 length between sequences generated in this study and subtype B sequences resulting in a longer V1/V2 and V4 length of subtype B than subtype G.agg. These results can be explained by the fact that subtype B sequences obtained from LANL database derived mostly from chronic viruses and seems to have longer amino acid length compared to our sequences obtained from patients recently infected.

**Table 2.2: Comparison of sequence lengths and PNGS between HIV-1 subtypes G, B and C gp120.**

G-agg, 47 subtype G sequences generated in this study and used in aggregate; G-db, 71 subtype G reference sequences from LANL database; B-db, 211 well-

Subtype	Number of PNGS										Length of variable loops									
	gp120		V1/V2		V3		V4		V5		gp120		V1/V2		V3		V4		V5	
	IQR	median	IQR	median	IQR	median	IQR	median	IQR	median	IQR	median	IQR	median	IQR	median	IQR	median	IQR	median
G-agg	25-28	26	4-6	5	1-1	1	3-5	4	1-1	1	496-511	506	53-68	62	35-35	35	25-28	27	12-12	12
G-db	25-27	26	4-6	5	1-1	1	4-5	4	1-2	1	502-511	506	59-66	61	35-35	35	24-29	26	11-12	12
B-db	23-27	25	4-6	5	1-1	1	4-5	5	1-2	1	507-517	512	59-66	62	35-35	35	30-34	32	12-12	12
C-db	22-27	26	4-6	5	1-1	1	4-5	4	1-1	1	499-513	507	56-66	60	35-35	35	26-32	29	12-12	12
Comparison	P value																			
G-agg vs G-db	>0.05		>0.05		>0.05		>0.05		<b>&lt;0.05</b>		>0.05		>0.05		>0.05		>0.05		>0.05	
G-agg vs B-db	>0.05		>0.05		>0.05		<b>&lt;0.05</b>		>0.05		<b>&lt;0.05</b>		>0.05		>0.05		<b>&lt;0.05</b>		>0.05	
G-agg vs C-db	>0.05		>0.05		>0.05		>0.05		>0.05		<0.05		>0.05		>0.05		>0.05		>0.05	

characterized subtype B database sequences; C-db, 206 well-characterized subtype C database sequences. Bolded P values (P<0.05) are significant differences analyzed by one way ANOVA after Bonferroni's correction.

### 2.4.7 V3 region analysis and Co-receptor usage

The V3 region was analyzed in details since it is an important determinant of the co-receptor usage (Briggs et al., 2000, Cann et al., 1992, Fouchier et al., 1992, Rizzuto et al., 1998). The V3 region was found to be highly conserved with the crown motif GPGQ present in 41 out of 42 sequences (Table 3). The GPGQ crown motif is known to be associated with CCR5 tropic in subtype C sequences (Cilliers et al., 2003). Another characteristic of the V3 loop associated with tropism determination is the property of amino acids at position 11 and 25 (Fouchier et al., 1992). No positively charged amino acid residues were found at positions 11 and 25. The neutral Serine and Glycine residues were present in most cases at position 11, whereas in most cases, an amino acid with a negatively charged side chain (E or D) was present at position 25. However, three sequences (12541.E11, 12541.D12, 12541.E2) had a deletion at position 25. The net charge value was calculated by subtracting the number of the negatively charged amino acids D and E from the number of positively charged amino acid R and K. The net charge value of the V3 loop was low and ranged from +2 to +6, which is in agreement with previous reports indicating that few numbers of positive charges in the V3 region is associated with CCR5 utilization (Cilliers et al., 2003). The R5 tropism genotypic predictions using WebPSSM and Geno2pheno based on the V3 region (Jensen et al., 2006, Sing et al., 2007) were confirmed in vitro by comparing pseudoviruses titers using co-receptor inhibitors on TZM-bl cells. TZM-bl cells express the receptor CD4 and both the CCR5 and CXCR4 co-receptors (Platt et al., 1998). Pseudoviruses expressing the R5 tropic IIB and the X4 tropic BalPS were used as control. All 28 subtype G clones had a CCR5 tropism as determined by a reduction or absence of TZM-bl cells infection in the presence of the CCR5 inhibitor TAK-779 (3.3  $\mu$ M), but not in the presence of CXCR4 inhibitor AMD-3100 (430 nM) (Table 2.3). Borrego et al (2012) reported recently similar  $IC_{50}$  (2.6 nM) for one clinical isolate but a 1000-fold higher  $IC_{90}$  (2.8  $\mu$ M) (Borrego et al., 2012) which is more similar to our results. One of the limitations in this experiment is that we did not calculate the  $IC_{50}$  or  $IC_{90}$  of TAK-779. However, the amount of virus added was the same for all viruses and the variation in sensitivity to TAK-779 may be related to the high genetic diversity of the envelope of these viruses that may affect the binding affinity of TAK-779. In addition, it is possible that CCR5 use may evolve during the course of HIV-1 infection such that CCR5 variants isolated from our subtype G patients which had low levels of CD4<sup>+</sup> T cells have reduced sensitivity to TAK-779.

**Table 2.3: co-receptor usage properties of the HIV-1 subtype G envelope sequences.**

Clone ID	Subtype	Web PSSM	Geno2 pheno	V3 net charge	Amino acid (11/25)	Crown motif	Co-receptor assay (TZM-bl)		
							Fold drop infectivity with 3.3uM TAK-779	Fold drop infectivity with 430nM AMD-3100	Co-receptor
10056_C12	G	CCR5	CCR5	4	GR	GPGQ	nd	nd	nd
10056_C1	G	CCR5	CCR5	4	GS	GPGQ	5.3	NR	CCR5
10056_C6	G	CCR5	CCR5	4	GS	GPGQ	nd	nd	nd
10056_E6	G	CXCR4	CCR5	6	SG	GPGQ	nd	nd	nd
10056_F5	G	CCR5	CCR5	4	GS	GPGQ	nd	nd	nd
10056_E8	G	CCR5	CCR5	4	GS	GPGQ	nd	nd	nd
10056_F2	G	CCR5	CCR5	4	GS	GPGQ	6.7	NR	CCR5
10056_D8	G	CCR5	CCR5	4	GS	GPGQ	6.7	NR	CCR5
11439_G7	G	CCR5	CCR5	4	SE	GPGQ	nd	nd	nd
11439_D4	G	CCR5	CCR5	4	SE	GPGQ	2.4	NR	CCR5
11439_H4	G	CCR5	CCR5	4	SE	GPGQ	4.3	NR	CCR5
11439_C5	G	CCR5	CCR5	4	SE	GPGQ	2.6	NR	CCR5
11439_F5	G	CCR5	CCR5	4	GE	GPGQ	nd	nd	nd
11439_F6	G	CCR5	CCR5	4	SE	GPGQ	nd	nd	nd
12541_C12	G	CCR5	CCR5	5	SA	GPGQ	5.9	NR	CCR5
12541_B2	G	CCR5	CCR5	5	SA	GPGQ	12.5	NR	CCR5
12541_E11	G	CCR5	CCR5	5	S-	GPGQ	nd	nd	nd
12541_D12	G	CCR5	CCR5	5	S-	GPGQ	nd	nd	nd
12541_E2	G	CCR5	CCR5	5	S-	GPGQ	nd	nd	nd
12541_C11	G	CCR5	CCR5	3	SD	GPGQ	nd	nd	nd
BS03_H3	G	CCR5	CCR5	4	SE	GPGQ	7.1	NR	CCR5
BS03_A1	G	CCR5	CCR5	4	SE	GPGQ	nd	nd	nd
BS03_G4	G	CCR5	CCR5	4	SE	GPGQ	5.3	NR	CCR5
BS03_E3	G	CCR5	CCR5	4	SE	GPGQ	nd	nd	nd
BS12_D9	G	CCR5	CCR5	3	SD	GPGQ	6.3	NR	CCR5
BS12_D2	G	CCR5	CCR5	3	SD	GPGQ	2.4	NR	CCR5
BS12_F6	G	CCR5	CCR5	3	SD	GPGQ	nd	nd	nd
BS12_A5	G	CCR5	CCR5	2	SD	GPGQ	nd	nd	nd
BS12_G8	G	CCR5	CCR5	3	SD	GPGQ	4.5	NR	CCR5
BS12_A6	G	CCR5	CCR5	2	SD	GPGQ	6.3	NR	CCR5
BS46_A2	G	CCR5	CCR5	4	DA	GPGQ	nd	nd	nd
BS46_E1	G	CCR5	CCR5	3	DA	GPGQ	16.7	NR	CCR5
BS46_mt	G	CCR5	CCR5	4	DA	GPGQ	nd	nd	nd
BS46_H4	G	CCR5	CCR5	4	DA	GPGQ	nd	nd	nd
BS46_E2	G	CCR5	CCR5	3	DA	GPGQ	6.3	NR	CCR5
BS48_F5	G	CCR5	CCR5	4	SE	GPGQ	25.0	NR	CCR5
BS48_H10	G	CCR5	CCR5	4	SE	GPGQ	5.3	NR	CCR5
BS48_E1	G	CCR5	CCR5	4	SE	GPGQ	4.2	NR	CCR5
BS48_E4	G	CCR5	CCR5	4	SE	GPGQ	20.0	NR	CCR5
BS48_mt	G	CCR5	CCR5	4	SE	GPGQ	nd	nd	nd
BS48_F1	G	CCR5	CCR5	4	SE	GPGQ	25.0	NR	CCR5
BS48_E3	G	CCR5	CCR5	4	SE	GPGQ	14.3	NR	CCR5
BS51_B5	G	CCR5	CCR5	4	SA	GPGQ	5.6	NR	CCR5
BS51_B6	G	CCR5	CCR5	4	SA	GPGQ	7.1	NR	CCR5
BS51_H2	G	CCR5	CCR5	3	SE	GPGQ	5.6	NR	CCR5
BS51_A3	G	CCR5	CCR5	4	SA	GPGQ	7.7	NR	CCR5
BS51_D4	G	CCR5	CCR5	4	SA	GPGQ	nd	nd	nd

IIIB (CXCR4)	B	Controls	NR	250	CXCR4
BALPS (CCR5)	B	Controls	1.9	NR	CCR5

'nd'= not determined, NR= No Reduction, fold drop infectivity shown is ratio of percentage neutralization of each virus to control virus.

## 2.5 Discussion

In this study, 47 full length sequences were analyzed from 8 individuals infected with HIV-1 subtype G with the goal to investigate the genetic properties that are known to influence epitope exposure on the gp120 molecule and elucidate the co-receptor utilization by HIV-1 subtype G viruses in Cameroon. Phylogenetic analysis of the full length *env* has revealed that all these viruses belong to HIV-1 subtype G. *env* sequences were interspersed among all subtype G sequences showing a wide spectrum of diversity among our samples. In fact, 12541 sequences branched from near the root of the subtype G subtree in a poorly populated branch containing some sequences related to a segment of CRF06\_cpx. Sequences from within one sample were genetically related to each other, while samples were genetically distinct from each other. These results suggest that our samples capture a substantial amount of the diversity within the subtype G envelope sequences and are largely different from each other. These findings are therefore consistent with the results of a previous study on subtype G (Revilla et al., 2011).

This study also showed that in 4 individuals, the viral population was heterogeneous with large genetic distances (>1.5%) indicating a likely long period of time since infection. The three individuals failing ART were almost certainly infected for much longer, suggesting a reason for the increased diversity. Individual analysis showed that 10056 had the most variable sequences (3.45%), followed by 12541 (3.3%), 11439 (2.1%) and the rest of patients (<2%). The maximum values are based largely on one outlier, and this outlier may have been derived from a related virus. So, the maximum might be more related to how far apart the two viruses were than how long the person was infected. Four individuals had homogeneous viral sequences, with a much shorter genetic distance (<1.5%), suggesting a restricted viral evolution in those individuals. Consistent with a recent infection, one individual (BS48) exhibits the lowest intra-patient genetic distance with values similar to those normally observed in the first 90 days following infection with a single viral variant (Keele et al., 2008)

The length of amino acids and the N-linked glycosylation of the HIV-1 envelope are among the mechanisms used by the virus to evade antibody recognition, possibly by shielding underlying epitopes of envelope from antibodies recognition (Gray et al., 2007, Pinter et al.,

2004). Since several PNGS are relatively constant across HIV-1 subtypes, there is a great interest in developing a carbohydrate based antigen in order to elicit a humoral immune response. Previous studies have shown that viruses with shorter V1V2 lengths are preferably transmitted in some subtypes (Chohan et al., 2005, Derdeyn et al., 2004). We examined and statistically analyzed the difference in length of variable loops and the number of PNGS of the isolated envelope genes and found a variation in the glycosylation sites and variable loop lengths in the 8 patients. The V1V2 lengths from patients infected for < 6 months (BS03, BS46, and BS48) were not significantly shorter when compared to patients infected for > 6 months (BS12, BS51). The V1V2 and V4V5 had the most length variation within and between participants, whereas the V3 length was constant. This variation in length appears similar to previously observed in subtype C (Coetzer et al., 2007).

Several studies have reported that viruses from acute infection have shorter variable loops and fewer glycosylation sites than viruses from chronic infection (Kwiek et al., 2008, Russell et al., 2011, Derdeyn et al., 2004). Li et al. (2006) suggested that increased exposure of epitopes on the shorter, less-glycosylated gp120 of newly transmitted subtype C viruses could result in enhanced immunogenicity, greater antigenicity or both. This interpretation is based on evidence that difference in the length of variable loops and number and position of PNGS on gp120 may contribute to different neutralization properties (Li et al., 2006a). Results of the analysis among our subtype G samples showed no relationship between time since infection and length of variable region and glycosylation sites. Some patients (12541) known to be infected for a long period of time exhibits a relatively short V1V2 and V4V5 length. In addition, BS48 from the blood donor group had unusually low within-donor genetic distance; equivalent to that seen in very recent infection (<90 days), and had unusually high V4 lengths. This might be a feature of many subtype G envelope compared to subtype B and C. However, due to the small sample size, this analysis may not have a strong power and should be taken with caution. Therefore, a larger number of subtype G gp120 sequences from acutely and chronically infected individuals will be needed to resolve whether or not viruses from acute infection have unique feature in the context of subtype G.

The gp120 regions of envelopes from subtype C acute viruses have been reported to be shorter and less glycosylated compared to subtype C chronic viruses (Derdeyn et al., 2004). However, these differences are not seen in subtype B (Chohan et al., 2005). Subtypes B and C are the most extensively studied and well characterized HIV-1 subtypes and the majority of the sequences available from LANL database are from chronic patients. A similar analysis

was performed in this study by comparing subtype G sequences generated in this study (subtype G.agg) to subtype G, B and C gp120 in the LANL database, known to contain in majority sequences from chronic viruses. A general distinguishing feature was the shorter of gp120 and V4 length on subtype G-agg compared to subtype B. Furthermore, although the number of PNGS site on gp120 was not significantly different, subtype B had more PNGS site in the V4 region. These findings are similar to those of recent studies where the gp120 of subtype C and A expands and add loop sequences and PNGS over the course of infection (Chohan et al., 2005, Sagar et al., 2006, Derdeyn et al., 2004). A significant difference in the PNGS number in the V5 region was also found by analyzing our patient collectively compare to Subtype G obtained from the database (Subtype G-db, whereas, there were no difference compare to subtype C-db.

The *env* sequences were also tested for the co-receptor usage in TZM-bl cells and we determined that all our *env* clones were CCR5 tropic, which is consistent with previous studies on subtype G suggesting the use of CCR5 by the majority of viruses (Revilla et al., 2011, Tebit et al., 2002, Zhong et al., 2003, Brandful et al., 2007). It was surprising that four viruses (BS12.D2, 11439.C5, 11439.D4, and BalPS) had 3.4 fold less reduction in infectivity in the presence of such a high concentration of TAK-779 (3.3 $\mu$ M). Baba et al, (1999) reported that TAK-779 inhibited the replication of CCR5 clinical isolates as well as laboratory strains at a concentration of 1.6-3.7nM, which is less than the concentration we used to inhibit these HIV-1 subtype G viruses. The variability in the sensitivity to TAK-779 of these CCR5 viruses may not be due to alternative co-receptor usage but rather differential CCR5 binding and to the extreme heterogeneity of the HIV-1 envelope glycoproteins (gp120 and gp41) that may affect the susceptibility of variant HIV-1 strains (Torre et al., 2000).

In HIV-1 subtype G, there is little information on the genetic characteristics of the V3 loops that determine the tropism. The V3 sequences were therefore analyzed and found that almost all isolates had the GPGQ crown motif with a low net positive charge value. The V3 loops of R5 viruses generally have lower net positive charge than those of X4 (Jiang, 1997, Huang et al., 2005, Fouchier et al., 1992).

## 2.6 Conclusion

In conclusion, this study characterizes extensively the genotypic properties of HIV-1 subtype G circulating in Cameroon. This study shows that the heterogeneity of *env* diversity and evolution among our study participants differs with HIV-1 disease course (HIV-1 naïve and HAART patients). This study also highlights the identification of apparent recombination events in some individuals. Subtype G envelope gene exhibit lower gp120, and V4 length compared to the well characterized subtype B, and the majority of infection are caused by viruses that exclusively utilizes CCR5 co-receptor. These findings are likely to provide useful information for the design and the evaluation of HIV-1 vaccine and for a better understanding of the HIV-1 epidemic .

## **CHAPTER 3**

### **RESULTS**

# Characterization of neutralization antibody responses in HIV-1 subtype G infected patients in Cameroon

## 3.1 Abstract

### Background

The high genetic diversity of HIV-1 is a great obstacle for development of an effective vaccine against this pathogen. HIV-1 subtype G accounts for 5% of HIV-1 infection worldwide, centered on West and Central. However, immunity to viruses of this subtype, particularly strains that circulate in its main reservoir, Central and West Africa have been understudied. In this study, we characterized the neutralization capacities of 7 subtype G-infected plasma samples, including their ability to neutralize 28 pseudoviruses obtained from the same seven samples. Five study participants were blood donors later determined to be HIV-infected and the remaining two were undergoing testing for antiretroviral drug resistance.

### Results

The neutralization sensitivity of subtype G pseudoviruses to autologous neutralization (own plasma), and plasma pools was characterized using full length envelope clones to generate pseudoviruses for infection studies. Our data showed that autologous neutralization was generally low to undetectable among out study participants HIV-1 subtype G infected individuals, as observed previously for samples from other subtypes. However, strikingly, all four BS12.derived viruses exhibited substantial sensitivity to neutralization by their own plasma (geometric mean  $ID_{50} = 564$ ).

The ability of subtype G plasma to neutralize a panel of 14 HIV-1 *env* pseudoviruses was evaluated. The panel includes representatives of subtypes A, B, C, and CRF02\_AG which were from tier 2 (moderately resistant viruses) to tier 3 (highly resistant viruses). Three of the samples were from patients undergoing testing for antiretroviral resistance, and thus may have antiretroviral drugs in their blood plasma despite having detectable viral loads. However, plasma from two of these three patients failed to neutralize negative control pseudovirus (Murine Leukemia Virus envelope, MLV) or most other viruses tested, suggesting that levels of antiretroviral drugs in these two samples were too low to interfere with the neutralization assay. It may be that these donors were not taking their antiretroviral. The third sample showed evidence suggesting that antiretroviral drugs were present in the plasma, and this

sample was excluded from further analysis in the neutralization studies. Blood plasma from two out of the remaining seven study participants, including BS12, neutralized more than half of panel virus tested at >50% neutralization at a dilution of 1/50, indicating at least limited neutralization breadth in these two samples. Interestingly, BS12 serum, the samples that showed a substantial potent autologous activity, also neutralized 3/5 of the tier 3 isolates from the panel viruses (278-50 and 257-31 not neutralized; 33-7, 253-11, and PV0.4 neutralized). Mapping of the epitope targeted by neutralizing antibodies in BS12 plasma indicated that the dominant neutralizing antibody for virus CAP45.2.00.G3 in sample BS12 targeted the PG9/16 site. This site is the target in the V2 and V3 regions of the broadly neutralizing monoclonal antibodies PG9 and PG16. BS12 plasma also exhibited substantial neutralization of an HIV-2 chimeric virus displaying the MPER of 253-11, a CRF02\_AG virus, although we did not examine if these anti-MPER antibodies are capable of neutralizing HIV-1 viruses by recognizing MPER.

### **Conclusion**

Our data showed that one sample, BS12, was unusual in that the plasma sample itself neutralized viruses derived from sequences found in the same sample. Our data suggest that in study participant BS12 plasma, the V1V2, and MPER regions are the main immunodominant regions of the envelope targeted by neutralizing antibodies, indicating that its autologous neutralization and its limited neutralization breadth may be dictated by a limited number of target epitopes. We believe that a subset of these HIV-1 viral variants may be used for screening of HIV-1 vaccine candidate to assure that evaluation of vaccine-induced antibody includes some of the substantial diversity ground in subtype G.

## 3.2 Introduction

In 2011, UNAIDS and WHO estimated that there were 34 million people living with HIV, and over 2.5 million new infections with 69% of these new infections occurring in Sub-Saharan Africa (UNAIDS, 2012, WHO, 2013). An effective vaccine that prevents HIV-1 transmission is urgently needed. It is likely that an effective vaccine will need to include a component that induces neutralizing antibodies against a broad range of HIV-1 strains (Burton et al., 2012). Studies in nonhuman primates have shown that passively transferred neutralizing antibodies (nAbs) can protect against HIV-1 infection when present at the time of exposure (Barouch et al., 2012, Baba et al., 2000, Mascola et al., 1999, Mascola et al., 2000, Parren et al., 2001). Designing an effective vaccine has so far been unsuccessful, presumably due to the high sequence variability of the envelope glycoproteins (Env) (Burton et al., 2004), the shielding of critical neutralizing epitopes by N-linked glycans (Kwong et al., 2002) and the considerable evolution of the antigen binding site of antibodies that appears to be required for the production of neutralization breadth (Burton et al., 2012). Some HIV-1 individuals mount a strong autologous neutralization response within a few months to one year of initial infection (Li et al., 2006b, Richman et al., 2003, Wei et al., 2003, Gray et al., 2007). Antibodies capable of neutralizing heterologous viruses tend to develop later in infection; but, only a small proportion of chronically infected patients can develop broadly cross-reactive nAbs against multiple HIV-1 viruses (Braibant et al., 2006, Donners et al., 2002, Simek et al., 2009, Gray et al., 2011a).

A number of new candidate immunogens have been identified and it is important that the vaccine-induced responses be examined against different HIV-1 variants, including those circulating in endemic areas. It has been recommended that separate panels of well characterized viruses be developed for each major HIV-1 subtypes (Mascola et al., 2005). The availability and standard use of these reference subtypes should allow the acquisition of standardized measurements of the neutralizing antibody responses so that advancement in immunogen design can be identified (Li et al., 2005).

HIV-1 subtype G is the sixth most prevalent subtype of HIV-1 after subtypes A, B, C, CRF01\_AE and CRF02\_AG (Hemelaar et al., 2006). Subtype G is concentrated in West and Central Africa (Hemelaar et al., 2011b) however; very limited information is available on the neutralization properties of HIV-1 subtype G viruses. Systematic analysis of a wide range of HIV-1 envelope sequences (Brown et al., 2005) and neutralization patterns (Brown et al., 2008, Seaman et al., 2010) have been performed. However, subtype G viruses are severely

underrepresented in these works. Brown et al (2005) studied only two subtype G-associated sequences while Brown et al (2008) did not study the neutralization patterns of any subtype G viruses and Seaman et al (2010) evaluated the intrinsic neutralization resistance of only one putative subtype G virus. Only a recent study has one neutralization study of Subtype G viruses been published (Revilla et al., 2011). In this work, only two of the subtype G sequences were derived from individuals from West or Central Africa and no testing of autologous and heterologous neutralization capacity was performed, which is critical for evaluating which viruses should be included in test panels for vaccine evaluation and understanding to what extent particular immunogen design techniques are likely to cover subtype G viruses.

This study describes the characterization of the autologous and heterologous neutralization antibody response of viral envelopes generated from seven HIV-1 subtypes G infected individuals from Cameroon using conventional assays. We also examined the specificity of neutralizing antibodies in a patient exhibiting good autologous and heterologous neutralization capacity to understand how such antibodies might contribute to neutralization breadth.

### **3.3 Methods**

#### **3.3.1 Study participants and plasma samples**

Study participants and plasma samples used in this study are described in details in chapter 2.3.1 of this thesis.

The plasma samples were tested against viruses individually or as a pool containing an equal mixture of individual plasma samples from the same subtype. Three pools of plasma were made: The subtype C pool (South Africa; n= 68) from our laboratory study cohort in Khayelitsha Site B clinic, the CRF02\_AG pool (Cameroon; n=12) (Jacob et al., 2012), and the subtype G pool (Cameroon; n=8) from our study samples (Tongo et al., 2013). All plasma samples were heat inactivated for 1 hour at 56<sup>0</sup>C before use as a source of antibodies in the neutralization assay. This study received approval from the CIRCB Ethics Committee and the Human Research Ethics committee of the University of Cape Town, Faculty of Health Sciences (REC NO: 107/2011).

### 3.3.2 Cell lines

The TZM-bl cells were obtained from Drs. J Kappes and X Wu through the NIH AIDS Research Reagent Reference Program (ARRRP). These cells were derived from HeLa cell clone and expresses CD4, CCR5 and CXCR4 (Platt et al., 1998). TZM-bl cells contain two reporter genes: the luciferase and the *Escherichia coli*  $\beta$ -galactosidase under the control of the HIV-1 LTR promoter (Wei et al., 2002). The 293T cells used for transfection were obtained from Dr. A Rice through the ARRRP.

### 3.3.3 Virus envelope panel and chimeric viruses

A panel of 14 reference HIV-1 *env* pseudoviruses comprising representatives of different subtypes (A, B, C, and CRF02\_AG) was used in the heterologous neutralization assays. The panel of pseudoviruses was chosen based on subtype diversity, neutralization resistance (Seaman et al., 2010, Blish et al., 2009, Jacob et al., 2012) and geographic diversity of origin. All tier designations are as per Seaman *et al* (Seaman et al., 2010). The cloned envelope constructs used and their subtypes in parenthesis were: Q168.a2 (A), QG984 (A), QH343 (A), Q461.e2 (A), TRO11 (B), RHPA (B), REJO (B), SC 4226 (B), ZM249 (C), CAP45 (B), 255.34 (CRF02\_AG), 33-7 (CRF02\_AG), 278-50 (CRF02\_AG), 253-11 (CRF02\_AG) and were all obtained from ARRRP. The Murine Leukemia Virus (MLV) was provided by Dr. Lynn Morris, NICD, Johannesburg, South Africa. Samples were tested against MLV as a negative control. The 7312A parent HIV-2 construct and all HIV-2/HIV-1 chimeric constructs containing HIV-1 MPER sequences (C1, C1C) (Gray et al., 2007) (except 253-11 MPER) were provided by Dr. George Shaw, University of Pennsylvania, Philadelphia, USA. The 253-11 MPER construct was produced by mutagenesis from HIV-2 C1 by Rajesh Jacob in our laboratory using the Stratagene QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA).

### 3.3.4 Cloning of envelope genes, pseudoviruses production and titration

The cDNA extracted from the infected individual plasma samples were used to amplify the full-length *env* gene using the single genome amplification (SGA) assay as previously described (Salazar-Gonzalez et al., 2008). Briefly, the approximately 3kb PCR products generated using the HIV-1 subtype G specific PCR primers or the general primers were cloned into pcDNA3.1D/V5.His.TOPO vector (Invitrogen, Carlsbad, CA, USA) and bacterial colonies screened by PCR for insertion and correct orientation using T7 and BGH primers. Pseudoviruses were made by cotransfecting 293 T cells with 4  $\mu$ g of a chosen plasmid with an envelope insert with 8  $\mu$ g of SG3 $\Delta$ env HIV genome plasmid using X-tremeGENE 9 DNA

transfection reagent (Roche Diagnostics, Basel, Switzerland) as previously described (Montefiori, 2009). 48 and 72 hours after transfection, the culture supernatant containing pseudoviruses was harvested, filtered, aliquoted, and stored at  $-80^{\circ}\text{C}$ . The dilution of supernatant needed to obtain 50,000 relative light units (RLU) of readout for each pseudovirus was determined by infection in TZM-bl as described (Montefiori, 2009).

### **3.3.5 Neutralization assay**

TZM-bl-based neutralization by patient plasma or plasma pools were assessed as described (Montefiori, 2009). Patient plasma samples were evaluated for neutralization antibody activity against patient derived pseudoviruses (autologous neutralization). Testing against a virus panel for measuring neutralization breadth/capacity was performed by estimating the ID<sub>50</sub> as described in section 3.3.6 below. Otherwise, serial two-fold dilutions of plasma were distributed in duplicate in 96-well flat-bottom plates (Costar) in a total volume of 100  $\mu\text{l}$  per well and pre-incubated with 50  $\mu\text{l}$  of pseudoviruses for one hour. Freshly trypsinized TZM-bl cells were then added (10,000 cells/well in Dulbecco's modified Eagle's medium [DMEM] 10% fetal bovine serum [FBS] containing HEPES and 7.5  $\mu\text{g/ml}$  of DEAE-dextran). After 48 hours of incubation at  $37^{\circ}\text{C}$ , 100  $\mu\text{l}$  of cells/well were transferred to 96-wells black solid plate (Costar) and the luminescence measured using the Bright-Glo™ luciferase substrate (Promega, Madison, USA) and measured on a VERITAS microplate luminometer (Turner BioSystems). The 50% inhibitory dilution (ID<sub>50</sub>) was measured as the plasma dilution that cause a 50% reduction in relative luminescence units (RLU) compared to the level in the virus control wells after subtraction of cell control RLU. The ID<sub>50</sub> was calculated from testing neutralization for at least four dilutions of plasma using curve fit functions in Prism version 5 (GraphPad, La Jolla, USA). The neutralization score for each virus and for each plasma sample was defined as the geometric mean of all of the ID<sub>50</sub> values for that virus or plasma sample.

### **3.3.6 Estimation of ID<sub>50</sub> for each serum/virus pair and its validation**

For the purpose of estimating neutralization breadth, an ID<sub>50</sub> was predicted from percentage neutralization (measured at 1/100 serum dilution) based on a linear regression analysis developed by others in my laboratory that was performed on a subpopulation (n=240; “training set”) of the sample/virus combinations with known percentage neutralization of serum (at 1:100 dilution) and measured ID<sub>50</sub> values. The effect of percentage neutralization was modeled both linearly and non-linearly. Based on  $R^2$ , we measured the goodness of fit of the corresponding models and chose a linear model (Jacob et al., 2012). The equation was

used to predict the ID<sub>50</sub> from % neutralization at 1/100 serum dilution. The calculated relationship is:  $\ln(\text{predicted ID}_{50}) = 1.303 + 0.060962 * \% \text{ neutralization @ } 1/100$ . The serum/virus pairs in the training set included sample/virus combinations from previous studies in the laboratory (Jacob et al., 2012). The 1/100 serum dilution was the only dilution tested for all pooled of sera.

The model was validated on a separate subset of sample/virus combinations (“test set”) with known percentage neutralization of serum (at 1:100 dilution) and measured ID<sub>50</sub> values (n=234) from others in our laboratory. The predicted ID<sub>50</sub> values derived from the percentage neutralization for the “test set” correlated well with the corresponding measured ID<sub>50</sub> values ( $R^2=0.67$ ) (Jacob et al., 2012).

### **3.3.7 Statistical analysis**

ID<sub>50</sub> titers for both autologous and heterologous neutralization responses were performed using the nonlinear regression (curve fit) function in GraphPad prism 5 software programme (GraphPad, La Jolla, USA). Correlations were measured by Spearman’s rank test in GraphPad 5. Correlations with p-value  $\leq 0.05$  were considered as statistically significant.

## **3.4 Results**

### **3.4.1 Study participants**

The five blood bank samples and the three initial samples from patients undergoing testing for antiretroviral resistance were screened against the negative control pseudovirus MLV. Plasma from the blood bank samples and two of the three patients failing ART failed to neutralize MLV suggesting that levels of antiretroviral drugs in these two samples were too low to interfere with the neutralization assay. It may be that these donors were not taking their antiretroviral. One donor (11439) neutralized MLV and may have antiretroviral drugs in the plasma, and was excluded in the neutralization studies.

### **3.4.2 Variations in autologous neutralization sensitivity between study participants**

The sensitivity to autologous neutralization of subtype G viral variants was studied from eight patients by testing the ability of each participant’s plasma sample to neutralize their envelope variants. A total of 28 pseudoviruses (ranging from 2 to 6 per participant) were tested against their respective plasma. Figure 3.1 shows that the neutralizing antibody response varied in magnitude for all the eight subjects. Evidence for neutralization by autologous plasma was detected in 6 out of 7 patients (Figure 3.1). The sensitivity of different viral variants to

autologous neutralization within the same patient varied among participants. The neutralizing antibody response was not detectable in two patients (BS03 and BS51) and three patients (12541, BS46, and BS48) had a low AnAbs activity. Interestingly, two patients (10056 and BS12) developed autologous neutralization activity against all their respective viruses. This is best illustrated in one of these two participants (BS12), in which all four BS12-derived viruses exhibited substantial sensitivity to neutralization by their own plasma (geometric mean  $ID_{50}=564$ ) which is approximately four-fold higher than the neutralization potency of 10056-derived viruses (geometric mean  $ID_{50}=142$ ). These results suggest an effective neutralizing antibody pressure which is mounted against the HIV-1 viruses of these participants, although this pressure is not enough for the effective control of the infection. Unfortunately, BS12 was an anonymous blood donor and it is not possible to obtain further samples to trace the effect of this high level of neutralizing antibody upon subsequent evolution of the HIV population in this individual.

Sample ID	Virus	Autologous plasma ID <sub>50</sub>
10056	10056.C1	228
	10056.D8	150
	10056.F2	84
12541	12541.B2	103
	12541.C12	<50
BS03	BS03.G4	<50
	BS03.H3	<50
BS12	BS12.A6	555
	BS12.D2	754
	BS12.D9	608
	BS12.G8	632
BS46	BS46.A2	<50
	BS46.E1	185
	BS46.E2	215
	BS46.H4	52
BS48	BS48.E1	<50
	BS48.E3	131
	BS48.E4	<50
	BS48.F1	127
	BS48.F5	<50
	BS48.H10	85
BS51	BS51.A3	<50
	BS51.B5	<50
	BS51.B6	<50
	BS51.H2	<50

300-999
100-299
50-99
<50

**Figure3. 1: Neutralization of subtype G viral isolates by autologous plasma.** The patient ID and the pseudoviruses tested are shown to the left and the patient plasma tested is indicated at the top. The neutralization titer is also shown as the plasma dilution that causes the inhibition of 50% of virus infection when the virus is neutralized by the participant's plasma. The highest titer (>300) is shown in red as indicated at the right of the figure. The grey box indicated that <50% neutralization was observed at 1:50 which was the highest concentration of plasma tested, and the value of 50 was assigned.

### 3.4.3 Neutralization activity of plasma pools

The ability of various plasma pool samples to neutralize viruses expressing HIV-1 subtype G envelopes was next assessed. Three different panels of HIV-1 plasma pools were used for neutralization assays. The subtype C pool (South Africa), subtype G pool (Cameroon) and CRF02\_AG pool (Cameroon) were made using equal amount of plasma from at least eight HIV-1 infected individuals in order to provide a desired sample diversity of the antibody repertoire present in each individual plasma sample. As depicted in figure 2, most of these viruses shared similar level of sensitivity to neutralization by the various plasma pools, but

some exhibit either a highly sensitive or highly resistant phenotype. This finding is consistent with previous reports from Seaman et al, (2010) and Shang et al, (2011) (Seaman et al., 2010, Shang et al., 2011), where variable degrees of sensitivity to neutralization were also found among panel of viruses against different subtype-specific plasma. This finding suggests that viral sensitivity to plasma neutralization seems dependent more on viral characteristics than on the type of plasma used. The overall potencies of these plasma pools were ranked in the following order: subtype C (ID<sub>50</sub> 1:285) > subtype G (ID<sub>50</sub> 1:151) > CRF02\_AG (ID<sub>50</sub> 1:90). Potency of the subtype C pool was significantly higher than that of the CRF02\_AG pool (P<0.001) and subtype G pool (P<0.009). The most parsimonious explanation could be the fact that samples from subtype C pool were selected from patients with moderate neutralization activity against a panel of 24 different HIV-1 viruses tested in another study in our laboratory (R.A.Jacob unpublished data), while the subtype G sample were not selected based upon neutralization activity. Differences in potency was also significant for the subtype G pool compared to CRF02\_AG (P<0.0001), suggesting that CRF02\_AG pool used in this study, which was also used in a previous study (Jacob et al., 2012) did not contain high levels of heterologous neutralizing antibody, including antibody specific for subtype G viruses. However, there was no significant correlation between neutralization activity of subtype G pool and the autologous neutralization activity (P=0.16), suggesting that the subtype G pool used in this study still contain nAbs present in the autologous plasma even at a lower concentration.

Sample ID	Virus	C pool	AG pool	G pool	Virus GMT
10056	10056.C1	346	62	95	127
	10056.D8	285	59	111	123
	10056.F2	61	<50	66	63
12541	12541.B2	200	110	198	163
	12541.C12	578	105	174	220
BS03	BS03.G4	465	100	221	218
	BS03.H3	<50	100	221	149
BS12	BS12.A6	397	118	228	220
	BS12.D2	869	174	361	379
	BS12.D9	338	116	257	216
	BS12.G8	872	76	118	199
BS46	BS46.A2	481	136	225	245
	BS46.E1	286	95	360	214
	BS46.E2	509	98	246	230
	BS46.H4	313	97	249	197
BS48	BS48.E1	746	210	914	523
	BS48.E3	546	97	315	255
	BS48.E4	540	111	51	145
	BS48.F1	490	161	236	265
	BS48.F5	191	54	<50	101
	BS48.H10	660	182	668	431
BS51	BS51.A3	713	122	196	258
	BS51.B5	332	102	160	176
	BS51.B6	403	95	157	182
	BS51.H2	547	82	151	189
	Serum GMT	410	105	203	

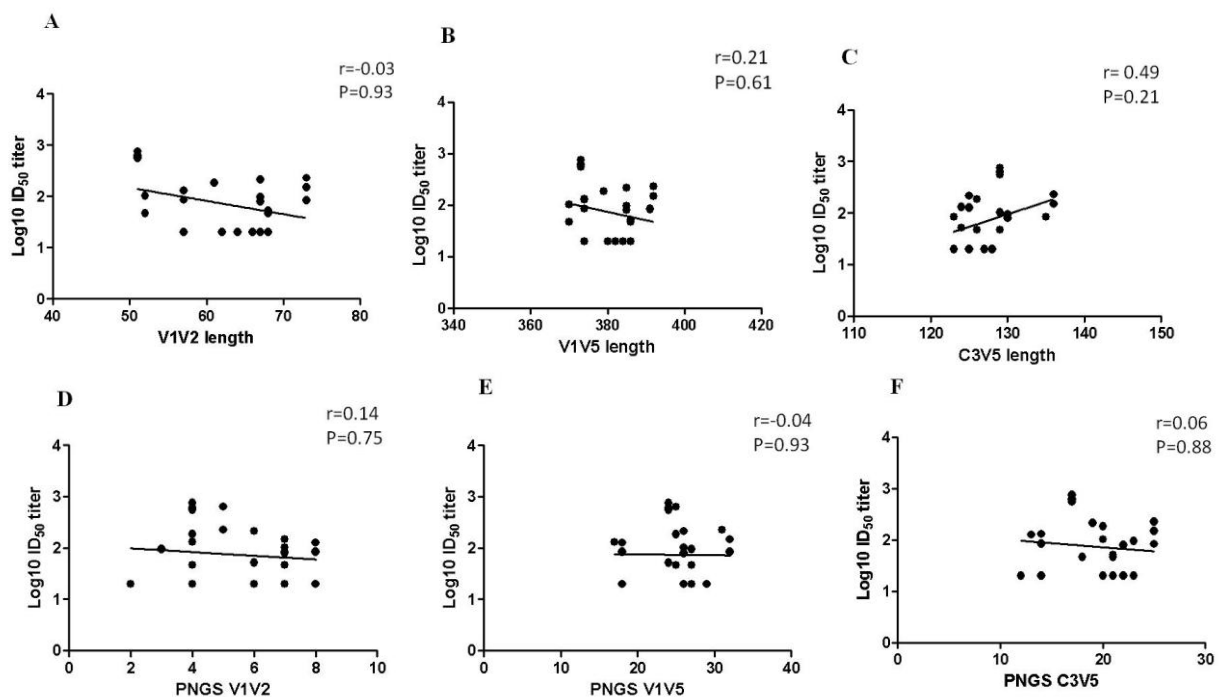
>1000
300-999
100-299
50-99
<50

**Figure3. 2: Neutralization of subtype G viral isolates by pool of plasma.** The patient ID and the pseudoviruses tested are shown on the left and the plasma pools tested are indicated at the top. Plasma pool were made using equal amount of plasma from HIV-1 infected individuals in order to provide a desired sample diversity of the antibody repertoire present in each individual plasma sample such as subtype C (n=68), CRF02\_AG (n=12), and subtype G (n=8). The neutralization titer is also shown as the plasma dilution that causes the inhibition of 50% of virus infection when the virus is neutralized by the plasma pools. The highest titer (>1000) is shown in red as indicated at the bottom of the figure. The grey box indicated that <50% neutralization was observed at 1:50 which was the highest dilution of plasma tested.

### 3.4.3 Subtype G Env autologous neutralization (ID<sub>50</sub> titers) does not correlate with the length and N-linked glycosylation site of the variable loops

Several recent studies in subtype C have demonstrated an inverse association between the neutralization sensitivity and length of variable loops (V1V2) and number of potential N-linked glycosylation sites (PNGS) (Rong et al., 2009, Gray et al., 2007, Sagar et al., 2006, Rong et al., 2007a, Pinter et al., 2004). Gray et al (2007) reported that the magnitude of nAbs responses was associated with shorter V1V5 envelope length and fewer glycosylation sites,

particularly in the V1V2 region (Gray et al., 2011a). In addition, Rong et al (2007) reported that the V1V2 lengths have affects on autologous neutralization sensitivity in patient derived subtype C envelopes (Rong et al., 2009). We therefore analyzed whether the length of variable loops and the number of potential N-linked glycosylation sites (PNGS) in all 7 subtype G-infected patient viruses was correlated with autologous neutralization titers using the Spearman rank correlation test. Figure 3.3.A, 3.B, and 3.C shows that there were no significant correlations between the amino acid lengths of V1V2, V1V5 and C3V5 with autologous neutralization titers ( $p=0.93$ ,  $p=0.61$ , and  $p=0.21$  respectively). The same analysis was extended for the PNGS, and again, there were no significant correlations with autologous neutralization titers ( $p=0.75$ ,  $0.93$ , and  $0.88$  respectively) (Figure 3.3.D-F).



**Figure 3.3: Correlations between the length of V1.V2, V1.V5, and C3.V5 of envelope and autologous neutralization titer.** The autologous neutralization titer for each of the 8 study participant was plotted against the length of the V1V2 (A), V1V5 (B), C3V5 (C) regions or the number of PNGS in the V1V2 (D), V1V5 (E), C3V5 (F) regions. The Spearman correlation coefficient (r), the p-values and a line fit with 95% confidence interval are shown. r values and p-values are shown. The correlation was significant when  $p < 0.05$ .

#### **3.4.4 Cross-neutralizing activity HIV-1 Subtype G plasma samples**

The heterologous neutralizing activity of subtype G plasma has not been previously reported. Plasma samples from HIV-1 subtype G infected individuals studied here was therefore evaluated on whether they could neutralize a panel of 14 pseudoviruses chosen upon subtype diversity and neutralization resistance (Seaman et al., 2010, Blish et al., 2009). The virus panel included four subtype A, four subtype B, two subtype C and four CRF02\_AG. The plasma were evaluated by a “geometric mean titer” that was the geometric mean of all the percentage neutralization values for that serum against all panel viruses that neutralized at 1/100 dilution. The Proportion of viruses neutralized was calculated by the number of viruses neutralized by each plasma sample divided by the total number of viruses tested.

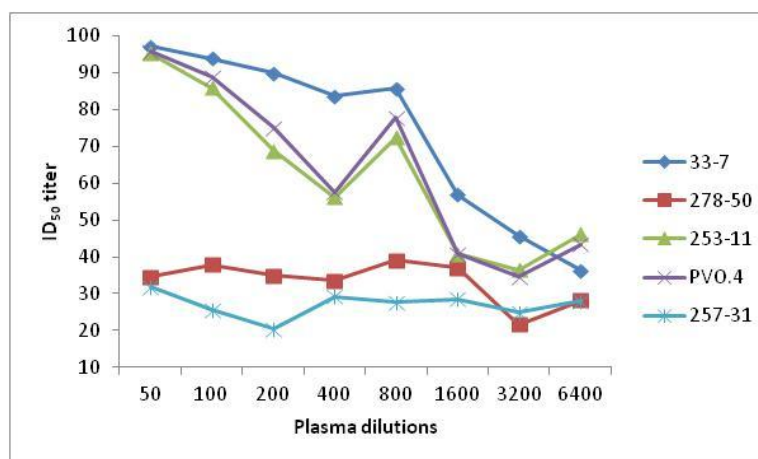
Blood plasma from two out of the seven study participants neutralized more than half of panel virus tested at >50% neutralization at a dilution of 1/50, indicating at least limited neutralization breadth in these two samples. One participant 10056 was excluded from further analysis in the neutralization studies because of evidence suggesting the presence antiretroviral drug in the plasma. Interestingly, the other participant BS12 neutralized 2 tier 3 viruses (33-7 and 253-11) (figure. 3.4.A). We therefore tested BS12 blood plasma with all tier five panel viruses available in the laboratory. We found that BS12 plasma neutralized 3/5 of the tier 3 isolates from the panel viruses (278-50 and 257-31 not neutralized; 33-7, 253-11, and PV0.4 neutralized) (figure. 3.4 B).

A

>90
70-89
50-69
<50

Heterologous neutralization																				
Samples plasma ID		14 virus panel																		
		Subtype A Kenya						Subtype B, various locations					Subtype C southern Africa		CRF02_AG				Serum geometric mean titer	No of virus neutralized
		Tier 2	unk	unk	Tier 2/3	Tier 2					Tier 2		Tier 2		Tier 3					
MLV	SF162	Q168.a2	QG984	QH343.21	Q461.e2	TRO11	RHPA 4259.7	REJO 4541.67	SC 422661.8	ZM249 M.PL1	CAP45.2.00.G3	255-34	33-7	278-50	253-11					
10056	11	97	24	98	52	16	92	66	47	55	40	21	60	35	26	20	62	6		
12541	9	95	0	30	44	0	21	0	52	24	34	28	47	15	15	37	17	1		
BS03	4	93	0	0	0	0	0	0	5	0	0	0	11	0	0	0	4	0		
BS12	4	100	35	76	70	6	88	7	47	50	32	0	44	64	2	76	50	6		
BS46	1	88	0	15	0	0	29	0	17	5	6	0	34	4	0	0	6	0		
BS48	0	84	0	0	0	0	0	3	0	0	0	0	0	0	0	0	4	0		
BS51	1	97	25	8	0	0	24	13	37	6	10	7	38	12	9	0	8	0		

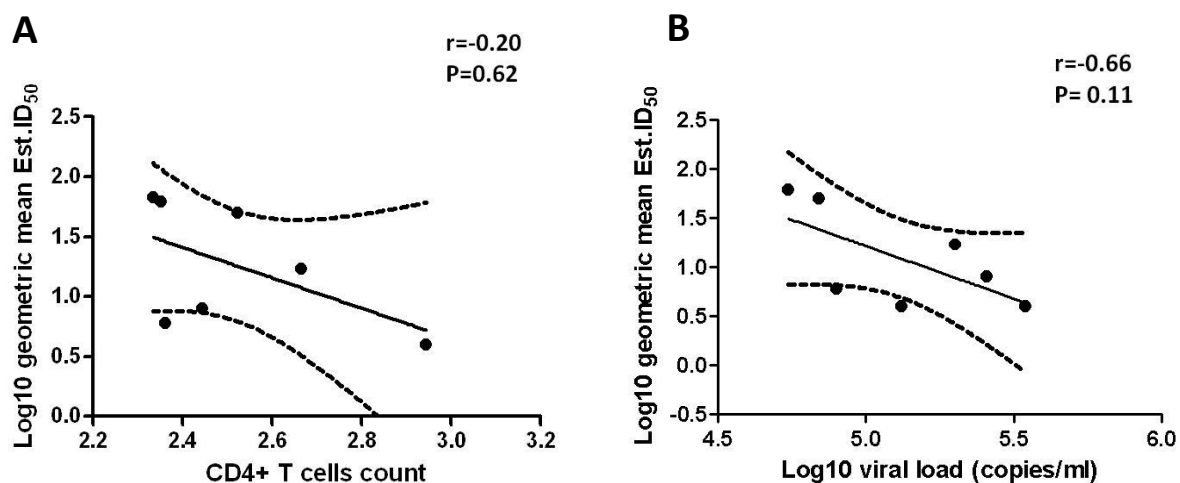
B



**Figure 3. 4: Heterologous neutralization capacity of participant’s plasma.** A) Neutralization capacity of subtype G plasma against a panel of reference HIV-1 pseudoviruses chosen for subtype diversity and neutralization resistance, including subtypes A, B, C, and CRF02\_AG. The participant’s plasma analyzed is shown to the left and virus panel tested is indicated at the top. The percent neutralization of the indicated pseudovirus by the indicated plasma at a screening dilution of 1/100 is also shown. The highest percent neutralization value (>90) is shown in red as indicated on the top of the figure. The grey box indicated that <50 percent neutralization was observed at a screening dilution of 1/100. Samples were tested against Murine Leukemia Virus (MLV) as a negative control and against the highly neutralization-sensitive subtype B SF162.2 as a positive control. unk, tier unknown; virus not analyzed in Seaman et al (Seaman et al., 2010). B) Neutralization of BS12 plasma against a panel of five tier 3 reference HIV-1 pseudoviruses known to be highly resistant to neutralization. The tier 3 viruses include four CRF02\_AG (33-7, 278-50, 253-11, and 257-31) and one subtype B (PV0.4) viruses. The ID<sub>50</sub> titer is plotted vertically and plasma dilutions are shown on the horizontal axis.

### 3.4.5 Correlation of neutralization breadth with CD4+ T cell counts and viral loads

There are many previous studies that showed an inverse relationship between CD4+ T cell counts and neutralization breadth, and a positive correlation with viral loads (Euler et al., 2010, Gray et al., 2011a, Piantadosi et al., 2009b, Sather et al., 2009). In order to assess the relationship between neutralization and clinical disease markers in our study participants, we correlated the participant's viral loads and CD4+ T cell counts to geometric mean titers (GMT). These analyses revealed as shown in figures 3.5A and 3.5B, there were no significant correlations between CD4+ T cell counts or viral loads and the geometric mean titers. One of the caveats of this study was the small sample size, which means that we may not have had sufficient statistical power to find this correlation in our samples.



**Figure 3. 5: Correlation of geometric mean titer (Neutralization breadth) with CD4+ T cell counts and Viral loads (A&B). Spearman r values and p-values are shown. Each dot represents a single individual.**

### 3.4.6 Mapping of the antibody specificity in broadly neutralizing plasma

One of the great interests in the antibody vaccine development is the target of broadly neutralizing antibodies. One patient in our study (BS12), which was ART naïve and that showed a substantial potent autologous activity as well as a good breadth of neutralization against a panel of different viruses (subtypes A, B, C, and CRF02\_AG), was further investigated. A recent study by Walker et al, (2010) elucidated a number of key sites targeted by individuals with neutralization breadth. The sites included N160, L165 in V2 region and

N332 glycan in C3 region (Walker et al., 2010). To assess the presence of this type of antibodies in BS12 infected plasma, we tested mutants viruses containing single point mutation introduced in V2 (N160A, T162A, K169E) for PG9/PG16-like epitopes, in C3 (N332A) for 2G12-like epitopes, into CAP45.2.00.G3, CAP255, Du156.12, and Q23.17 compared to wild type virus and tested for loss of neutralization sensitivity against BS12 plasma. We also tested for anti-MPER activity using three HIV-2 chimeric viruses containing HIV-1 MPER fragments: C1 containing subtype B (Yu2 sequence) MPER, C1C a consensus subtype C MPER, and 253-11 containing CRF02\_AG MPER from 253-11 virus.

As shown in Table 3.1.A, there was a 9.3 fold drop in neutralization titer compared to the wild type neutralization titer when BS12 plasma was tested against the CAP45 K169E mutant. This suggested that the dominant neutralizing antibody for virus CAP45.2.00.G3 in sample BS12 targeted the PG9/PG16 site. This site is the target in the V2 and V3 regions of the monoclonal antibodies PG9 and PG16. In addition, BS12 plasma also exhibited substantial neutralization of an HIV-2 chimeric virus displaying the MPER of 253-11, a CRF02\_AG virus (Table 3.1.B), although we did not examine if these anti-MPER antibodies are capable of neutralizing HIV-1 viruses by recognizing MPER. Taken together, these results suggested that BS12 most likely has PG9/PG16.site antibodies, and that these antibodies were responsible for most of the neutralization activity against virus CAP45.2.00.G3, and the sample also contained detectable anti-MPER antibodies. However, we could not follow the evolution of the virus in response to these antibodies because this study participant was an anonymous blood donor.

**Table 3.1: Mapping data to define antibody specificities found in plasma sample BS12.****A**

Parent virus and mutation	ID <sub>50</sub> (parent)	ID <sub>50</sub> (mutant)	Fold reduction mutation ratio <sup>a</sup>	Target tested
CAP45 N160A	1127	437	2.6	PG9/16 site
CAP45 T162A	1127	442	2.5	PG9/16 site
CAP45 K169E	1127	121	<b>9.3</b>	PG9/16 site
CAP255 N332A	4764	2997	1.6	PGT/2G12 site
Q23.17 N332A	1528	650	2.3	PGT/2G12 site
Q23.17 N160K	1528	2978	0.5	PG9/16 site
Du156 N332A	592	990	0.6	PGT site

**B**

Parent virus and mutation	ID <sub>50</sub> (parent)	ID <sub>50</sub> (mutant)	Fold effect neutralization of MPER HIV-2 chimera <sup>b</sup>	Target tested
C1	50	59	1.2	MPER
C1C	50	197	<b>3.9</b>	MPER
253-11	50	3882	<b>78</b>	MPER

<sup>a</sup> Calculated as wild type IC<sub>50</sub>/mutant IC<sub>50</sub> for the plasma. <sup>b</sup> Calculated as HIV-2 MPER chimera IC<sub>50</sub>/HIV-2 IC<sub>50</sub> (7312A) for the plasma and ID<sub>50</sub>>1000 is an indicator of anti-MPER activity (Gray et al., 2007; Gray et al., 2011). Changes in titer of >3.fold are shown in bold.

### 3.5 Discussion

As HIV-1 continue to spread and initiate new infections especially in high endemic areas, understanding the specificities of neutralizing antibodies developed in HIV-1 infected individuals may be important in identifying possible targets for an effective HIV-1 vaccine. Some recent studies in subtype C, B, and few from other subtypes have suggested that the contemporaneous viruses tend to be less sensitive to neutralization by contemporaneous plasma (Li et al., 2006a, Moore et al., 2009b, Rong et al., 2009, Richman et al., 2003, Frost et al., 2005). We therefore undertook this study with the goal of characterizing neutralizing antibody patterns in the context of HIV-1 subtype G, one of the most circulating strains in West Africa. The ultimate goal of this study was to create a well characterize panel of subtype G gp160 reference clones to facilitate the evaluation of vaccine-elicited neutralization antibody responses.

We found that nAbs responses against contemporaneous autologous virus was generally low in the majority of viruses, including viruses from recently infected individuals (individuals with BED value <0.8) and individuals with longer infection (ART failing individuals and individuals with BED value >0.8). This results are not surprising because previous studies with untreated primary HIV-1 infections have reported that, nAbs responses against contemporaneous autologous viruses were generally lower than the responses against earlier viruses (Richman et al., 2003, Gray et al., 2011a, Rong et al., 2009, Moog et al., 1997). However, in one apparently long-term HIV-1 infected individual (BED assay>0.8), BS12 exhibits a substantial potent AnAbs activity against all four isolated contemporaneous viruses. Although low levels ( $ID_{50}<200$ ) of neutralizing titers to contemporaneous autologous virus were observed in the majority of the participants, neutralizing titers varied between and within individuals. The most possible explanation may be that some viruses that we isolated just arose, and then we would expect no neutralization (Moog et al., 1997), or perhaps some are on the way out from antibody pressure that has been there for a while because the autologous neutralization might be high (Rong et al., 2009).

Our study has demonstrated that many subtype G viral variants exhibit a variable level of sensitivity to neutralization by the various plasma pools, with a small proportion of viruses showing a more sensitive or resistant phenotype. This finding is consistent with Seaman et al, (2010) in which a rank order approach was used to classify viral neutralization sensitivity to pooled plasma into three-tier (Seaman et al., 2010). The neutralization activity observed between the HIV-1 subtype G viruses and subtype G plasma pool (subtype-specific neutralization) (Seaman et al., 2010, Brown et al., 2008, Jacob et al., 2012) was less potent as viral variants were more sensitive to subtype C pool than subtype G pool. The most parsimonious explanation is that the subtype C samples were selected for good neutralizers, while the subtype G and CRF02\_AG were not. Therefore, subtype C viruses used in this study had high levels of heterologous neutralizing antibody, including antibodies specific for subtype G and CRF02\_AG. We also observed a within-subtype neutralization with our subtype G infected plasma samples that neutralizes subtype G viruses better than CF02\_AG indicating the sensitivity of subtype G viruses to subtype G specific neutralizing antibodies. Furthermore, the sensitivity of one virus to a particular plasma pool was often observed to other plasma pools suggesting that the neutralization was dependent on the virus characteristics rather than the type of plasma pool used.

Previous studies have reported that the antibody response increase in potency over time, capable of neutralizing heterologous HIV-1 variants (Moore et al., 1996, Moog et al., 1997, Donners et al., 2002, Binley et al., 2008, Piantadosi et al., 2009a, Sather et al., 2009, Simek et al., 2009, Gray et al., 2011a). Our results show that nAbs in plasma from long-term HIV-1 infection is associated with BnAbs responses against heterologous viruses than are plasma from recently infected individuals. BED assay value of 1.28 identified BS12 as a long-term HIV-1 individual, and 10056 is a patient under ART, therefore with a long-term HIV-1 infection. Blood plasma from the two (10056 and BS12) out of seven (29%) study participants, neutralized more than 50% of panel virus tested at  $ID_{50}>50$ , indicating at least limited neutralization breadth in these two samples. This frequency agrees to those previously described in other studies reporting up to 30% of samples with this activity (Doria-Rose et al., 2009, Euler et al., 2010, Gray et al., 2009b, Piantadosi et al., 2009b). One of the two plasma samples remaining from patients failing ART (10056) showed broad neutralization activity against almost 50% of panel reference viruses that covered HIV-1 subtype A, B, C and CRF02\_AG, which is consistent with the results from Medina-Ramirez, (2011) done in HIV-1 subtype B infected patients under ART, where there was broadly neutralizing antibody activity despite undetectable viral loads (Medina-Ramirez et al., 2011). Therefore, the good neutralizing antibodies in the patient 10056 could be a consequence of improve B cell function associated with antiretroviral treatment. On the other hand, it is possible that the good neutralizing antibodies in the patient BS12, which is a naive infected patient with a long-term HIV-1 infection, could be the fact that maturation of neutralizing antibodies in this patient is very efficient.

In this study we did not see any significant correlation between in one hand, the autologous neutralization titer and the length and PNGS in V1V2, V1V5 and C3V5 regions, and in another hand the neutralization breadth and the markers of disease progression (CD4+ T-cell counts and viral loads) among our HIV-1 subtype G infected individuals. Gray and colleagues (2011) found a significant correlation between the CD4 T-cell count and viral loads with neutralization breadth at six months post-infection only and not at later time points (Gray et al., 2011). The small sample size has severely limited our statistical power to address those issues.

Previous studies have shown that V1V2 and C3 regions are the main targets of autologous neutralizing antibodies in subtype C (Moore et al., 2009b, Rong et al., 2009, Moore et al., 2008, Lynch et al., 2011). The fine mapping of the plasma from the participant BS12 who

displayed potent autologous neutralization and broadly Cross-neutralizing antibodies indicated that nAbs recognized the lysine (K) in position 169 in the V2 and V3 regions, indicating possible quaternary epitopes that are targeted by PG9/PG16.site antibodies. In addition, the same participant had antibodies against the MPER in the gp41 region, indicating that the high autologous neutralization and the relatively high neutralization breadth may be dictated by antibodies directed at more than one epitope. However, we could not establish whether these antibodies were present early in the infection or whether they evolved, as the patient was a blood donor and plasma sample availability at different time point was a limitation. Despite this limitation and based on our data we propose that in BS12 HIV-1 subtype G infected plasma, the V1V2, and MPER regions are the main immune-dominant region of the envelope targeted by neutralizing antibodies.

Certain limitations of our study deserve to be mentioned. Firstly, we only tested a limited number of viral clones derived from the SGA amplicons from each patient, it is hard to be sure that these samples are not biased compared to the population of HIV-1 in each individual that we tested. Secondly, we do not know the time since initial HIV-1 infection of any of the individuals that we studied, although, the low diversity in BS48 suggests a very recent time since infection. Thirdly, our sample size was too small, making this study underpowered regarding the significance of different correlations analyzed in this study. Fourthly, the unavailability of plasma samples at different time point was one of our major disappointments. Therefore, it was difficult to follow-up the immunological data and the evolution of immune response in these participants, particularly BS12, the one that we found to have very high neutralization against contemporaneous viral isolates.

### **3.6 Conclusion**

In summary, this study provides the first characterization of neutralization phenotype in HIV-1 subtype G viral variants using a set of HIV-1 infected individual plasma, plasma pools from different subtypes, and a panel of 14 different viruses. Our data showed that one sample, BS12, was unusual in that the plasma sample itself neutralized viruses derived from sequences found in the same sample. Our data suggest that in study participant BS12 plasma, the V1V2, and MPER regions are the main immune-dominant region of the envelope targeted by neutralizing antibodies. We believe that a subset of these HIV-1 viral variants may be used for screening of HIV-1 vaccine candidate. Last, our characterization of these viruses added detailed information about subtype G, which is historically understudied.

# CHAPTER 4

## RESULTS

# **HIV-1 subtype G envelope variants have similar sensitivity to new generation of anti-CD4-binding site and anti-MPER broadly neutralizing monoclonal antibodies, but sensitivity to inhibitors of viral entry varies**

## **4.1 Abstract**

### **Background**

HIV-1 subtype G is the sixth most prevalent subtype and infects approximately 1.5 million individuals worldwide, especially in West and Central Africa. The development of neutralizing antibody-based vaccine against HIV-1 may need to be effective against different subtypes that are transmitted globally. One of the main components of this effort has been the identification and characterization of broadly neutralizing antibodies (BnAbs). In this study, we assessed the neutralization breadth and potency of a panel of eleven (11) first and second-generation of BnAbs against 26 HIV-1 subtype G viruses generated from 8 HIV-1 infected individuals. The sensitivity of these viruses against soluble CD4 (sCD4), TAK-779, a CCR5 inhibitor, and T20, a fusion inhibitor was also evaluated.

### **Results**

We found that subtype G viruses were highly sensitive to the CD4-binding site monoclonal antibodies (mAbs) NIH45–46<sup>G54W</sup> and VRC01 as well as MPER mAb 10E8, neutralizing all 26 viral variants. In addition, subtype G viruses showed sensitivity to 4E10 which was able to neutralized 25 of 27 (92%) of viruses and median IC<sub>50</sub> of 0.42ug/ml. These viruses were more sensitive to 4E10 than a large panel of worldwide viruses published last year (IC<sub>50</sub>=1.93ug/ml) and of all subtype G viruses with published sensitivity to 4E10 (median IC<sub>50</sub> =17ug/ml). However, neutralization by the mAbs b12, 2G12, Z13e1 was generally poor, while 2F5, PG16, PG9 and VRC03 have moderate activity neutralizing 42%, 65%, 27%, and 50% of viral variants respectively. Subtype G viruses were highly sensitive to TAK-779 and T20 but exhibit more variable sensitivity to sCD4, a phenotype that have been observed frequently among freshly isolated viruses because sensitivity to sCD4 protein is thought to be associated with exposure of the CD4 binding site.

### **Conclusion**

NIH45–46<sup>G54W</sup>, VRC01 and 10E8 which are directed against the CD4-binding site and the MPER region showed significant activity against HIV-1 subtype G viral isolates. These

results confirm the broadly neutralizing activities of NIH45-46<sup>G54W</sup>, VRC01, 10E8 and 4E10 across group M and reveal the resistance of most subtype G viruses to mAbs 2G12, b12, and Z13e1. Despite the mapping hit in BS12 plasma for PG9/PG16 site antibodies against CAP45.2.00.G3 described in chapter 3 of this thesis, BS12 derived viruses were not highly sensitive to neutralization against PG9 and PG16 and it is not clear if these viruses are neutralized by the autologous plasma sample by recognition of this site. In particular, these viruses were not more sensitive to PG9 and PG16 compared to other viruses. This group of subtype G viruses could also be useful for screening T20 and CCR5 inhibitors that are being tested as potential microbicides in regions where HIV-1 subtype G predominate. In that regard, the relative sensitivity of the viruses examined here to T20 and CCR5 inhibitors is encouraging, because freshly isolated viruses are representatives of circulating viruses that are the critical targets for such interventions. This characterization is important and should feed into the design of immunogens for HIV-1 vaccine, particularly as a global vaccine will be used in regions where HIV-1 subtype G and related recombinant forms predominate.

## 4.2 Introduction

Multiple studies have demonstrated the potential of HIV-1 broad neutralizing antibodies to protect against HIV-1 infection using nonhuman primate models (Burton et al., 2012, Baba et al., 2000, Mascola et al., 1999, Mascola et al., 2000, Parren et al., 2001). However, one of the greatest obstacles for the development of an effective HIV-1 vaccine is the high sequence variability of HIV-1 isolates. An important component for the development of HIV-1 vaccine is the identification and characterization of neutralization antibody specificities that are effective against the majority of circulating HIV-1 strains, particularly those common in Sub-Saharan Africa such as subtype G, in order to use their epitope for immunogen design (Burton, 2002). Several studies have shown that HIV-1-specific neutralizing antibodies and inhibitors of HIV-1 entry used as microbicides can also prevent infection when used as a microbicide in a non-human primate model (Veazey et al., 2003, Veazey et al., 2005).

Until 2009, only a small number of BnAbs isolated from HIV-1 subtype B infected individuals have been identified and known to potently neutralize different HIV-1 viral isolates: b12 recognizes the CD4 binding site (Burton et al., 1994), 2G12 targets a conserved cluster of oligo-mannose glycans on gp120 (Trkola et al., 1996), and mAbs 2F5 (Barbato et al., 2003), 4E10 (Stiegler et al., 2001) and Z13e1 (Zwick et al., 2001) recognizes three adjacent epitopes in the membrane-proximal external region (MPER) of gp41. These mAbs have limited activity in non-B subtypes, with the exception of 4E10 which neutralizes most strains with low to moderate potency (Gray et al., 2006). Therefore, there was an urgent and important need to isolate new BnAbs from non-subtype B infected individuals. Since 2009, several new BnAbs have been identified with improved breadth and potency activity against several different HIV-1 strains and are gaining more attention, although any of these BnAbs would be desirable to induce with HIV-1 vaccine (Hraber et al., 2013). These new BnAbs were derived from donors infected with different HIV-1 subtypes: subtype A (PG9 and PG16), subtype B (VRC01, VRC02, and VRC03, NIH45-46<sup>G54W</sup>) and subtype C (HJ16 and PGT) (Tomaras et al., 2011). The success of this effort was based on the combination of three strategies: a) the selection of chronically infected individuals with potent and cross-subtype reactive serum antibodies; b) the use of novel selection of screening approaches; and c) the development of efficient methods to isolate human monoclonal antibodies (Corti and Lanzavecchia, 2013). PG9 and PG16 recognize a quaternary epitope involving the V1V2 and V3 loop of the gp120 (Walker et al., 2009, Doores and Burton, 2010) MAbs VRC01, NIH45.45W which is an engineered form of NIH45.46, a clonal variant of VRC01, and HJ16

recognize the CD4 binding site (Zhou et al., 2010, Wu et al., 2010a, Corti et al., 2010), while the mAb 10E8 recognize the MPER in the gp41 region (Huang et al., 2012).

HIV-1 subtype G is the sixth most prevalent subtype of HIV-1 after subtypes A, B, C, CRF01\_AE and CRF02\_AG (Hemelaar et al., 2006). Subtype G circulates mainly in West and Central Africa (Hemelaar et al., 2011a) however; very limited information is available on the neutralization properties of HIV-1 subtype G viruses. To date, no study of which we are aware have study the sensitivity of subtype G viruses to new generation of BnAbs. Revilla et al, (2011) studied the sensitivity of subtype G against the first generation of mAbs b12, 2F5, 4E10 and 2G12 (Revilla et al., 2011). It is unclear how effective new generations of BnAbs are specifically against HIV-1 subtype G viruses. Therefore a better understanding of a relative breadth and potency of these new BnAbs against HIV-1 subtype G viruses will allow the evaluation of the neutralization phenotype of subtype G viral variants.

In this study, we determined differences in neutralization sensitivity of HIV-1 viral variants generated from 8 HIV-1 subtype G infected individuals from Cameroon using TZM-bl neutralization assay against eleven BnAbs and three entry inhibitors: soluble CD4 (sCD4), TAK-779, a CCR5 inhibitor, and T20, a fusion inhibitor . We sought to determine what BnAbs had the highest potency and broadest coverage against our panel of HIV-1 subtype G viruses.

## **4.3 Methods**

### **4.3.1 Study participants, plasma samples, entry inhibitors, and monoclonal antibodies**

Study participants and plasma samples used in this study are described in section 2.3.1 of this thesis.

The human monoclonal antibodies 2F5, 4E10, 2G12, IgG1b12, PG9, PG16, VRC01, VRC03, Z13e1, NIH45-46<sup>G54W</sup>, and 10e8, the soluble human CD4 (sCD4-183), the CCR5 inhibitor TAK-779, the fusion inhibitor T20 were all obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (ARRRP). The pSG3Δenv HIV-1 plasmid was also obtained from Drs. J. Kappes and Xiaoyun Wu through ARRRP.

### **4.3.2 Cell lines**

The TZM-bl cells were obtained from Drs. J Kappes and X Wu through the ARRRP. These cells were derived from HeLa cell clone and expresses CD4, CCR5 and CXCR4 (Platt et al.,

1998). TZM-bl cells contain two reporter genes: the luciferase and the *Escherichia coli*  $\beta$ -galactosidase under the control of the HIV-1 LTR promoter (Wei et al., 2002). The 293T cells used for transfection were obtained from Dr. A Rice through the ARRRP.

#### **4.3.3 HIV-1 *env* gene single genome amplification, cloning, and pseudoviruses production**

The cDNA extracted from the infected individual plasma samples were used to amplify the full-length *env* gene using the single genome amplification (SGA) assay as previously described (Keele et al., 2008). Briefly, the 3kb PCR products generated using one of two sets of primers (as described in section 2.3.4 methods in chapter 2) were cloned into pcDNA3.1/D/V5/His/TOPO vector (Invitrogen, Carlsbad, CA, USA) and bacterial colonies screened by PCR for insertion and correct orientation using T7 and BGH primers and plasmid were sequenced and compared to SGA PCR products. Pseudoviruses were made by cotransfecting 293 T cells with 4  $\mu$ g of each cloned viral envelopes with 8  $\mu$ g of SG3 $\Delta$ env HIV genome plasmid using X-tremeGENE 9 DNA transfection reagent (Roche Diagnostics, Basel, Switzerland) as previously described (Montefiori, 2009). 48 and 72 hours after transfection, the culture supernatant containing pseudoviruses was harvested, filtered, aliquoted, and stored at  $-80^{\circ}\text{C}$ . The TCID<sub>50</sub> for each pseudoviruses was determined by infection in TZM-bl as described (Montefiori, 2009).

#### **4.3.4 Neutralization assay**

Neutralization by mAbs, T20, and sCD4 were assessed as described (Montefiori, 2009). The monoclonal antibodies were used at a maximum concentration of 20 $\mu$ g/ml. briefly; serial two-fold dilutions of the appropriate inhibitor in Dulbecco's modified Eagle's medium (DMEM) were pre-incubated in duplicate in 96-well flat-bottom plates (Costar) with pseudovirus in a total volume of 150  $\mu$ l per well for one hour. Freshly trypsinized TZM-bl cells were then added (10,000 cells/well in [DMEM] 10% fetal bovine serum [FBS] containing HEPES and 7.5  $\mu$ g/ml of DEAE-dextran). Inhibition of HIV-1 infection by TAK-779 was assessed by adding the inhibitor to TZM-bl cells for 1 hour prior to the addition of pseudovirus stocks. After 48 hours of incubation at 37 $^{\circ}\text{C}$ , 100 $\mu$ l of cells/well were transferred to 96.wells black solid plate (Costar) and the luminescence measured using the Bright-Glo<sup>TM</sup> luciferase substrate (Promega, Madison, USA) and measured on a VERITAS MicroPlate Luminometer (Turner BioSystems).

Percentage neutralizations were determined by the following calculation

$$\left( \frac{\text{Difference in average RLU between virus control and sample}}{\text{Difference in average RLU between virus control and cell control}} \right) \times 100\%.$$

ID<sub>50</sub> values were calculated from curve fits to titration data using Prism 5.0 (Graphpad, La Jolla, USA)

#### 4.3.5 Statistical analysis

IC<sub>50</sub> titers for various monoclonal antibodies neutralization responses were calculated using the nonlinear regression (curve fit) function in GraphPad prism 5 software programme (GraphPad, La Jolla, USA). Comparisons between level of neutralization and other variables were measured by the Spearman's rank test in GraphPad 5. Correlations with p-value ≤0.05 were considered as statistically significant.

## 4.4 Results

### 4.4.1 Neutralization sensitivity of HIV-1 subtype G viral variants to BnmAbs

A total of 26 pseudoviruses (ranging from 2 to 6 per participant) were tested against 11 BnmAbs. This includes the older set of BnAbs (first generation) that generally have lower potency and breadth but are still considered broadly neutralizing 2G12, 2F5, b12, 4E10 and Z13e1, as well as some of the more potent recently isolated BnAbs (second generation) such as PG9, PG16, VRC01, VRC03, NIH45–46<sup>G54W</sup>, and 10E8.

There was a variation in the neutralizing activities of the BnAbs against subtype G viruses, with IC<sub>50</sub> values ranging from 0.01 µg/ml to resistant (>20 µg/ml) (Figure 4.1.A). Subtype G viruses were highly sensitive to the CD4-binding site monoclonal antibodies (mAbs) NIH45–46<sup>G54W</sup> and VRC01 as well as MPER mAb 10E8, neutralizing all (100%) viral variants with a geometric mean IC<sub>50</sub> of 0.07, 0.11, and 0.20µg/ml respectively. In addition, subtype G viruses showed sensitivity to 4E10 which was able to neutralized 25 of 27 (92%) of viruses and median IC<sub>50</sub> of 0.42ug/ml. These viruses were more sensitive to 4E10 than a large panel of worldwide viruses published recently (IC<sub>50</sub>=1.93ug/ml) (Huang et al., 2012) and of all subtype G viruses with published sensitivity to 4E10 (median IC<sub>50</sub> =17ug/ml) (Revilla et al., 2011, Binley et al., 2004, Huang et al., 2005). The activity of BnAb PG9 was relatively low 27%, with a geometric mean of 13.28µg/ml. However, PG16 and VRC03 have moderate

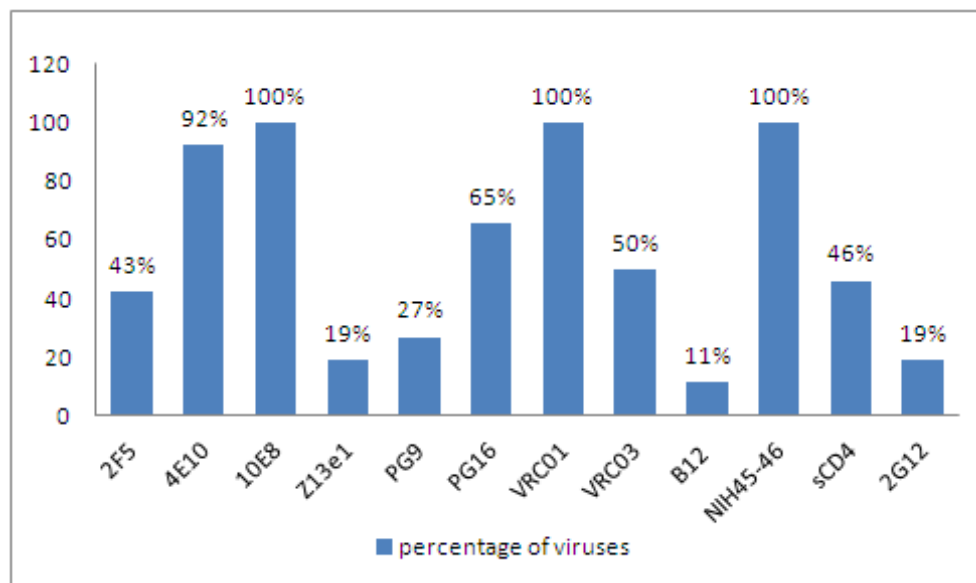
breadth and potency neutralizing 65% and 50% with geometric mean IC<sub>50</sub> of 2.10 and 13.25µg/ml respectively. Despite the mapping hit in BS12 plasma for PG9/PG16 site antibodies against CAP45.2.00.G3 described in chapter 3 of this thesis, BS12 derived viruses were not highly sensitive to neutralization against PG9 and PG16. Moreover, these viruses were not more sensitive to PG9 and PG16 compared to other viruses. All viral variants were sensitive to at least three of the BnAbs tested, and none were neutralized by all eleven BnAbs tested; however, one virus (BS12.D9) was neutralized by 10 of the 11 (91%) BnAbs tested including b12 and 2G12 and was only resistant to mAb VRC03. It was also resistant to the soluble CD4.

The majority of subtype G viruses were resistant to b12, 2G12, and Z13e1 with respectively 11%, 19%, and 19% neutralization activity at highest concentration tested 30µg/ml (Geometric mean IC<sub>50</sub> of 19.40, 20.86, and 17.29µg/ml respectively). 2F5 and 4E10 showed moderate and high neutralization activities with 43% and 92% respectively with geometric mean IC<sub>50</sub> of 7.63 and 0.57µg/ml respectively (Figure 4.B). Last, virus 12541.B2 was the most resistant virus and was resistant to 8 of 11 BnAbs tested, while BS12.D9 was the most sensitive and was neutralized by 10 of 11 BnAbs tested.

**A**

Patient ID	Clones ID	Anti-MPER mAbs							Anti-CD4 binding site mAbs					sCD4
		2G12	2F5	4E10	10E8	Z13e1	PG9	PG16	VRC01	VRC03	NIH45-46	B12		
10056	10056.C1	>20	>20	0.18	0.81	>20	>20	3.7	<1.87	1.8	0.03	>20	>20	>20
	10056.F2	>20	<1.25	<0.62	<0.15	>20	>20	>20	<19	<18.5	1.2	>20	>20	10-20
	10058.D8	17	13	0.03	0.004	>20	>20	1.25	0.02	<1.87	0.01	>20	>20	2.5-10
11439	11439.C5	>20	<2.5	<2.5	0.44	>20	>20	>20	0.8	>20	0.13	1.21	0.25	0.1-2.5
	11439.D4	>20	0.44	>20	0.36	>20	>20	>20	4.02	>20	0.35	>20	>20	<0.1
	11439.H4	>20	1.15	<2.5	0.37	>20	>20	0.009	0.72	>20	0.27	>20	>20	<0.1
12541	12541.B2	>20	>20	>20	0.05	>20	>20	>20	0.06	>20	0.02	>20	10.5	>20
	12541.C12	19	>20	0.23	0.21	>20	>20	5.8	0.04	12.7	0.03	>20	4.72	>20
BS03	BS03.G4	>20	>20	0.32	0.2	13	>20	>20	0.07	13.45	0.07	5.2	>20	>20
	BS03.H3	>20	>20	0.39	0.22	>20	>20	>20	0.07	18.76	0.05	>20	>20	>20
BS12	BS12.A6	18.01	>20	0.42	0.27	>20	>20	>20	0.07	>20	0.07	>20	>20	>20
	BS12.D2	>20	>20	0.12	0.05	>20	12.5	<0.15	0.04	5.2	0.07	>20	15.5	>20
	BS12.D9	<2.5	<2.5	1.5	0.83	6.18	20	1.16	<0.15	>20	0.09	3.4	>20	>20
	BS12.G8	>20	>20	0.55	0.56	>20	>20	>20	0.09	>20	0.07	>20	3.8	>20
BS46	BS46.E1	>20	2.8	13.26	7.9	>20	16	0.02	0.06	3.68	0.06	>20	2.5	>20
	BS46.E2	>20	>20	0.28	0.12	>20	>20	5.28	0.03	9.75	0.04	>20	17.1	>20
BS48	BS48.E1	>20	>20	0.36	0.18	>20	>20	6.9	0.07	>20	0.07	>20	15.8	>20
	BS48.E3	>20	14.5	0.8	0.19	>20	6.2	<0.15	0.05	>20	0.08	>20	>20	>20
	BS48.E4	12.1	0.52	0.49	0.09	>20	1.3	<0.15	0.05	>20	0.06	>20	2.5	>20
	BS48.F1	>20	0.78	0.41	0.1	0.3	0.009	0.08	0.06	>20	0.07	>20	0.9	>20
	BS48.F5	>20	0.9	0.76	0.19	>20	1.5	<0.15	0.05	>20	0.05	>20	>20	>20
	BS48.H10	>20	>20	0.29	0.24	2.74	>20	>20	<0.15	>20	0.07	>20	13.5	>20
BS51	BS51.A3	>20	>20	0.66	0.31	>20	>20	8.1	0.07	14.76	0.03	>20	>20	>20
	BS51.B5	>20	>20	0.05	0.03	10.12	>20	0.72	0.07	3.22	0.07	>20	>20	>20
	BS51.B6	>20	>20	0.43	0.62	>20	>20	3.76	0.07	13.5	0.02	>20	17.4	>20
	BS51.H2	>20	>20	0.31	0.3	>20	>20	7.5	0.07	15.6	0.07	>20	>20	>20

**B**



**Figure 4. 1: Neutralization sensitivity of HIV-1 subtype G viruses against broadly monoclonal antibodies.** A) The patient ID, and viruses analyzed are shown to the left. Each row shows the virus name, and the concentration that is required to achieve 50% neutralization [IC<sub>50</sub> (μg/ml)] for the mAbs tested. The red shading indicates more potent neutralization, as indicated below the figure. Gray shading indicates that 50% neutralization was not achieved at highest concentration of mAb tested (20 μg/ml). B) Summary of neutralization breadth and potency of BnAbs against subtype G viral isolates. The percentage of viruses neutralized is indicated for each BnAb on the top of the graph.

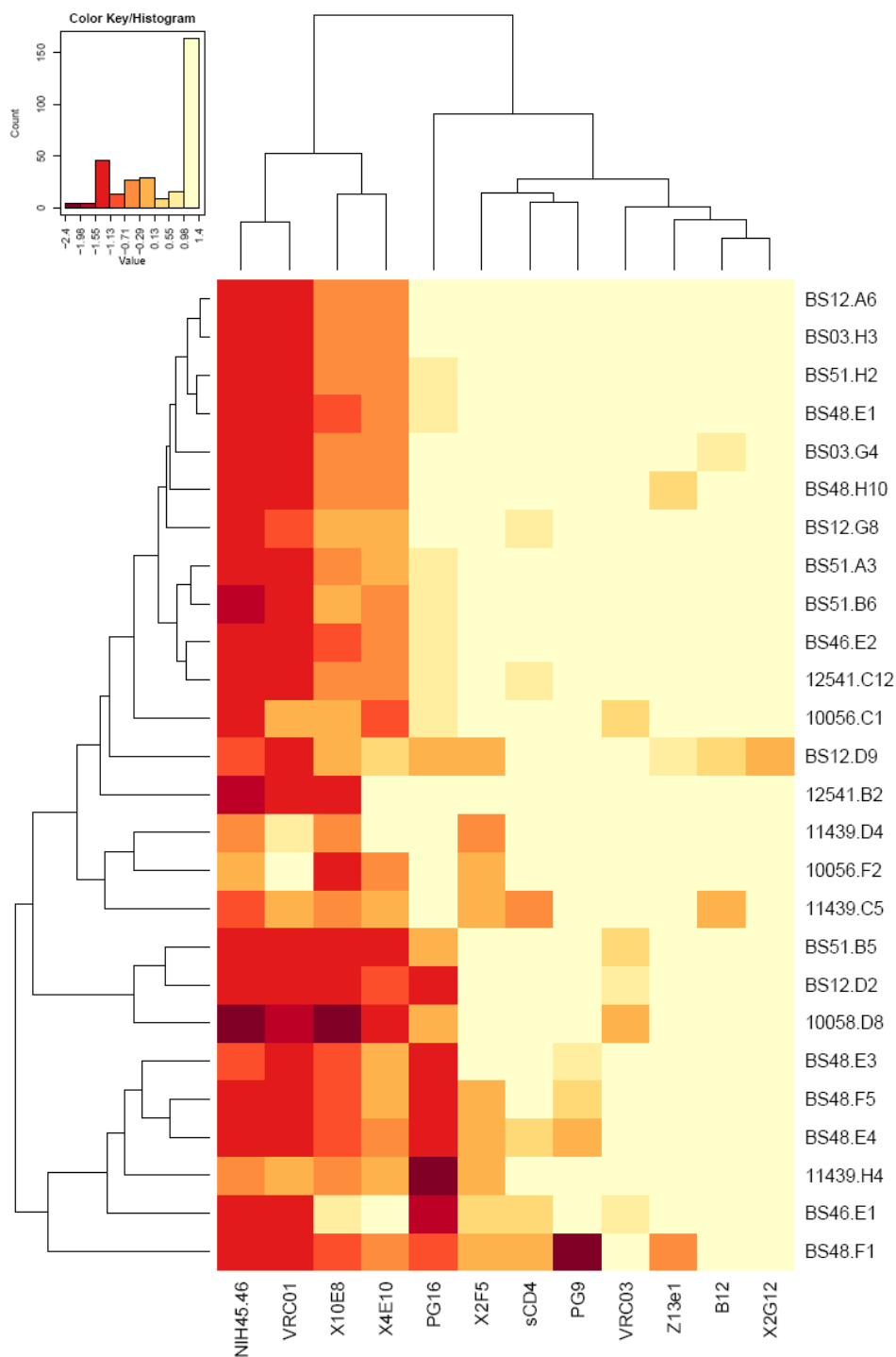
#### 4.4.2 Clustering analysis of the neutralization activities of BnAbs against HIV-1 subtype G variants

We generated heat maps to study the hierarchical clustering patterns of neutralization by BnAbs and to group viruses with similar neutralization sensitivity to BnAbs (Figure 4.2). The dendrogram at the top of the figure shows three distinct clusters of BnAbs, with those on the left being more broad and potent. The second cluster showed moderate neutralization activity and the third cluster a low neutralization activity. The dendrogram on the left shows two groups of viruses identified based on their sensitivity to BnAbs. Viruses generally clustered by their overall neutralization sensitivities. Four of the BS48 samples cluster together in the cluster of six viruses at the bottom (Figure 4.2), but two (BS48.E1 and BS48.H10) clustered with the resistant viruses. Interestingly, BS48 is the sample that looks like it was taken within 30 days of seroconversion as described previously (Keele et al., 2008). Bar et al., (2012) suggested that the low ID<sub>50</sub> titers at this stage are associated with antibody-mediated selection pressure. Indeed, despite the low autologous ID<sub>50</sub> titer (ID<sub>50</sub> from <50 to 1:131), the BS48 patient can produce neutralizing antibody responses as early as within 30 days of seroconversion. Selection at this early stage has been observed in other infected individuals in which early antibody can select for virus escape, even at very low ID<sub>50</sub> (Bar et al., 2012).

#### 4.4.3 Analysis of epitope sequences recognized by BnAbs

The epitope sequences recognized by BnAbs were analyzed, since mutagenesis studies have revealed that mutations in the BnAbs epitopes can affect the neutralization phenotype (Blish et al., 2008, Zwick et al., 2005, Moore et al., 2011, Tomaras et al., 2011).

Because 2G12 failed to neutralize the majority of viral variants, we investigate whether these viruses lacked the PNGS at position 295, 332, 339, 386, and 392 required for neutralization (Sanders et al., 2002, Trkola et al., 1996, Scanlan et al., 2002). As shown in Table 1, all viruses lacked one or more potential N-linked glycosylation sites (PNGS) at these positions, which is likely to explain their resistance to 2G12.



**Figure4. 2: Hierarchical clustering of mAbs (bottom) and subtype G viruses (right).** A heatmap of IC<sub>50</sub> values for each virus-mAb combination is shown, with darker shading indicating increasing potency, as indicated on the top. The yellow indicates that 50% of neutralization was not achieved at the highest concentration of mAb tested (20 µg/ml). The heatmap was generated using hierarchical clustering and Euclidean distance method and the log<sub>10</sub> values of the IC<sub>50</sub> neutralization data for each viruses and nAbs that are displayed on the horizontal axes ([www.hiv.lanl.gov](http://www.hiv.lanl.gov)).

2F5 recognize the core epitope ELDKWA at the MPER region with LDKW residues (at positions 664-667) being critical for binding and neutralization activity (Zwick et al., 2005, Tian et al., 2002) and amino acid mutation at Lys.665 appeared to be the key determinant of resistance (Binley et al., 2004).

Among the fourteen 2F5 resistant viruses, 10 had mutations at the LDKWA motif consistent with their phenotypic resistance (Table 4.1). Four maintained the LDKWA motif, although all were resistant.

4E10 binds to the core epitope NWFDIT (at positions 671-676), located at the C terminus of the 2F5 epitope. Mutagenesis studies have shown that mutations at residues Trp-672, Phe-673, and Trp-680 are critical for binding and result in resistance to neutralization by 4E10 (Zwick et al., 2005). Analysis of epitope sequences of the two 4E10 resistant viruses revealed that none had mutation at these three key residues, however substitutions at positions 671, 674, and 676 may contribute to their resistance to 4E10. In addition, it has been documented that many viruses that contain the amino acids that correspond to the full epitope may be resistant (Zwick et al., 2005, Binley et al., 2004), and therefore must be reasons other than changing of the epitope sequence that may result in 4E10 resistance. In fact, Sun Zy et al, (2008) have reported that residues Trp-672 and Phe-673 are connected via a short hinge to a flat C-terminal helical segment (at position 675-683). This meta-stable L-shaped structure is immersed in viral membrane and, therefore, less accessible to immune attack (Sun et al., 2008). b12 targets the CD4 binding site on gp120 (Burton et al., 1994). Site-directed mutagenesis have shown that gp120 amino acid substitutions D185N, N276A, S364H, S365A, P369L, and T373R reduced the susceptibility or resulted to neutralization resistance to b12 (Mo et al., 1997, Pantophlet et al., 2003, Duenas-Decamp et al., 2008). All clones contained one or more mutations known to impact b12 activity at residues 369, 185, and 364 which may explain their overall resistance to b12.

PG9 and PG16 recognize a quaternary epitope involving the V1V2 and V3 loop of the gp120 (Walker et al., 2009, Doores and Burton, 2010). As reported previously by walker et al, (2009), the absence of the key residues at positions 156 and 160 in the V2 region is associated with resistance to neutralization to both PG9 and PG16 (Walker et al., 2009). Only one virus (11439.D4) did not have the N160 residue normally required for recognition of PG9. More mutations were observed at position 158, 169, and 181 which may account for the

neutralization resistance of some variants to PG9 (Walker et al., 2009, Tomaras et al., 2011, Moore et al., 2011). Few variants were resistant to PG16. PG16-resistant viruses were likely the result of substitutions K305R and I309F. It is striking that resistance to PG9 and PG16 appeared distinct among our viruses. This effect was large enough that PG9 and PG16 appear in distinct cluster in the hierarchical clustering in Figure 4.2.

**Table 4.1: Amino acid sequences in positions of BnAb epitopes important for binding or neutralization**

Virus	clade	2G12(HXB2)					PG9/PG16(HXB2)						PG16(HXB2)					b12(HXB2)					2F5: ELDKWA(HXB2)						4E10: NWFDIT(HXB2)										
		295	332	339	386	392	156	158	159	160	162	169	173	176	181	299	305	307	309	317	318	185	276	364	365	369	662	663	664	665	666	667	671	672	673	674	675	676	680
		N	N	N	N	N	N	S	F	N	S/T	K*	Y	F	I	P	K	I	I	F	V	D	N	S	S	P	E	L	D	K	W	A	N	W	F	D	I	T	W
10056.C1	G	.	T	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	Y	G	.	.	.	L	A	.	.	.	.	.	.	S	.	.	.	.	S	.
10056.D8	G	.	T	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	Y	G	.	.	.	L	A	.	.	.	.	.	.	S	.	.	.	.	S	.
10056.F2	G	.	T	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	Y	G	.	.	.	L	A	.	.	.	.	.	.	S	.	.	.	.	S	.
11439.C5	G	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	E	.	.	.	L	A	.	.	.	.	.	.	.	.	.	.	.	S	.
11439.D4	G	.	.	K	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	Y	E	.	.	.	L	A	.	.	.	.	.	.	.	.	.	.	.	S	.
11439.H4	G	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	E	.	.	.	L	A	.	.	.	.	.	.	.	.	.	.	.	S	.
12541.B2	G	K	.	S	D	.	.	T	.	.	.	.	.	L	.	.	.	.	F	Y	.	.	.	.	L	A	.	.	Q	.	.	S	.	.	S	.	S	.	S
12541.C12	G	K	.	S	D	.	.	T	.	.	.	.	.	L	.	.	.	.	F	Y	.	.	.	.	L	A	.	.	Q	.	.	S	.	.	S	.	S	.	S
BS03.G4	G	I	.	K	.	.	.	.	.	.	R	.	.	.	.	.	.	.	F	Y	.	.	.	.	I	A	.	.	.	.	T	S	.	.	.	.	.	S	.
BS03.H3	G	I	.	K	.	.	.	.	.	.	R	.	.	.	.	.	.	.	F	Y	.	.	.	.	I	A	.	E	.	.	T	S	.	.	.	.	.	S	.
BS12.A6	G	.	.	D	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	Y	.	.	.	.	A	L	A	W	.	.	.	S	.	.	.	.	.	S	.
BS12.D2	G	.	.	D	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	Y	.	.	.	.	A	L	A	W	.	.	.	S	.	.	.	.	.	S	.
BS12.D9	G	.	.	D	D	.	.	.	.	.	R	.	.	.	.	.	.	.	.	Y	.	.	.	.	A	L	A	W	.	.	.	S	.	.	.	.	.	S	.
BS12.G8	G	.	.	D	.	.	.	.	.	.	R	.	.	.	R	.	.	.	.	Y	.	.	.	.	A	L	A	W	.	.	.	S	.	.	.	.	.	S	.
BS46.E1	G	.	.	T	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	Y	.	.	.	.	T	L	A	.	.	.	.	T	.	.	.	.	.	S	.
BS46.E2	G	.	.	T	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	Y	.	.	.	.	T	L	A	.	.	.	.	.	.	.	.	.	.	S	.
BS48.E1	G	.	D	Y	D	.	.	.	.	.	.	.	.	V	.	.	.	.	.	Y	.	.	T	A	L	A	.	.	.	.	.	.	.	.	.	.	.	.	
BS48.E3	G	.	.	Y	D	.	.	.	.	.	.	.	.	V	.	.	.	.	.	Y	.	.	T	A	L	A	.	.	.	.	.	.	.	.	.	.	.	.	
BS48.E4	G	.	.	Y	D	.	.	.	.	.	.	.	.	V	.	.	.	.	.	Y	.	.	T	A	L	A	.	.	.	.	.	.	.	.	.	.	.	.	
BS48.F1	G	.	D	Y	D	.	.	.	.	.	.	.	.	V	.	.	.	.	.	Y	.	.	T	A	L	A	.	.	.	.	.	.	.	.	.	.	.	.	
BS48.F5	G	.	D	Y	D	.	.	.	.	.	.	.	.	V	.	.	.	.	.	Y	.	.	T	A	L	A	.	.	.	.	.	.	.	.	.	.	.	.	
BS48.H10	G	.	D	Y	D	.	.	.	.	.	.	.	.	V	.	.	.	.	.	Y	.	.	T	T	L	A	.	.	.	.	.	.	.	.	.	.	.	.	
BS51.A3	G	.	.	.	Y	.	.	.	.	.	.	.	.	V	.	.	.	.	.	Y	.	.	.	.	A	L	A	.	.	.	.	T	.	.	.	S	.	S	.
BS51.B5	G	.	.	.	Y	.	.	.	.	.	.	.	.	V	.	.	.	.	.	Y	.	.	.	.	A	L	A	.	.	.	.	T	.	.	.	S	.	S	.
BS51.B6	G	.	.	.	Y	.	.	.	.	.	.	.	.	V	.	.	.	.	.	Y	.	.	.	.	A	L	A	.	.	.	.	T	.	.	.	S	.	S	.
BS51.H2	G	.	K	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	Y	.	.	.	.	T	L	S	.	.	.	.	.	.	.	.	S	.	S	.

All positions correspond to the HXB2 envelope protein. Amino acid positions correspond to sites for which mutations have been shown to affect the neutralization phenotype

#### **4.4.4 Sensitivity of subtype G viruses to HIV-1 entry inhibitors**

The sensitivity of subtype G viral variants was further evaluated against three HIV-1 entry inhibitors (Figure 4.3). The inhibitors used were sCD4, T20 a fusion inhibitor, and TAK-779 a CCR5 antagonist. As depicted in figure 4, Subtype G viruses were highly sensitive to TAK-779 (based upon the fold drop in infectivity with the drug added) and T20 (geometric mean  $IC_{50}$  of 1.44  $\mu\text{g/ml}$ ) but exhibit more variable sensitivity to sCD4, a phenotype that have been observed frequently among freshly isolated viruses because sensitivity to sCD4 protein is thought to be associated with exposure of the CD4 binding site. Soluble CD4 neutralizes 12 of 26 (46%) of viral variants, while no neutralization activity against viruses generated from 10056 and BS03 individuals was observed at highest concentration tested ( $IC_{50}$  of 20  $\mu\text{g/ml}$ ). The susceptibility to T20 and TAK-779 was similar for almost all variants did not vary substantially among the different viral variants.

Patient ID	Clones ID	T20	sCD4	Fold drop infectivity with 3.3uM TAK-779
10056	10056.C1	1.07	>20	5.3
	10056.F2	<0.31	>20	6.7
	10058.D8	0.14	>20	6.7
11439	11439.C5	0.04	0.25	2.6
	11439.D4	0.09	>20	2.4
	11439.H4	0.07	>20	4.3
12541	12541.B2	0.18	10.5	12.5
	12541.C12	0.006	4.72	5.9
BS03	BS03.G4	1.05	>20	5.3
	BS03.H3	1.04	>20	7.1
BS12	BS12.A6	0.49	>20	6.3
	BS12.D2	0.1	15.5	2.4
	BS12.D9	1.5	>20	6.3
	BS12.G8	0.9	3.8	4.5
BS46	BS46.E1	0.06	2.5	16.7
	BS46.E2	0.66	17.1	6.3
BS48	BS48.E1	0.74	15.8	4.2
	BS48.E3	0.09	>20	14.3
	BS48.E4	0.06	2.5	20
	BS48.F1	0.05	0.9	25
	BS48.F5	0.07	>20	25
	BS48.H10	0.53	13.5	5.3
BS51	BS51.A3	0.7	>20	7.7
	BS51.B5	0.49	>20	5.6
	BS51.B6	0.57	17.44	7.1
	BS51.H2	0.56	>20	5.6



**Figure4. 3: Sensitivity of HIV-1 subtype G viruses to entry inhibitors.** The patient ID, and viruses analyzed are shown to the left. Each row shows the virus name, and the concentration that is required to achieve 50% neutralization [IC<sub>50</sub> (µg/ml)] for the entry inhibitor tested. Viruses were obtained as described previously (Montefiori et al., 2009) and entry inhibitors was determined using pseudovirus infection of TZMbl cells. We couldn't determine IC<sub>50</sub> for TAK-779 due to substrate limitations and TAK-779 values represent the fold drop infectivity used in the coreceptor assay by comparing percentage neutralization in well containing co-receptor inhibitors to control wells to determine if either agent led to a reduction of infectivity. The red shading indicates more potent neutralization, as indicated below the figure. Gray shading indicates that 50% neutralization was not achieved at highest concentration of mAb tested (20 µg/ml).

## 4.5 Discussion

This study was aimed to characterize the sensitivity of subtype G viruses generated from 8 infected individuals in Cameroon against BnAbs and inhibitors of viral entry. Subtype G is a HIV-1 subtype circulating mostly in West and Central Africa. Several new BnAbs with great breadth and potency have been identified, but their neutralization profile against subtype G viruses from a West and Central African country has not been defined. We analysed the neutralization profile of 26 HIV-1 subtype G envelope variants, against 11 BnAbs targeting different epitopes and HIV-1 entry inhibitors.

Most subtype G variants displays high sensitivity profile to BnAbs tested. NIH45–46<sup>G54W</sup>, VRC01, and 10E8 which are directed against the CD4-binding site and the MPER region showed a remarkable combination of breadth and potency. Interestingly, we observed that the neutralization sensitivity to these three BnAbs results in 100% coverage of subtype G variants tested. Most subtype G viruses were also highly sensitive to 4E10 (92% coverage). These results are similar to the pattern of neutralization observed using these BnAbs in recent studies (Hraber et al., 2013, Mabuka et al., 2013, Euler et al., 2011, Goo et al., 2012), and confirm that these BnAbs may be among the most effective against different circulating HIV-1 variants.

Many subtype G viruses were resistant to one or more BnAbs, including those recently identified such as PG9 and VRC03, known to possess a remarkable breadth and potency against different HIV-1 strains. Most subtype G viruses were resistant to b12, 2G12, and Z13e1 up to a concentration of 20 µg/ml, and exhibit a moderate sensitivity against 2F5, and PG16. The resistance profiles found for the subtype G viruses described here are similar to two previous reported studies using 15 plasmids expressing subtype G Env libraries (Walker et al., 2009, Revilla et al., 2011). Those studies showed the resistance of subtype G to mAbs B12 and 2G12, high frequency neutralization by 2F5, 4E10, PG9 and PG16 although, in contrast to Walker study, the susceptibility of our viruses showed low frequency to mAb PG9. We were somewhat surprise that although the mapping of BS12 plasma indicated that the dominant neutralizing antibody for virus CAP45.2.00.G3 in sample BS12 targeted the PG9/16 site, these viruses were not unusually sensitive to the PG9 and PG16 monoclonal antibodies themselves. We expected BS12.derived viruses to be better neutralized by the mAbs PG9 and PG16. Therefore some resistance to PG9 and PG16 may be more common among subtype G viruses. However, having antibodies that target the PG9/PG16 site does not necessarily mean

they bind it the same way that PG9 or PG16 do. It is possible that the autologous antibodies recognize the site differently and thus can see the BS12 viruses even though the PG9 and PG16 cannot.

The relatively poor neutralization by the mAbs b12, Z13e1, and 2G12 was not surprising, it is known that some of the subtypes A, C, and the few G viruses previously characterized circulating in Sub-Saharan Africa, display limited sensitivity to first-generation antibodies such as b12 and 2G12 (Revilla et al., 2011, Walker et al., 2009, Wu et al., 2010a, Blish et al., 2007). Our results point to subtype G as showing a wide resistance to four of the five first generation BnAbs raised against subtype B used in this study with only 4E10 which target the MPER in the gp41 region, being able to neutralize 92% of the viruses.

It was interesting to evaluate how conserved the epitopes targeted by these BnAbs were among HIV-1 subtype G. It is clear that, while substitutions in the conserved core epitopes can predict neutralization resistance, the presence of the correct core sequence cannot predict neutralization (Binley et al., 2004). We found that viruses that are resistant to these BnAbs are largely involved in mutations at residues known to be critical for mAb binding and neutralization. However, some BnAbs-resistant viruses had mutations to the core epitope, though other BnAbs-resistant maintained the core epitope (Table 1), a phenomenon reported previously by several authors (Binley et al., 2004, Blish et al., 2009, Blish et al., 2007, Zwick et al., 2001). This was observed for example in PG9-resistant viruses in which five of the nineteen resistant viruses maintained the core epitope. Strikingly, this resistance to PG9 was not reflected in similar resistance to PG16. This provides additional evidence that conformational changes controlled by sequences not in the core epitope may influence the resistance phenotype in these viruses, as suggested previously (Revilla et al., 2011).

We further characterized the sensitivity of these subtype G viruses to HIV-1 entry inhibitors since no study of which we are aware have been reported. All subtype G viruses were highly sensitive to T20, the fusion inhibitor, and TAK-779, a CCR5 inhibitor. These results correlated with previous reported studies with subtype C viruses (Cilliers et al., 2004, Blish et al., 2007, Kishko et al., 2011). Although some subtype G virus were sensitive to sCD4, most were resistant and thus apparently lack the b12 epitope or is not accessible to b12 nAbs, a phenotype that has been reported by previous studies with many subtype A viruses (Blish et al., 2007) and some subtype B viruses (Reeves et al., 2002). Sensitivity to sCD4 protein is thought to be associated with the exposure of the CD4 binding site (Pugach et al., 2004).

## 4.6 Conclusion

In summary, this study highlights the fact that it may be possible to neutralize HIV-1 subtype G viral variants with the recently identified BnAbs targeting the CD4 binding site such as NIH45-46<sup>G54W</sup> and VRC01, and the anti MPER BnAb 10E8. Our results point to HIV-1 subtype G as showing a wide resistance to four of the five first generation BnAbs raised against subtype B used in this study with only 4E10 which target the MPER in the gp41 region, being able to neutralize the majority of the viruses. This group of subtype G viruses could also be useful for screening T20 and CCR5 inhibitors that are being tested as potential microbicides in regions where HIV-1 subtype G predominate. In that regard, the relative sensitivity of the viruses examined here to T20 and CCR5 inhibitors is encouraging, although the partial resistance of several of our viruses to TAK-779 may indicate that drug pressure will select more resistant viruses quickly from the partially resistant ones. We believe that these results will facilitate in inclusion of subtype G viruses in test panels to evaluate responses to candidate vaccines and in choices needed for the design of immunogens for HIV-1 vaccine in regions where HIV-1 subtype G predominate.

## **CHAPTER 5**

### **CONCLUSION AND PERSPECTIVES**

## Conclusion and perspectives

HIV-1 subtype G accounts for 5% of HIV-1 infection worldwide, centered on West and Central Africa. It has also spread to several European countries, Cuba and is found among Russian intravenous drug users. Subtype G presumably spread outside West Africa with migration of infected individuals. In addition, 80% of the recombinant strains circulating worldwide contain segments attributed to subtype G (Brennan et al., 2008). However, historically, much of HIV-1 vaccine research and immunogen design to date has focused on subtype B, largely due to the ease of availability of this subtype in the high income countries of North America and Europe where most basic studies on HIV-1 immunogen design were done. Therefore there is urgency, despite the challenges of developing an HIV-1 vaccine, to study these understudied subtypes such as subtype G in order to develop a globally relevant vaccine. During the course of this study, we characterized the genetic properties of subtype G *env* sequences and evaluated the neutralization sensitivity and vulnerabilities of HIV-1 subtype G viruses.

In the first part of this work, we analyzed in total 47 *env* sequences, 45 of which I isolated by single genome analysis (SGA) PCR and two of which were isolated by colleagues (Tongo et al., 2013). Our samples were chosen based upon subtype analysis of *gag* and *nef* genes (Tongo et al., 2013) or of *pol* genes (not shown). Although recombinants were possible, phylogenetic analysis of the full length *env* has revealed that all virus sequences belong to HIV-1 subtype G. 12541-derived sequences branched from near the root of the subtype G subtree in a poorly populated branch containing some sequences related to a segment of CRF06\_cpx. However, the 12541 sequences did not contain the J-like sequences at the 3' end of *env* normally expected in CRF06\_cpx and the sequences without the J-like region did not cluster within CRF06\_cpx sequences, suggesting that these 12541 sequences are within subtype G and not CRF06\_cpx. This suggests that the full extent of the diversity of Subtype G has not yet been described, and provides additional evidence for the hypothesis that subtype G itself may be older than has been calculated (Abecasis et al., 2009). We did not find any significant difference between the V1V2 lengths from patients likely to be infected for < 6 months (BS03, BS46, and BS48, based upon BED assay results (Parekh et al., 2011) with patients likely to be infected for > 6 months (BS12, BS51). The V1V2 and V4V5 had the most length variation within and between participants, whereas the V3 length was constant. This variation in length appears similar to previously observed in subtype C (Coetzer et al., 2007). This study also illustrated the fact that all of our HIV-1 subtype G infections are

caused by viruses that exclusively utilizes CCR5 co-receptor. However, we found that three of our subtype G viruses (BS12.D2, 11439.C5, and 11439.D4) had only a 2.3 fold less reduction in infectivity in the presence of a high concentration of TAK-779 inhibitor (3.3 $\mu$ M). Although this may suggest use of another receptor, all viruses were resistant to the CXCR4 inhibitor. The variability in the sensitivity to TAK-779 of these CCR5 viruses may not be due to the extreme heterogeneity of the HIV-1 envelope glycoproteins (gp120 and gp41) that may affect the susceptibility of variant HIV-1 strains (Torre et al., 2000). CCR5 inhibitors have an antiviral effect in people who are predominantly infected with HIV that is adapted to using the CCR5 receptor, typically patients with higher CD4 counts (Torre et al., 2000). Unfortunately, we did not calculate IC<sub>50</sub> so our results are not comparable with those others.

We next characterized the neutralization sensitivity of subtype G viruses and the neutralizing capacities of antibodies induced by the viruses. However, a number of interesting findings were apparent. Firstly, as expected, we found that nAbs responses against contemporaneous autologous virus was generally low in either individuals with longer infection or recently infected individuals. We understand this using the following scenario: Within a few months of HIV-1 infection, nAbs against the autologous virus develop. In response to the immune pressure nAbs exert on the virus, escape mutants appear. Over the course of acute to chronic infection the immune response matures, as a consequence, nAbs from a specific time point are able to neutralize early autologous virus but generally not contemporaneous circulating viruses (Richman et al., 2003, Gray et al., 2011a, Rong et al., 2009, Moog et al., 1997). However, BS12 exhibits a substantial potent autologous nAbs activity against contemporaneous viruses. This has also been observed in at least one study (Rong et al., 2009). Secondly, the sensitivity to neutralization by the various plasma pools varied, and viral variants were more sensitive to subtype C pool than subtype G pool suggesting that the neutralization was dependent on the virus characteristics or the neutralization potency of the serum/plasma pool, rather than the subtype infecting the serum/plasma pool used. Thirdly, blood plasma from two study participants neutralized more than 50% of panel virus tested at a low threshold of ID<sub>50</sub>>50, indicating at least limited neutralization breadth in these two samples. In addition, BS12 serum, the sample that showed a substantial potent autologous activity, also neutralized 3/5 of the tier 3 isolates from the panel viruses, suggesting substantial heterologous neutralizing activity in this plasma sample. Fourthly, mapping of BS12 plasma, the sample that display a potent autologous and heterologous neutralization capacities suggests that nAbs likely recognized epitopes overlapping the quaternary epitopes

targeted by PG9/PG16.site antibodies (Moore et al., 2011, Gray et al., 2011b). In addition, the same participant had antibodies against the MPER in the gp41 region. Antibodies to other epitopes were not found, suggesting that the breadth and the high level of autologous neutralization may be dictated by a limited number of target epitopes. In subtype C infection, the V1V2 and C3 regions are the immunodominant regions commonly targeted by autologous neutralizing antibodies particularly during the early stage of infection (Moore et al., 2008, Moore et al., 2009a, Rong et al., 2009, Lynch et al., 2011). Our results do indicate that the V1V2 and MPER regions are the immunodominant regions and the focus of the nAb responses resulting in broadly Cross-neutralizing antibodies in the plasma sample from the study participant BS12. Unfortunately, BS12 was an anonymous blood donor and follow up samples cannot be obtained to observe the effect of this high autologous neutralization on viral dynamics.

We further characterized the sensitivity of HIV-1 subtype G viruses to eleven first and second-generation of BnAbs and three HIV-1 entry inhibitors. We found that subtype G viruses were highly sensitive to the CD4-binding site mAbs NIH45-46<sup>G54W</sup> and VRC01 as well as MPER mAb 10E8, neutralizing all (100%) viral variants. In addition, subtype G viruses showed sensitivity to 4E10 which was able to neutralized 92% of viruses. These viruses were more sensitive to 4E10 than a large panel of worldwide viruses published last year and of all subtype G viruses with published sensitivity to 4E10. Our results point to subtype G as showing a wide resistance to four of the five first generation BnAbs raised against subtype B used in this study with only 4E10 which target the MPER in the gp41 region, being able to neutralize 92% of the viruses. The relatively poor neutralization by the mAbs B12, Z13e1, and 2G12 was not surprising, it is known that several other subtypes display limited sensitivity to first-generation antibodies such as b12 and 2G12 (Revilla et al., 2011, Walker et al., 2009, Wu et al., 2010a, Blish et al., 2007). We generated heat maps to study the hierarchical clustering patterns of neutralization by BnAbs and to group viruses with similar neutralization sensitivity to BnAbs. Four of the BS48 samples cluster together in the cluster of six viruses at the bottom of the heat map, but two (BS48.E1 and BS48.H10) clustered with the resistant viruses. Interestingly, BS48 is the sample that looks like it was taken within 30 days of seroconversion as described previously (Keele et al., 2008). This results suggests that despite the low autologous ID<sub>50</sub> titer (ID<sub>50</sub> from <50 to 1:131), the BS48 patient may already be producing escape variants to autologous antibody that we can only

observe by a shift in their sensitivity to neutralization. Selection at this early stage has been observed in other infected individuals (Bar et al., 2012).

Subtype G viruses were highly sensitive to TAK-779 and T20 but exhibit more variable sensitivity to sCD4, a phenotype that have been observed frequently among freshly isolated viruses because sensitivity to sCD4 protein is thought to be associated with exposure of the CD4 binding site.

Taken together, our characterization of these viruses added detailed information about subtype G, which is historically understudied. The results obtained in this thesis have contributed toward additional understanding of the genetic properties, co-receptor usage and vulnerabilities of HIV-1 subtype G against nAbs, new generation of human BnAbs, and entry inhibitors.

Based on these results, the following are recommended:

- 1- The characterization of full length sequences of HIV-1 subtype G generated by single genome analysis (SGA) in order to better characterize the origin of subtype G.
- 2- A study with a large sample size with a proper follow-up should be explored. This type of study will be valuable for the characterization of antibodies, understanding of the characteristics of escape variants among subtype G-infected individuals and hopefully the definition and the discovery of new target immunogens.
- 3- Investigate the contribution of antibody dependant cell-mediated cytotoxicity (ADCC) and antibody dependent cell-mediated viral inhibition (ADCVI) in controlling or attenuating HIV-1 subtype G progression, since B-cells producing BnAbs may have to compete with non-neutralizing antibodies to immunodominant epitopes and perhaps with non-neutralizing antibodies to the same epitope (Alam et al., 2008).

# **CHAPTER 6**

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# Appendices

## Appendix A: RNA extraction

The QIAamp Viral RNA Mini Kit (Qiagen, Dusseldorf, Germany) was used according to the protocol as written in the manufacturer's handbook.

1- 560µl of prepared buffer AVL containing Carrier RNA was pipetted into a 1.5 ml micro centrifuge tube.

2- 140µl of plasma was added, mixed by pulse vortexing for 15 seconds.

3- Thereafter, the mixture was incubated at room temperature (RT) for 10 minutes.

4- This was brief centrifuged to remove drops from the inside of the lid.

5- 560µl of 96.100 % ethanol was added to the tubes, pulse.vortexed for 15 seconds and brief centrifuged.

6- 630µl of the resulting solution was applied to QIAamp spin column in a 2ml collection tube without wetting the rim and centrifuged at 6000 x g; 8000 rpm for 1 minute. The spin column was placed into a clean 2ml collection tube and tube containing the filtrate was discarded.

7- The QIAamp spin column was carefully opened and step 6 repeated.

8- 500µl of buffer AWI was added and centrifugation performed at 6000 x g; 8000 rpm for 1 minute. The column was placed into another clean 2ml collection tube and the filtrate was discarded.

9- 500µl of buffer AW2 was added and centrifuged at full speed 20 000 x g; 14 000 rpm for 3 minutes. Spin column was placed into a clean 2ml collection tube and centrifuged at full speed for 1 minute.

10- The QIAamp spin column was placed into a clean 1.5ml micro centrifuge tube and 50µl of elution buffer AVE was added, incubated for 1 minute at room temperature and then centrifuged at 6000 x g; 8000 rpm for 1 minute.

## Appendix B: Reverse transcription and cDNA synthesis

The Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, USA) was used in order to generate the cDNA through reverse transcription (components of the kit included: SSIII RT (200 U/ $\mu$ l), 5X First-Strand Buffer, 0.1 M DTT.) and 10 mM dNTP (Fermentas, Canada). This procedure was performed in a RNA-only clean room. The condensate from the previous step was spin down briefly, after the heat incubation steps.

The following components were added into a 0.2 ml RNase-free tube for each cDNA synthesis as depicted in table B.1.

**Table B.1 Mastermix for the cDNA synthesis**

Reagent	Volume ( $\mu$ l)/tube
Sterile water	8.75
Oligodt primer (20 $\mu$ M)	1.25
dNTP (10mM each)	5
RNA template	50
Final volume/tube	65

The tubes were placed in a thermo-cycler at 65°C for 3.5 minutes, removed and placed on ice for 1 minute. The following components were added from the kit to the mix from above according to the method depicted in table B.2.

**Table B.2 Mastermix for the cDNA synthesis**

Reagent from kit (SSIII RT)	Volume ( $\mu$ l)/tube	Stock from kit	Final dilution
5x Buffer	20	5x	1x
DTT	5	100mM	5
RNaseOUT	5	40U/ $\mu$ l	2
SSIII RT	5	200U/ $\mu$ l	10
Final volume/tube	35		

The reaction mixture was then gently mixed and left to incubate at 50°C for 1 hour and then increased to 55°C for 1 hour. Inactivation of the SSIII RT was achieved by heating at 70°C for 15 min. To each tube, 1 $\mu$ l of RNase H was added and left to incubate at 37°C for 20 minutes.

### Appendix C: First round amplification PCR reaction

Serial dilutions of cDNA were performed to obtain 20% positivity as illustrated above in figure 2.1. PCR reaction was made for one-100 reactions (94 tests + 2 negative controls) as depicted in table C.1.

**Table C.1 Mastermix for the first round PCR**

Reagent	Volume (µl)/tube	X100 reactions (µl)
DEPC H2O	15	1500
10X Buffer	2	200
MgCl <sub>2</sub>	0.8	80
dNTP	0.4	40
Taq (Faststart Taq DNA polymerase)	0.4	40
Primer: OFM19 (20µM)	0.2	20
Primer: VIF1.10W (20µM)	0.2	20
Final volume	19	1900

Once the correct cDNA dilution was determined through the dilution, 1µl of diluted cDNA was added into each well containing the 19µl of the master mix and mixing was achieved by pipetting up and down. The plate was placed in a thermal cycler and run with following PCR parameters: 94°C for 2 minutes followed by 35 PCR cycles of 94°C for 15 sec - denaturation, 55°C for 30 sec - annealing, and 68°C for 4 minutes – elongation with a final extension of 68°C for 15 minutes and held at 4°C upon completion.

From all positive wells, the amplicons from the first round were stored at -20°C for future envelope cloning once the second round products were resolved, visualized and confirmed using gel electrophoresis.

### Appendix D: Second round (Nested) PCR reaction

PCR reaction mixture was prepared for one-100 reactions (94 tests + 2 controls) as depicted in table D.1.

**Table D.1 Mastermix for the second round PCR**

Reagent	Volume (µl)/tube	X100 reactions (µl)
DEPC H2O	15	1500
10X Buffer	2	200
MgCl <sub>2</sub>	0.8	80

dNTP	0.4	40
Taq (Faststart Taq DNA polymerase)	0.4	40
Primer: ENVA (20µM)	0.2	20
Primer: ENVN.7R10T17W19T (20µM)	0.2	20
Final volume	19	1900

19µl of the master mix was pipetted into each well of a 96-well plate and 1µl from each of the first round PCR reaction samples was added to the corresponding well of the nested PCR plate. Mixing was achieved by pipetting up and down. The 96 well plate was placed in a thermal cycler and PCR performed with the following cycling parameters: 94°C for 2 minutes followed by 45 PCR cycles of 94°C for 15 sec - denaturation, 55°C for 30 sec - annealing, and 68°C for 4 minutes – elongation with a final extension of 68°C for 15 minutes and held at 4°C upon completion.

#### Appendix E: Table of primers used for PCR reactions

Primer name	Primer sequence	HXB2 position
VIF1.10W	GGGTTTATTWCAGGGACAGCAGAG	4900→4923
OFM19	GCACTCAAGGCAAGCTTTATTGAGGCTTA	519←547
ENV.A	GGCTTAGGCATCTCCTATGGCAGGAAGAA	5954→5982
ENVN.7R10T17W19T	CTGCCARTCTGGGAAGWATCCTTGTGT	9171←9145

→ denotes a forward primer. ← denotes a reverse primer.

#### Appendix F: Table of primers used to sequence HIV-1 subtype G Env gp160

Primer name	Primer sequence	HXB2 position
EF00	GGGAAAGAGCAGAAGACAGTGGCAATGA	6204→6228
For14	TATGGGACCAAAGCCTAAAGCCATGTG	6556→6582
For16	TTAATTGTGGAGGAGAATTTTCTA	7350→7375
EF170	AGCAGGAAGCACTATGGG	7799→7816
EF200	GGGATAACATGACCTGGATGCAGTGGG	8095→8118
EF260	TTCAGCTACCACCGATTGAGAGACT	8523→8544
EF175	TTAGCATCTGATGCACAGAATAG	6378←6398
Rev15	CTGCCATTTAACAGCAGTTGAGTTGA	6990←7015
EF115	AGAAAAATTCTCCTCTACAATTAA	7351←7371
EF55	GCCCCAGACCGTGAGTTGCAACATATG	7914←7940
EF15	CTTGCTCTCCACCTTCTTCTTC	8424←8445
Rev19	ACTTTTTGACCACTTGCCACCCAT	8797←8820

→ denotes a forward primer. ← denotes a reverse primer.

## **Appendix G: Post PCR clean-up**

Before sending samples for sequencing, post PCR clean-up was performed to each PCR products using either DNA clean and concentrator kit (Zymo Research) or the Wizard SV Gel and PCR clean-up system (Promega) according to the protocol as written in the manufacturer's handbook.

- 1- An equal volume of Membrane Binding Solution was added to the PCR amplification.
- 2- The Minicolumn was inserted into 2 ml collection tube.
- 3- Therefore, the prepared PCR product was transferred to the Minicolumn assembly and incubated at room temperature for 1 minute.
- 4- This was centrifuged at 16 000 x g for 1 minute. The flow through was discarded and the Minicolumn was reinserted into collection tube.
- 5- 700µl of membrane wash solution (containing ethanol) was added into Minicolumn and centrifuged at 16 000 x g for 1 minute. The flow through was discarded and the Minicolumn was reinserted into collection tube.
- 6- The step 5 was repeated with 500µl of membrane wash solution and centrifuged at 16 000 x g for 5 minutes.
- 7- The flow through was discarded and the column assembly was recentrifuged for 1 minute with microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.
- 8- The minicolumn was transferred to a clean 1.5 ml microcentrifuge tube, and 50µl of nuclease.free water was added to the minicolumn, incubated for 1 minute at room temperature and then centrifuged at 16000 x g for 1 minute.
- 9- The DNA was stored at 4°C or -20°C.

## **Appendix H: Directional cloning using pcDNA3.1 Expression Vector**

PCR fragments, generated using EnvA-rx and ENVN.7R10T17W19T primers, were cloned into the pCDNA 3.1/V5.His TOPO vector (Invitrogen, Carlsbad, USA) into **One Shot TOP10 Chemically-Competent cells** (Invitrogen, Carlsbad, USA) and bacterial colonies were screened by PCR for insertion and correct orientation using T7 and BGH (supplied in the pcDNA 3.1/V5.His TOPO expression kit).

## **Cloning procedure**

Sterile tubes were chilled on ice in order to be adequately chilled for downstream cloning. The cloning reaction mastermix consisted of the following as depicted in table H.1.

**Table H.1 Mastermix for ligation of PCR product into pcDNA3.1 Expression Vector**

Reagent	Volume (µl)/tube
Sterile water	add to a final volume of 5
Salt	1
pcDNA3.1/V5.His TOPO Vector	1
DNA	0.5 to 4
Final volume/tube	6

This reaction was mixed gently and incubated for 5 min at room temperature. Tubes were placed on ice for 10 minutes and One Shot TOP10 Chemically-Competent cells were thawed on ice for 10 minutes. Thereafter, 2µl of the cloning reaction above was added into the tubes containing One Shot TOP10 Chemically-Competent cells and left to incubate for 30 minutes. Cells were then heat shocked for 30 seconds at 42°C in a water-bath without shaking. The tubes containing the cells were then placed on ice for 2 minutes and to each tube of cells, 250 µl of SOC medium (from the pcDNA 3.1 kit) was added. The tubes were then shaken for 1 hour at 220 rpm at 32°C in a shaking incubator. Hundred.150µl of bacteria cells were then spread on Luria Bertani (LB) agar plates containing 100µg/ml Ampicillin (Sigma) and incubated overnight at 32°C. To screen for the presence of inserted *env* gene, 10 colonies were picked randomly for each sample and added into 5 ml of LB broth medium with 100 µg/ml ampicillin in an individual 14 ml polypropylene round-bottom tube (BD Falcon) and incubated in a shaking incubator at 200 rpm overnight at 32°C. Bacteria were lysed and minipreps of plasmid DNA were prepared using GeneJet plasmid Miniprep Kit (Thermo Scientific) according to the manufacturer’s instructions. A PCR was therefore set up at a final volume of 20 µl to identify the plasmid containing the insert using 1 µl of plasmid DNA (100 ng/µl), T7 (5’-TAATACGACTCACTATAGGG-3’), and BGH (5’.TAGAAGGCACAGTCGAGG.3’), both primers found on the vector and provided with the kit. The PCR conditions were as follows: 94°C for 5 minutes followed by 35 PCR cycles of 94°C for 30 sec - denaturation, 51°C for 30 sec - annealing, and 72°C for 3 minutes – elongation with a final extension of 72°C for 10 minutes and held at 4°C upon completion.

**Table H.2 Mastermix for colony PCR**

Reagent	Volume (µl)/tube
DEPC H2O	9.96
10X Buffer	1.25
MgCl <sub>2</sub>	1
dNTP	0.2
Primer: T7	0.013
Primer: BGH	0.013
Faststart Taq DNA polymerase	0.038
Final volume	12.5

PCR products were run on a 0.8% Agarose gel electrophoresis and a visualization of a band at 3kb was considered as positive clone. To confirm the presence of the insert in the plasmid, a restriction digestion with BamH1 and Xho1 (New England Biolabs) was also performed as depicted in table H.3. Plasmids with fragments of the correct size after restriction digestion were sequenced to confirm sequence identity to the original *env* amplicon. A bacterial stock of each positive clone was made and kept at -80°C and the plasmid DNA was kept at -20°C until further analysis.

**Table H.3 Restriction enzyme digestion using BanH1 and Xho1**

Reagent	Volume (µl)/tube
Sterile water	16
Xho1(New England Biolabs, Ipswich, USA)	1
BamH1(New England Biolabs, Ipswich, USA)	1
DNA	10 (~0.2µg)
10X Fastdigest Buffer	2
Final volume	30